The Phase Change from Vegetative to Reproductive Growth in *Agaricus bisporus*

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Declaration

The results presented in this thesis are the work of the author unless specified.

Sources of information have been acknowledged by reference.

None of this work has been previously used to apply for a degree.

Summary

The phase change from vegetative to reproductive growth in *Agaricus bisporus* is a complex process involving changes in morphology at least in part regulated genetically and influenced by various environmental signals. This work was aimed at understanding how the morphology changes, and the specific environmental parameters are involved, and which genes show changes in transcription during the phase change process in *A. bisporus.*

Different resources and methodologies were developed and applied to investigate this process including digital time-lapse photography, genome database assembly, design, validation and normalisation of a custom oligonucleotide gene expression microarray and analysis of microarray-generated gene expression profiles showing the response of this fungus under stimulatory and non-stimulatory environmental conditions.

Key stages that occur during reproductive differentiation and development were identified and defined. It was found that temperature and the mushroom volatile, 1-octen-3-ol, act as an on/off switches as they block specific stages of the phase change while carbon dioxide acts as a quantitative regulator as high amounts of this molecule reduce the number of primordia and fruit bodies that develop.

Gene expression profiles were constructed showing the changes in gene expression in peat-based *A. bisporus* samples, grown under commercial cultivation conditions which were designed to stimulate reproductive growth, and experimental cultivation conditions which were designed to separate out the effects of the three environmental parameters mentioned previously. It was found that 52 genes were differentially expressed in *A. bisporus* during the phase change from vegetative mycelium into fruit body primordia. A comparison with the gene expression profiles constructed for the experimental growth conditions, in correlation with morphological observations enabled the separation of these 52 genes into 3 clusters. One cluster contained 4 genes that are likely to be involved in the regulation of the "early" phase change, a second cluster contained 11 genes that are likely to be involved in the regulation of the "late" phase change and the third cluster contained 37 genes that are likely to be involved with physiological processes supporting the phase change.

Abbreviations

aCGH	array-based comparative genome hybridisations
AMP	adenosine monophosphate
BATS	bayesian analysis of time series
BC	base composition
BLAST	Basic Local Alignment Search Tool
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
cRNA	complementary ribonucleic acid
Ct	cycle threshold
CTAB	hexadecyltrimethylammonium bromide
Cy	Cyanine
DEPC	diethyl pyrocarbonate
Dnase	deoxyribonuclease
EC	enzyme commission
ECV	eight carbon volatile
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
G6PD	glucose-6-phosphate dehydrogenase
GC	guanine-cytosine
GO	gene ontology
h	hour
ID	identifier
L	liter
LiCl	lithium chloride
M	molar
MAANOVA	microarray analysis of variance
Mg	magnesium
MIC	minimal inhibitory concentration
ml	milliliter
MPa	megapascal
MPSS	massive parallel signature sequencing
mRNA	messenger RNA
N	nitrogen
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
ng	nanogram
nm	nanometer
O_2	oxygen

p.p.m	parts per million
pmol	picomol
PMT	photomultiplier tube
PVP	poly-1-vinylpyrrolidone-2
PVPP	polyvinylpolypyrrolidone
QC	quality control
qRT-PCR	quantitative real time reverse transcription polymerase chain reaction
r.p.m.	rotations per minute
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
s	second
S	svedberg
SAGE	Serial Analysis of Gene Expression
SD	standard deviation
SDS	sodium dodecyl sulphate
SSH	suppression subtractive hybridisation
TIFF	tagged information file format
Tm	melting temperature
Tris	tris(hydroxymethyl)aminomethane
v/v	volume/volume
XDR	eXtended Dynamic Range
µM	micromolar
µl	microliter
°C	degrees Celsius

Chapter 1. Introduction

1.1 *Fungal morphogenesis*

In the simplest form a fungal colony can be thought of as an interconnected network of branching hyphal cells spreading from a single point, usually termed vegetative growth. In addition to such networks, fungi have evolved numerous morphologies and co-ordinated growth forms that allow adaptation to different environments, for example mycelial cords, appresoria, haustoria and sclerotia. Furthermore, mitotic and meiotic spore production and release has seen the evolution of numerous growth forms from relatively simple conidiophores to the specialised basidiocarps (fruit bodies) of the higher fungi which develop during reproductive growth.

Fungal morphology and morphological development are regulated at physiological, biochemical and molecular levels. How fungi detect and respond to environmental stimuli to alter morphology is an exciting and complex topic of study; often the consequences of such stimuli are observed, but the underlying molecular mechanisms are poorly understood.

This study will use a well defined system to investigate the reproductive phase change in the basidiomycete fungi. The cultivated white button mushroom *Agaricus bisporus*, commercially produced under controlled conditions, provides an ideal model for understanding the fungal morphogenetic change in response to an environmental stimulus. Changes in substrate and environmental treatment cause the phase change from vegetative to reproductive growth leading through a series of morphological responses. Despite representing a multimillion pound industry the molecular mechanisms governing this process have not been identified. This study is focused on developing and applying molecular resources and methodologies in conjunction with morphological studies to identify genes that respond to different environmental triggers during the phase change to reproductive growth in *A. bisporus*. In order to achieve these goals we must first consider what is currently known about fungal growth and morphological development.

1.2 *Vegetative growth*

A significant part of the fungal life cycle consists of vegetative growth where the fungus explores its habitat and colonises nutrient rich areas to support its metabolism and general development. Fungi have become so successful in this that they dominate microbial biomass of forest soils and play a key role in sustaining forest ecosystems by scavenging and redistributing key nutrients such as nitrogen and phosphorous (Watkinson *et al.*, 2005). Fungi may also grow in areas with poor or complex nutrients and therefore efficient resource utilisation is required. These processes require the adaptation of specialised modes of growth.

1.2.1 Hyphae

Deacon (1997) described a hypha as a tube with a rigid wall containing a moving slug of protoplasm. It has a virtual infinite length as it is comprised of linked fungal cells separated by cross-walls. The diameter ranges around 5-10 µm. The hyphal cell walls are the only barrier to the (hostile) external environment and have to protect the fungus from osmotic lysis and regulate the exchange of various molecules such as enzymes and nutrients with its surroundings. Cell walls also protect the protoplasm against radiation or toxic chemicals. The hyphal wall comprises mostly of polysaccharides (chitin, glucans and/or chitosan), hydrophobins and lesser amounts of other proteins and lipids while a mesh of actin provides protection against osmotic turgor pressure (Wessels, 1996). The rigid composition of this cellular organisation has important consequences for the way fungi can expand: through polar, apical growth. Wessels (1996) suggested that the hyphal tip (apex) is plastic and flows outwards and backwards at the point of expansion while slowly rigidifying. Recent work by Harris (2009) showed that the Spitzenkörper, a phase-dark structure found at the apex of expanding hyphae, serves as a vesicle trafficking centre where the building blocks of the expanding cell wall arrive. Growing hyphae can branch in order to change the direction of growth. The mechanism controlling this process is not fully understood but Laing and Deacon (1991) found evidence of a regulatory mechanism in the mycoparasite *Pythium oligandrum* as it can form infectious branches at any location of a hyphal cell where it has been in contact with a host fungus. In addition to branching observed during colonisation of a substrate, hyphae can branch at an acute angle. In this case, the newly formed hyphae will grow

alongside the parent hyphae and can aggregate resulting in mycelial cords. As hyphae normally grow away from each other in culture, the formation of these mycelial cords indicates a close control at the molecular level and could be a different growth mode than normal, vegetative growth (Deacon, 1997; Hickey *et al.*, 2002; Trinci, 1984).

1.2.2 Nutrient acquisition and effect on fungal development

Fungi exhibit various (adaptive) foraging strategies, ranging from slow, but intensive exploration by a diffuse colony of fine hyphae, to more open systems with rapidly growing aggregates of hypae (mycelial cords) that are thought to be better suited to the discovery of large, sparsely distributed resources. These mycelial cords, described in some basidiomycetes, are thought to provide a higher capacity long-distance transport system compared to a vacuolar system (Tlalka *et al.*, 2008). Tlalka *et al.* (2008) reported that *Phanerochaete velutina*, a saprotrophic woodland fungus, exhibits a sparse and asymmetrical growth mode when exposed to nutrient limitation while a more dense and heavily branched growth occurs in nutrient rich areas. Addition of a cellulose resource to a colony of *P. velutina* resulted in a strong polarised growth in the area of the resource and cessation of extension in other parts of the colony. A focused N-allocation was found in that area to optimise the utilisation of the carbon-rich substrate. The mechanism controlling this is yet to be identified, but it was found to enhance the production of extracellular degradative enzymes through altered activity of various transcription factors (e.g. a CCAAT binding complex) (Bahn *et al.*, 2007). Changes in growth pattern in response to a stimulus (e.g. added resource) have been detected within 12 h of contact (Bahn *et al.*, 2007; Tlalka *et al.*, 2008). Watkinson (1999) previously observed this phenomenon in other fungi and suggested that this change in growth mode was controlled at a molecular level which sensed internal nutrient levels.

While the molecular mechanism controlling this change in growth mode is not yet identified, it has been shown that the carbon to nitrogen ratio and internal nitrogen concentration has distinct effects on fungal development and morphology as it is involved in mycelial cord formation and potentially in a switch from explorative growth to an intense localised growth during substrate colonisation and mycelial cord formation (Watkinson, 1999). The comparatively (to carbon) low availability of

nitrogen in terrestrial habitats such as forest soils means that this essential nutrient can be a limiting factor to fungal growth. However, fungi have evolved efficient nitrogen scavenging mechanisms such as recycling of nitrogen from old to new mycelium supported by translocation, selective depletion of cell materials under nitrogen starvation and the ability to utilise and select between many different nitrogenous compounds and sources (Boswell *et al.*, 2002; Watkinson *et al.*, 2006). It has been reported that fungal biomass can accumulate and immobilise as much as 20% of total soil nitrogen and that fungi can accumulate >95% dry weight of the available nitrogen when grown on nutrient agars (Griffin, 1972; Watkinson *et al.*, 2005). Mycelial cords have been observed in the casing layer of mushroom growth beds, as illustrated in Figure 1-1, but not in the compost layer (Masaphy *et al.*, 1987; Umar & Van Griensven, 1998). During commercial growing, a moist casing layer which mainly consists of peat and chalk is applied to the surface of the compost layer. This casing layer is required to stimulate fruit body production. Kues *et al.* (2000) suggested that the lower amount of available nutrients in the casing layer stimulates mycelial cord formation, on which mushroom primordia (developing fruit bodies) can develop, when the mycelium is exposed to specific environmental conditions.

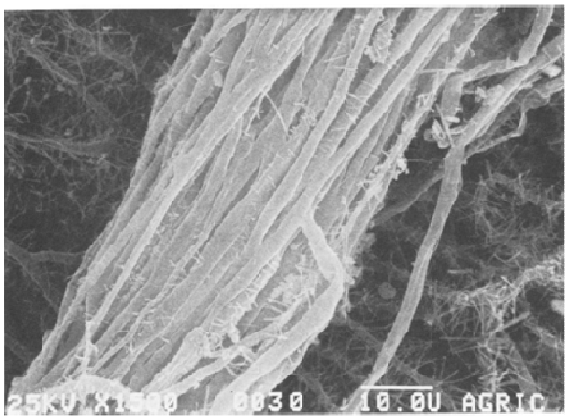

Figure 1-1 Multihyphal mycelial strand from the casing soil. Magnification, x 1,500. Scale bar = 10.0 µm (Masaphy *et al.*, 1987)

Nutrient acquisition in fungi is influenced by the structure of the hyphal cells. The cellular structure means that only simple molecules can be absorbed. Experiments with *Saccharomyces cerevisiae* and *Aspergillus niger* showed that digestive enzymes are transported in vesicles to the apex of the hypae where they are

actively released by exocytosis. Some enzymes will flow outwards driven by the expansion of the cell wall while others will become locked in the cell wall. The secreted enzymes will complete an initial breakdown of nutrients while the cell wall bound enzymes will complete the final stages of polymer breakdown so that the readily utilisable nutrients can be absorbed by the hyphae (Chang & Trevithi.Jr, 1974; Deacon, 1997). Substrates such as hydrocarbons, alcohols, chitin, keratin and lignin can be metabolised by a number of "specialist" fungi, while amino acids, saccharides, starch and cellulose are easily metabolised and therefore the vast majority of fungi can use one or more of these molecules as carbon source (Beguin, 1990; Cerniglia, 1992; Deacon, 1997; Jennings, 1988; Kunert, 2007). *A. bisporus* can utilise partially degraded leaf litter that is rich in complex lignocellulose polymers including cellulose, xylan and lignin and proteins (Xu *et al.*, 1997) by producing numerous degrading enzymes (De Groot *et al.*, 1998). These enzymes include endocellulase, cellobiohydrolase and β-glucosidase for cellulose degradation; glyoxal hydrolase, laccasse and manganese peroxidase for lignin degradation. Bacteriolytic enzymes such as β-Nacetylmuramidase have also been identified, indicating that *A. bisporus* can use dead biomass of micro-organisms that had previously colonised the growing substrate (De Groot *et al.*, 1998).

1.2.3 Specialised growth

Certain fungi develop specialised structures after receiving specific stimuli to assist with the colonisation of their habitat. Examples of this can be seen in *Magnaporthe grisea* and *Colletotrichum gloeosporioides* where appressoria and infection pegs develop when a hydrophobic plant surface is detected. These structures secrete a glue-like mucilaginous matrix to anchor the fungus to the host. A penetration hypae (infection peg) develops beneath these structures and the pressure inside the appressorium increases up to 8 MPa, caused by conversion of glycogen into osmotically potent products. This pressure forces the infection peg through the host cell walls after which hyphae invade and colonise the host tissue (Bahn *et al.*, 2007; Deacon, 1997). Parasitic fungi like *Uromyces appendiculatus* can sense the structure of its surroundings, using mechano-sensitive channels, in order to find stomata which it uses to enter the host (Bahn *et al.*, 2007).

1.3 *Reproductive growth*

At a certain point in the fungal life cycle, a change in metabolism occurs and reproductive growth is initiated. This change in growth is also defined as the phase change and can be mitotic (asexual) or meiotic (sexual).

1.3.1 Mitotic reproductive growth

A wide range of fungi exhibit asexual reproduction commonly in nature, for example, the cereal blast pathogen *Magnaporthe grisea*. Similarly example of this can be found in *Coprinus cinereus* which can develop asexual oidiophores and chlamydospores when exposed to certain conditions. Development of these structures on monokaryons of this fungus is under light control as oidiophore development is stimulated by light while chlamydospore development is inhibited by light. Germinating oidiospores attract hyphae of other colonies and act as spermatia in matings with compatible *C. cinereus* hyphae while they can also fuse with hyphae of other coprophilous fungi, resulting in the death of the foreign hyphae (Kues, 2000). In contrast to *C. cinereus,* no evidence of asexual spores has been documented for *A. bisporus*.

1.3.2 Meiotic reproductive growth

Fungi exhibit both homothallic and heterothallic sexual reproduction. Homothallic forms are self-fertile and possess both mating types. In heterothallic forms, sexual reproductive growth generally requires the merger, or anastamosis, of two monokaryotic hypae of different and compatible mating types. The resulting dikaryotic hyphae can produce a reproductive structure when specific environmental conditions occur. Well defined examples of this are apothecia in ascomycetes such as *Neurospora crassa* and the large basidiocarps (fruit bodies) of basidiomycetes such as *C. cinereus*, which produce the sexual spores.

Fungal spores are the fungus in a state of interrupted growth. In basidiomyctes, basidiospores develop on the apex of basidial cells in the hymenium (spore bearing layer). These spores are typical survival structures as the cytoplasm is denser, the walls are thicker, and the metabolic rate is slower compared to hyphal cells. Most sexual spores are also dormant. Spore germination starts with rehydration

followed by non-polar growth. At a certain point (e.g. a specific temperature in *Aspergillus niger*), an apex is formed and a germ-tube starts to develop and the newly formed hyphae can start a new session of the fungal life cycle (Deacon, 1997).

1.3.2.1 Genetic control of mating types in basidiomycetes

Mating genes can affect cellular morphology dramatically and have an important role in the prevention of self-mating to sustain viability of a fungal colony. Mating type loci in basidiomycetes contain similar genes and compatible mates generally have different allelic variants of these genes. *Ustilago maydis*, a maize pathogen, exhibits yeast-like vegetative growth mode. At a certain point, cells start to produce pheromones that bind to receptors of cells with compatible mating types. The pheromones initiate the growth of thin mating filaments. Once the filaments of cells with compatible mating types have fused (anastamosis), a hyphal growth mode is initiated. The resulting dikaryotic hyphae invade host cells which are used to accommodate the production of spores. The mating filament formation is dependent on "A" genes while dikaryotic growth depends on compatible "B" genes.

Similarly, compatible monokaryotic hyphae of *C. cinereus* need to fuse with hyphae with different alleles of "B" genes to initiate dikaryotic growth. Unlike *U. maydis*, mentioned previously, fusion of compatible hyphae is not dependent on pheromones. Pheromones are only produced after fusion of these compatible hyphae. An exchange of nuclei follows, after which the donor nuclei migrate to the hyphal tip where a clamp cell fusion creates a dikaryotic, fertile cell (Casselton & Challen, 2006). The "A" locus in *C. cinereus* comprises pairs of genes that are transcribed in opposite directions and encode specific DNA binding domains (homeodomain). Only when homeodomains of compatible mating types fuse, a subset of genes required for sexual fertility is activated. The "B" locus genes in *C. cinereus* encode mating pheromones and their 7-transmembrane domain receptors. Similar to "A" locus genes, compatible mating partners have different versions of these genes that encode pheromones that can activate each other's receptors, which in turn activates a second subset of genes required for sexual fertility (Li *et al.*, 2004). The dikaryotic mycelium can then start to develop fruit bodies when optimal environmental conditions occur (Kamada, 2002).

Within the *A. bisporus* group, various breeding styles have been observed. Both heterothallic (self-sterile) and homothallic (self-fertile) individuals are identified. The cultivated *A. bisporus* strain produces basidiophores yielding two-spores. The unifactorial "A" mating type affects compatibility. Both type "A" and "B" genes are thought to be found in the same mating-type locus. Haploid nuclei of compatible mating type are combined in each of the two spores which form a fertile heterokaryon, a growth mode defined as secondary homothallism.

Figure 1-2 illustrates the secondarily homothallic life cycle of *A. bisporus*. The majority of meiotic divisions (63%) result in basidia carrying two spores. Each spore contains two nuclei of compatible mating types and is therefore self-fertile (homothallic). Fungal colonies originating from these spores can develop fruit bodies when exposed to phase change stimulatory conditions. Approximately 32 % of the meiotic divisions of basidia yield binucleate spores that contain nuclei of the same (incompatible) mating type and therefore do not develop further. A small proportion of the meiotic divisions results in basidia holding 3 spores (4.5%) and 4 spores (0.5%). Monokaryotic spores resulting from these "aberrant" divisions are viable and can each produce a new fungal colony. Similarly to the wild type of *A. bisporus*, hyphae of these colonies need to fuse with other monokaryotic hypae (of a compatible mating type) before reproductive growth can be initiated (Li *et al.*, 2004) The commercial strain of *A. bisporus* has been developed through selective breeding, aimed to increase the frequency at which binucleate (compatible) spores develop in the basidium. For this reason, the commercial strain of *A. bisporus* currently used is classified as "secondarily" homothallic.

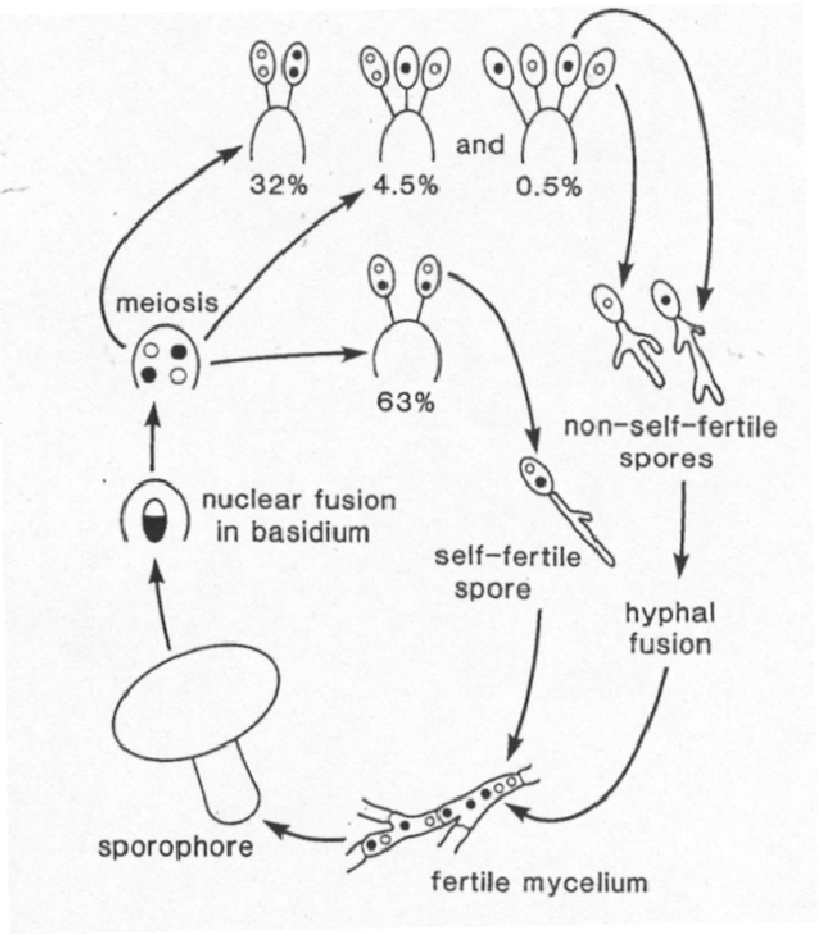

Figure 1-2 The life-cycle of *A. bisporus* illustrating the secondary homothallic growth mode. The majority of spores (63%) contain two nuclei of compatible mating type and are therefore self-fertile (Flegg *et al.*, 1985)

Li *et al.* (2004) have attempted to clone and analyse the mating-type genes of an *A. bisporus* homokaryon. While 14 different mating types have been identified from wild and commercial *A. bisporus* strains, they only found evidence of 3 alleles of this pair of homeodomains ("A" locus genes). No genes encoding pheromones and receptors ("B" locus genes) have been identified yet. This was not unexpected as *C. cinereus* "B" locus genes have a function in ensuring equal distribution of genetically different nuclei in a hyphal cell (heterokaryon), while in *A. bisporus* the cells are multinucleate. The secondarily homothallic commercial strains might have lost the "B" locus gene function (Li *et al.*, 2004). The commercial, secondarily homothallic

strains of *A. bisporus* have significant value for research into the environmental control of the reproductive phase change as the mycelium is capable of reproductive growth at any time as compatible mating type genes are present in each cell, but reproductive growth is held-back until specific environmental conditions occur.

1.3.2.2 Reproductive growth

Once specific environmental conditions occur, a phase change is triggered in *A. bisporus* and subsequently, development of various morphological changes can be observed, ultimately resulting in the development of a reproductive structure that holds the fungal spores. Harris (2009) suggested that the detection of optimal conditions for reproductive growth is coordinated by the Spitzenkörper at the tip of an extending hyphae, which serves as a signal trafficking organ. The earliest morphological sign of reproductive growth in basidiomycetes such as *A. bisporus* and *C. cinereus* is seen when mycelial cords exhibit heavy, localised branching. Localised aggregation of these newly formed branches is referred to as primary nodules or hyphal knots (Kues, 2000; Kues & Liu, 2000; Umar, M. Halit & Van Griensven, Leo J. L. D., 1997; Wood, 1976). The newly formed, short hypae in these hyphal knots often have a globose and inflated cellular morphology and are embedded in a mucilaginous material. Further growth and aggregation gradually results in the formation of a fluffy, spherical association of about 1-2 mm, commonly referred to as a secondary nodule, initial or primordium (Kues, 2000). Figure 1-3 (A) illustrates the fluffy appearance of hyphae and a developing primordium of *A. bisporus* after exposure to phase change stimulating conditions. Generally, a cluster of parallel orientated hyphae will aggregate inside the developing primordium (mainly horizontally organised hyphae) and ultimately forming the stipe. A cap region develops in the upper part of this developing primordium and contains dome-shaped features (hymenia) (Kues & Liu, 2000). In *C. cinereus*, cell differentiation occurs early in the development of the primordium and a bipolar fruiting body primordium develops which contains all tissue types of a mature fruit body as illustrated in Figure 1.3 (B).

Differentiation in an *A. bisporus* primordium is initiated only late in the development of the primordium and continues during the first stages of fruit body development. Umar and Van Griensven (1997) reported that a spherical primordium

of *A. bisporus* (~ 4 mm diameter) lacks internal differentiation. They found that this structure consists of a somatic undifferentiated hyphal mass enveloped by a universal veil. During further development of the primordium, the hyphal organization in this structure took on its final mushroom-like characteristics from whereby hyphae that will form the stipe were predominantly orientated vertically, and those that will form the cap were orientated radially. Simultaneously, a circular, "perforated" area appeared at the upper half of the primordium, which was approximately 10 mm in diameter at that stage.

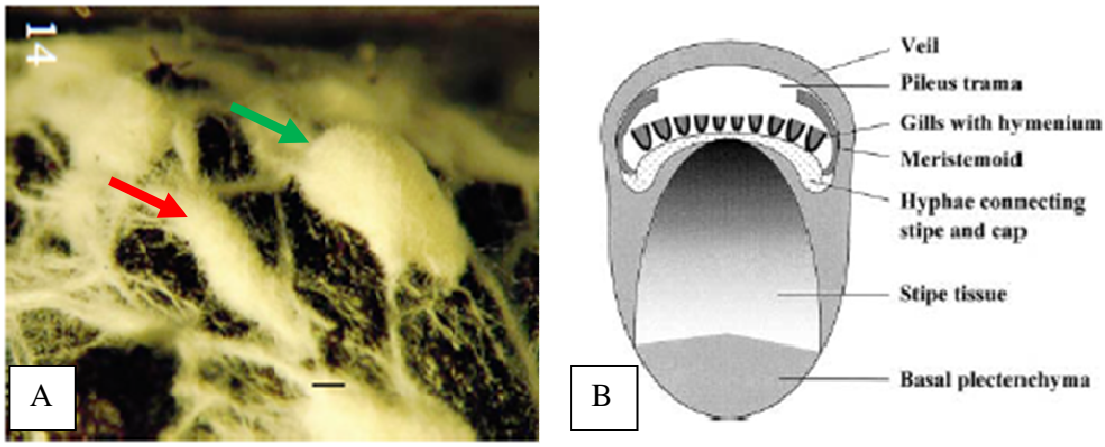

Figure 1-3 (A) First stages of reproductive growth in *A. bisporus*. Vegetative hyphae exhibit intense localised branching (red arrow) when exposed to phase change stimulating conditions. At certain locations (e.g. overlapping hyphae), aggregation of these short branches occurs and a hyphal knot is formed. The hyphal knot gradually develops into a three-dimensional spherical appearance defined as a primordium (green arrow) Scale bar = 0.3 mm (Umar, M. Halit & Van Griensven, Leo J. L. D., 1997). (B) Schematic representation of a *C. cinereus* primordium (Kues, 2000).

Umar & Van Griensven (1999) reported that an overwhelming majority (up to 95%) of the primordia observed on a the surface of a growth tray did not develop further when they were approximately 10 mm in diameter and ultimately regressed and decayed by increasing microbial colonisation. The mechanism that controls which primordia will develop further and which primordia will not develop further has not been identified yet. Gill tissue differentiated in stage 2 fruit bodies as defined by Hammond & Nichols (1976). Craig & Gull (1977) reported that cells in the basal part of the stipe expand gradually up to 100 µm followed by a more rapid expansion

of the cells in the mid and top part of the stipe. Craig & Gull (1977) suggested that the initial expansion has a significant role in pushing the fruit body above ground level while second elongation step, potentially driven by a hormone produced in the gill tissue, raises the cap further from the surface so that the spores can be optimally dispersed.

In contrast to early primordium development, maturation of the fruit body of *A. bisporus* is well documented. Hammond & Nichols (1976) have defined seven stages of fruit body development, starting at the pin (primordium) stage. These stages are illustrated in Figure 1-4. A mature fruiting body comprises a base which is connected to one or more mycelial strands, a stipe and a pileus (cap) which contains the gills (lamellae).

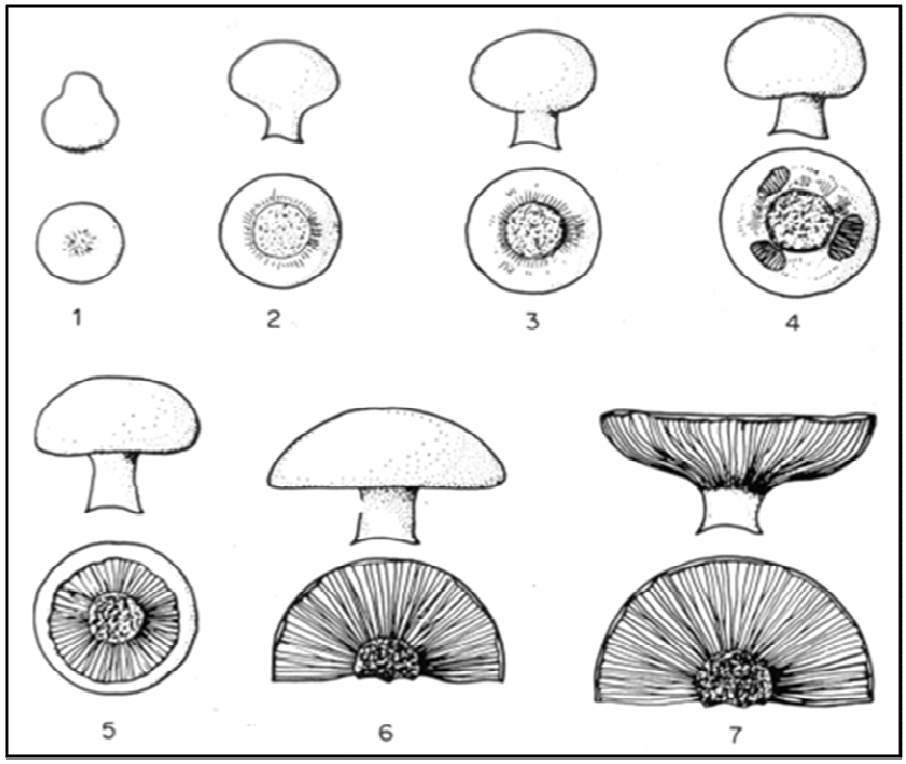

Figure 1-4 Developmental stages of *A. bisporus* fruit body development as defined by Hammond & Nichols (1976)

Research by Hammond (1985) and Hammond & Nichols (1976) into the biochemistry of the reproductive phase change identified clear differences in the carbohydrate metabolism during this process. Trehalose, glycogen and glucose-6-phosphate dehydrogenase (G6PD) activity levels were found to peak over a period of

one day or less during the phase change. This induces an increased pentose phosphate pathway flux and NADPH production, which provides the target substrate for mannitol dehydrogenase. As a consequence, mannitol levels increase significantly in a developing fruit body (up to 50 % of dry weight) compared to vegetative mycelium (1.5- 4.5 % of dry weight). This creates an osmotic difference in the fruit body resulting in the in-flow of water which drives the expansion of the stipe and cap. A similar but less dramatic increase is seen for urea levels due to increased arginase and reduced urease activity during fruit body development (Ewaze *et al.*, 1978). The reason for the increase in urea is not yet understood but it could enhance the osmotic gradient created by the mannitol accumulation. Elongation of the stipe of *C. cinereus* requires active chitin synthase. Similarly, newly synthesised chitin is incorporated in the expanding cell wall during stipe elongation in *A. bisporus* (Craig & Gull, 1977; Flegg *et al.*, 1985; Kues, 2000). Chitin synthase gene (*chs1*) transcripts were found to be higher in developing fruit bodies of *A. bisporus* (Sreenivasaprasad *et al.*, 2000).

1.4 ***Exogenous influences of mushroom development***

Studies into the controls of the phase change from vegetative to reproductive growth revealed that fungi have evolved to use a specific set of exo- and endogenous parameters to assess the optimal position and conditions for the development of reproductive structures. The cause for this variation is most likely to be found in the differences in the habitats in which these fungi are found. Previously identified parameters include the nature of the substrate, light, temperature and composition of the gaseous headspace. It is well recognised that the commercial production of *A. bisporus* requires a reduction in temperature and modification of the gaseous headspace to initiate the reproductive phase change. Figure 1-5 gives a schematic representation of our current knowledge of the environmental controls of the reproductive phase change of *A. bisporus* and key developmental stages.

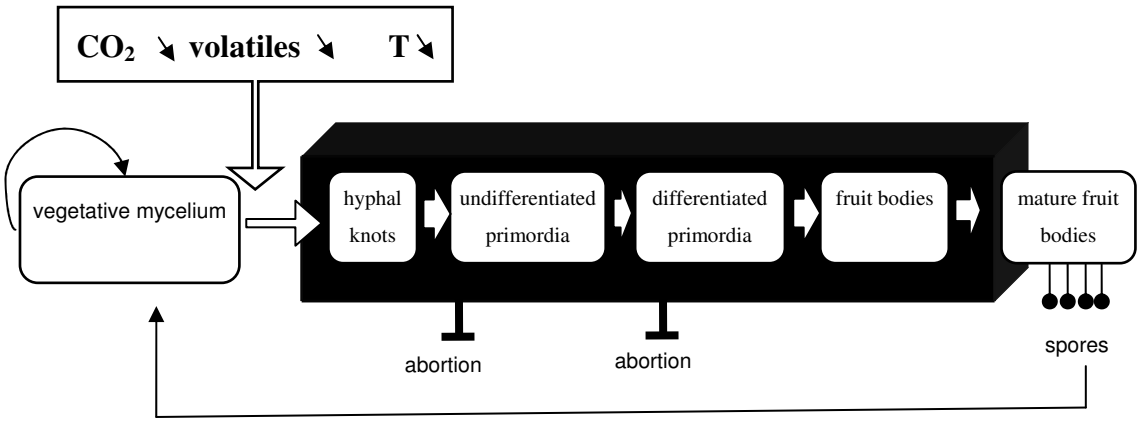

Figure 1-5 Schematic representation of the phase change in *A. bisporus* from vegetative mycelium to fruit bodies. Little is known of the molecular mechanism which controls and supports this process, hence the representation as a "black box". Commercial cultivation of *A. bisporus* requires a reduction in temperature (T) and carbon dioxide is required to initiate the phase change. Inhibotory mushroom volatiles are thought to be metabolised by bacteria present in the casing layer (e.g. *Pseudomonas putida*).

Little is known of the molecular mechanism which controls the phase change from vegetative to reproductive growth in response to a change in environmental conditions in *A. bisporus,* hence the representation of this process as a "black box". The next paragraphs will give a review on the effect of temperature (section 1.4.1), carbon dioxide (section 1.4.2.1), mushroom volatiles (section 1.4.2.2) and light (section 1.4.3) on basidiomycete fruiting.

1.4.1 Temperature

The average temperature of a habitat will affect the composition of the fungal population. The majority of fungi grow optimally between 10-35 °C. In contrast to bacteria, few fungi can survive at temperatures above 37 °C while 62-65 °C is the upper limit at which living fungi have been isolated. These thermophilic fungi are mainly (human) parasites or live inside compost heaps, bird nests or sun-heated soils, for example, *Aspergillus fumigatus* grows well under mesophilic and thermophilic temperatures and it can utilise compost, moulding grain, hydrocarbons and human lungs or transplantation tissue illustrating once again versatility of fungal organisms (Deacon, 1997). The fruit bodies from *Flammulina velutipes* are frost-tolerant and can survive repeated freezing and thawing (Margesin *et al.*, 2007).

Similarly, a change in temperature or a specific temperature regime can induce a change in growth mode. The human parasite *Histoplasma* has a hyphal growth while colonising bird or bat droppings (between 25-30 °C) and an infectious yeast like growth at 37 °C while infecting human lung tissue after inhalation of spores (Deacon, 1997). Commercial strains of *F. velutipes* require an initial growing period at 15 °C (30 days) in the dark while fruit body maturation required growth at 8 °C (20 days) under continuous light (Yamada *et al.*, 2006). Flegg (1979) found that continued exposure of *A. bisporus* mycelium to temperatures above 22-24 °C (in ventilated growth rooms) interfered with the phase change as primordia developed but no fruit body maturation occurred. A reduction of temperature for 2-3 days to 16 °C, in combination with a change in the composition of the gaseous headspace which will be discussed in section 1.4.2 was required to complete the maturation of the fruit bodies.

These examples show that temperature is an exogenous factor that has a distinct effect on fungal growth and morphogenesis. The mechanism used by fungi to sense and respond to specific temperature levels or regimes during phase change has yet to be identified. Gene expression of *F. velutipes* during the reproductive phase change identified a number of differentially expressed genes such as hydrophobins and Cytochrome P450 but none could be linked to a (putative) temperature-controlled pathway (Yamada *et al.*, 2006). Gange *et al.* (2007) have reviewed the rapid changes in fungal fruiting patterns in response to changes in the climate. They concluded that changes (delays) in fungal fruiting mirror the changes in British temperatures that have occurred since 1975. A significant part (59%, 47) of the monitored mycorrhizal fungi found in deciduous woods showed a delay in fruiting, in contrast to mycorrhizal fungi found in coniferous woods where no delay in fruiting was observed. Climate warming also appears to initiate fruiting in numerous species in spring as well as in autumn. As fruiting requires active mycelium growth, this can imply that the mycelium of these species must also be active in late winter and early spring as well as late summer and autumn, which will in turn cause increase decay rates in forests. Similar changes were observed in Norway by Kauserud *et al*. (2008).

1.4.2 The gaseous headspace

The complex mixture of gases and volatile metabolites comprising the atmosphere around fungi has a direct impact on the growth and development.

1.4.2.1 Carbon dioxide

Pathogenic fungi such as *Candida albicans* depend on changes in the gaseous headspace in order to detect their host. Increases of CO_2 levels produced by the host result in a change from yeast-like growth to virulent hyphal growth (Bahn *et al.*, 2007; Mock *et al.*, 1990). Elevated CO_2 levels can inhibit sexual reproduction in *Cryptococcus neoformans* by altering the pheromone metabolism (Granger *et al.*, 1985). The adenylyl cyclase/cyclic AMP, carbonic anhydrase and mitogen-activated protein kinase cascade signalling pathway activity is influenced by the carbon dioxide/ bicarbonate balance in various fungi. The mechanism involved with sensing and transporting of CO_2 in cells and the targets of the protein kinase are not yet understood (Bahn *et al.*, 2007).

Decomposing organic matter generally has a higher CO_2/O_2 balance than the atmospheric composition. This is an advantage for fungi as hyphae are relatively CO_2 tolerant and increasing CO_2 concentrations up to 100,000 p.p.m of the gaseous headspace has a stimulatory effect on growth rates (Lipson *et al.*, 2005). This is in contrast to the effect seen in bacteria where increasing CO_2 levels does not influence growth rate or affect it negatively, which presumably gives fungi an advantage in the colonisation of these substrates (Lipson *et al.*, 2005). Higher levels of this gas (100,000- 200,000 p.p.m), however, have a growth inhibitory effect. This number is approximate as other factors such as temperature, nutrient status and growth phase can affect the level at which this gas becomes inhibitory (Hutchinson, 1973).

Flegg *et al*. (1985) reported that the different growth modes of *A. bisporus* respond differently to specific CO_2 levels. The vegetative mycelium shows little to no growth in the absence of the gas while growth is optimally stimulated by levels up to 6,600 p.p.m. Initiation of the reproductive phase change is however more sensitive to CO_2 as concentrations over 2,000 p.p.m. are inhibitory to this process in *A. bisporus*. Tschierpe (1959) reported that developing fruit bodies exposed to CO_2 concentrations between 3,000- 5,000 p.p.m. result in an abnormal elongation of the

stipes. The molecular mechanism involved with CO_2 sensing and response has not been described in *A. bisporus*.

1.4.2.2 Mushroom volatiles

Research into fungal volatiles is still ongoing but it has been established that these molecules and the by-products of their synthesis have an effect on the growth metabolism in many fungi (Nemcovic *et al.*, 2008). Nemcovic *et al*. (2008) reported that volatiles produced by *Trichoderma* spp. (1-octen-3-ol, 3-octanol and 3-octane) are individually capable of inducing conidiation in these fungi. Of these volatiles, 1-octen-3-ol has also been shown to be a self-inhibitor and inducer of microconidiation in *Penicillium paneum* (Chitarra *et al.*, 2004).

The volatiles and other molecules originating from the breakdown of fatty acids in *A. bisporus* are known to influence development, however, the mechanism involved with their production and detection is poorly documented. Combet *et al*. (2006; 2009) found that linoleic acid, stearic acid and palmitic acid were the major constituents of the fatty acid fraction in *A. bisporus*. The ketone 3-octanone was the most abundant eight-carbon volatile (ECV) in non-homogenised samples while 1-ocent-3-ol was the most abundant volatile in homogenised (damaged) samples. Damaging mushrooms also resulted in increased ECV production. The pathways involved in the formation of these ECV have yet to be confirmed but Chen and Wu (1984) suggested conversion of 1-octen-3-one to 1-octen-3-ol and 3-octanone by a reductase as a possible pathway. It has yet to be established if 3-octanone is the product of 1-octen-3-ol conversion, or its precursor (Combet *et al.*, 2009). The effects of the non-volatile components of this linoleic acid breakdown pathway might also play an important role in fungal development. One of these "by-products" of the production of the ECVs in *A. bisporus,* 10-oxo-trans-8-decenoic acid is thought to have growth regulating and anti-fungal properties (Combet *et al.*, 2006; Mau *et al.*, 1992; Okull *et al.*, 2003). This suggests that the accumulation and breakdown of fatty acids such as linoleic acid forms a complex regulatory network for fungal development but the details of this mechanism have not been clearly identified.

As described previously, a casing layer is applied on the surface of the compost to stimulate mycelial cord formation. Hayes *et al.* (1969) found that *Pseudomonas putida* is the most prominent bacteria in the casing colonised with *A. bisporus*

mycelium. Noble *et al.* (2003) reported that no fruit bodies developed on axenic casing material. Activated charcoal allowed fruit body development on axenic casing material, suggesting that *P. putida* may reduce the inhibiting effect of the ECV produced by *A. bisporus* on the initiation of the phase change. The function of *P. putida* in the stimulation of the phase change by reducing levels of volatiles is currently under discussion based on work performed as part of a larger project including this PhD programme and reported by Noble *et al.* (2009). It has been shown that *Pseudomonas* isolates tested in axenic casing were less efficient in stimulating fruit body development compared to naturally occurring microbiota or absorbents such as activated charcoal. The inhibitory effect of the mushroom volatiles could also be eliminated by ventilation which reduces their levels, as shown during this project.

1.4.3 Light

Even though photomorphogenesis has been observed in many fungi such as *C. cinereus* (Kues, 2000) and *F. velutipes* (Yamada *et al.*, 2006) and *Pleurotus ostreatus* (Danay *et al.*, 1998), it does not appear to affect growth and fruit body development in *A. bisporus* (Couvy, 1973). Kues (2000) reported that NADP-linked glutamate dehyrdogenase and chitinase production and activity in the cap of *C. cinereus* are dependent on a specific light regime which initiates maturation of fruitbody primordia. Similarly, commercial strains of *F. velutipes* require an initial growing period of 30 days in the dark followed by 20 days at 8°C under continuous light exposure to initiate the phase change (Yamada *et al.*, 2006).

1.5 *Gene expression analysis*

Changes in gene expression of thousands of genes between two or more samples such as a time-course representing development can be investigated with a high degree of accuracy due to the maturation of specific scientific tools.

1.5.1 Gene expression analysis technologies

A selection of gene expression analysis tools will be presented here. The main difference between these tools is the number of genes that can be analysed

simultaneously, availability of genomic information and dynamic range of the difference in gene expression.

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1.5.1.1 Direct cDNA sequencing and whole, single molecule transcriptome sequencing

Direct cDNA sequencing was the first "digital" tool to measure gene expression. This process involved the creation of a cDNA library and sequencing of the individual cDNA fragments. The number of times the sequence of a specific transcript is generated provided a "rough" indication of the level of expression of that gene transcript. More recently, complete genome and transcriptome sequencing technologies have been presented. The single molecule "Digital Gene Expression" platform by Helicos Biosciences (Lipson *et al.*, 2009) and other next generation sequencing platforms are currently undergoing validation. Their main advantage to direct cDNA sequencing is that no cDNA library has to be prepared as thousands of transcript fragments are being sequenced simultaneously. Expressed sequence tags (ESTs) were generated to study development in *A. bisporus* (Ospina-Giraldo *et al.*, 2000) as described in section 4.2.1 and *Pleurotus ostreatus* (Lee *et al.*, 2002).

1.5.1.2 Differential screening and suppression subtractive hybridisation (SSH)

These two applications require the construction of a representative library containing millions of clones, similar to the cDNA library required for direct cDNA sequencing but differ from cDNA sequencing as they allow the identification of expressed sequence tags[1] (EST's) that are differentially expressed between two cDNA samples. An advantage of this approach is that it directly leads to the identification and sequencing of genes that are differentially expressed. . These applications were used previously to study development in *A. bisporus* (Browning *et al.*, 2004; De Groot *et al.*, 1997), *F. velutipes* (Ando *et al.*, 2001; Masato *et al.*, 2006), *L. edodes* (Leung *et al.*, 2002) and *P. ostreatus* (Sunagawa & Magae, 2005).

[1] An Expressed Sequence Tag is a short sub-sequence from an expressed cDNA and is created by short, single-strand sequencing of the parent cDNA.

1.5.1.3 Serial analysis of gene expression (SAGE) and massively parallel signature sequencing (MPSS)

Both technologies require the insertion of short and unique fragments of cDNA molecules into a vector. For SAGE, the fragments are ligated to a unique tag prior to insertion in the vector while for MPSS, each fragment is inserted in a vector containing a unique 17 nt tag. The number of times a specific tag is detected is an indication for gene expression levels. The MPSS vectors containing the sequence fragments are amplified, isolated, linearised and bound to microbeads carrying sequences complementary to the tags. These beads are than scanned to quantify the number of bound fragments to allow quantification and a fragment from each bead is then sequenced. These technologies can detect single nucleotide differences but are not high-throughput, compared to upcoming next generation sequencing platforms (Patino *et al.*, 2002).

1.5.1.4 Macro- and microarray technology

Macroarrays are nylon membranes on which denaturated double-stranded DNA fragments are bound. Expression levels of a sample is measured by estimating the amount of ^{33}P labelled cDNA fragments bound to complement sequences on the macroarray. Modifications of this system include the use of non-radioactive single or dual colour detection systems in which gene expression levels are represented by colour intensities generated by colourimetric reactions. The resolution of the macroarrays is significantly lower than current microarray-platforms, but does not require any previous knowledge of the transcripts to be sequenced.

Microarrays are generally glass slides that hold single-stranded DNA fragments on their surface. cDNA or cRNA samples are fluorescently labelled and hybridised to the arrays. A microarray scanner is then used to acquire the fluorescence levels at specific positions of each slide. These values are then converted to expression data. In contrast to next generation sequencing, microarray technology has matured and can be used to simultaneously screen the expression levels of more than 2 million genes from multiple different samples. The reliability and significance of well designed microarray experiments has resulted in the adaptation of the technology by various medical institutions involved with the diagnosis of genetic diseases (Ogilvie *et al.*, 2009). Depending on the production method and available sequence information, the complementary strand of whole

transcripts (cDNA) or transcript fragments (oligonucleotide) are bound on a substrate, generally glass or silica or are synthesised *in-situ* (oligonucleotide). *In-situ* synthesis of oligonucleotides is commonly achieved using photolithographic masks or ink-jet printer head deposition. Ink-jet technology has higher yields per stage so that longer oligonucleotides and higher stringency hybridisations are achieved, compared to microarrays created using photolithographic masks (Hughes *et al.*, 2001).

1.5.2 Gene expression studies into fungal developmental biology

Various fungal gene expression studies have been performed previously, using technologies mentioned in section 1.5.1. Hirano *et al.* (2004) have identified various genes that are uniquely expressed in the fruit bodies of the basidiomycete *Lentinula edodes.* They compared cDNA derived from the gill tissue of the fruit body with cDNA representing mycelia by differential screening and found 6 genes uniquely expressed in the fruit body. Two genes were unknown but the other 4 genes showed significant similarity to known proteins. These were: 1) cytochrome P450, 2) riboflavin aldehyde-forming enzyme, 3) cyc07 and 4) histone H3. This was complemented by the work of Chum *et al.* (2008) who performed SAGE on dikaryotic mycelium and primordia of *L. edodes*. They identified various genes that were differentially expressed between these two developmental stages: two hydrophobins, each specific to mycelium or primordia, an acyl carrier protein coding gene, a metallothionein, riboflavin aldehyde forming enzyme, the priA gene, DNA-binding transcription factor which has a zinc-binding motif, various ribosomal proteins. Other research into the molecular mechanisms involved with vegetative and or reproductive development in this fungus further identified a lectin that was highly expressed only in the mycelium (Oguri *et al.*, 1996), a gene encoding for a DNA-binding protein (Endo *et al.*, 1994) and two laccasse genes (Zhao & Kwan, 1999).

Masato *et al.* (2006) performed fluorescence differential display analysis on mycelial, primordial and fruit body samples of the basidiomycete *F. velutipes*. They identified various genes that were differentially expressed between these stages including G-protein species, adenosine deanimase growth factor, an ubiquitin-binding protein, ubiquitin ligase, Cytochrome P450 and a hydrophobin.

Nowrousian and Kuck (2006) performed comparative gene expression analysis of fruiting body development in two filamentous, homothallic and fruit body forming fungi: the ascomycetes *Pyronema confluens* and *Sordaria macrospora*. Similar expression patterns were observed in both fungi in relation to reproductive development. Results from gene expression analysis of vegetative growth and reproductive growth yielded 58 and 61 unique genes respectively. Of the unique genes found in the vegetative samples, only 2 % appear to be involved in DNA synthesis, cell signalling and communication while this increased up to 10% in the reproductive growth samples. Known genes, upregulated in both *P. confluens* and *S. macrospora* during reproductive development are: β-1,3-exoglucancase, a putative transporter protein, and a COP9 signalosome.

Kamada (2002) found that *ich1* prevents differentiation of the cap in *Coprinus cinereus* and a recessive mutation of this gene also interferes with basidiospore development. Dominant mutations of *eln2* resulted in primordia with short stipes. This *eln2* gene is part of the Cytochrome P450 family.

Various European and American research groups have used a range of approaches such as differential screening (Browning *et al.*, 2004; De Groot *et al.*, 1997), ESTs (Ospina-Giraldo *et al.*, 2000), and SSH (Dr. Daniel Eastwood, Warwick HRI, unpublished data) to investigate the growth and developmental processes in *A. bisporus*. The results from these investigations will be further considered in relation to the results of this project.

1.6 *Aims and objectives*

Previous research has established that fungi can detect changes in their environment and use these changes to guide their development. This is also valid for the phase change from vegetative to reproductive growth. In addition to the effect of external stimuli, fungi also produce (volatile) molecules that can stimulate or inhibit their own development. While the external conditions required to stimulate this phase change have been well established, our understanding of the molecular mechanisms involved with sensing the changes in the environment and the subsequent responses is still very basic.

The leaf litter degrading fungus *A. bisporus* is secondarily homothallic which means that the mycelium is capable of meiotic sexual growth without the need of anastamosis of hyphae of compatible mating types. This implies that the phase change is suppressed or initiated when specific environmental triggers occur. Research performed by the mushroom industry identified the optimal environmental conditions required for reproductive growth, in a well controlled environment.

This makes this fungus a valuable candidate for investigating the molecular mechanisms underlying the effects of specific environmental conditions (temperature, carbon dioxide and mushroom volatiles) in stimulating the phase change from vegetative to reproductive growth. The availability of microarray technology makes it possible to study the effects of each of these endogenous and exogenous factors on the expression level of thousands of genes during fungal development.

This project was aimed at understanding the process of the phase change from vegetative to reproductive growth of higher fungi such as *A. bisporus* and to investigate the regulation of this process by temperature, CO_2 and mushroom volatiles. In order to achieve this I have:

- developed a range of molecular and imaging methodologies and resources required to carry out the investigations;
- identified key stages of the reproductive phase change and investigated the combined and individual effect of changes of temperature, carbon dioxide and mushroom volatiles levels on this process;
- investigated the gene expression profile of *A. bisporus* grown under commercial and experimental growth conditions in order to get a better understanding of the molecular mechanism controlling and supporting the phase change process.

Chapter 2. General material and methods

2.1 *Biological material*

Agaricus bisporus Sylvan A15 (Sylvan Spawn Limited, Peterborough, Cambridgeshire, UK) was used for all experiments during this project. Compost was prepared according to commercial practice at Warwick HRI. Casing material (85% peat and 15% sugar beet lime) is supplied commercially by Tunnel Tech Ltd.

2.2 **A. bisporus** *growth experiments*

2.2.1 Commercial mushroom cultivation conditions

2.2.1.1 Phase I compost

Compost used for mushroom cultivation is generally a mixture of chopped straw, manure and gypsum. This mixture is placed in long stacks, watered thoroughly and then mixed frequently. Gypsum is used as a method to control the pH of the casing which tends to be acidic. This compost mixture is stacked in rows, turned with a compost machine and wetted to achieve 70-75% moisture content. Higher moisture levels will create anaerobic conditions in the compost, allowing the metabolism of valuable nutrients by anaerobic bacteria while lower moisture contents will stimulate the development of spore-forming organisms and slow down the breakdown of the compost. Microbial oxidation of organic compounds during the composting process results in temperature rises up to 65 °C, favourable for the development of thermophilic organisms. The activity of these increases the compost core temperature up to 80°C while mesophillic bacteria develop in the colder borders. The most readily available compounds (soluble carbohydrates) are utilised first and converted into CO_2 or microbial biomass. The majority of nitrogen and mineral elements are allocated to biomass and extracellular enzyme production although some are volatised as ammonia. This first phase takes approximately 10-14 days during which the compost is mixed frequently in order to get uniform compost decomposition.

2.2.1.2 Phase II compost

The phase I compost is transferred to a tunnel for peak heating. Firstly, the compost is heated to 57-60 °C for 6-8 hours using steam to destroy pathogens and pests which survived the first step of the composting process. Although the pasteurised compost is free of pathogens and pests, it must be conditioned further to reduce ammonia levels. The temperature is therefore dropped to 45-48 °C for 6-10 days so that thermophilic organisms can make the substrate usable and selective for mushroom growth by converting the remaining ammonia and nitrates into proteins. It is important that the temperature is not above 60 °C for excessively long periods as this would slow down the dissipation of ammonia and might destroy microbes, releasing their cellular nutrients, which promotes the growth of other organisms that compete with *A. bisporus*. Therefore the selectivity of compost is dependent on an intact but dormant biomass while being colonised by *A. bisporus*.

2.2.1.3 Phase III compost

Phase II compost is now ready for inoculation with mushroom spawn. This is typically rye grains that have been pre-wetted (40% water content), mixed with gypsum and powdered chalk which have been autoclaved and inoculated and colonised with mycelium. The compost is inoculated with the grain spawn on a 1-2 % weight for weight basis and kept in a controlled environment room at 25 °C for 14-21 days during which mycelia can be seen colonising the compost.

2.2.1.4 Casing of colonised mushroom compost

A casing layer of approximately 55 mm is then placed on top of the inoculated compost in order to prevent water loss and to create a suitable substrate for fruit body development. The casing material is a mixture of a water retaining component (e.g. peat, soil or clay), a pH buffering component (chalk, lime or sugar beet lime) and water. The cased trays are then placed at 25 °C and 95% humidity for approximately 1 week until the mycelium is visible on the top of the casing layer.

2.2.2 **A. bisporus** ***grown under standard and experimental growth conditions in mushroom growth trays***

Vegetative mycelium of *A. bisporus* was inoculated in phase two compost as described in section 2.2.1.3 (phase III compost) and placed in mushroom trays (diameter 0.91x 0.61x 0.18 m, 50 kg inoculated compost per tray). The trays were then incubated for 14 days at 25 °C and 95 % humidity so that the *A. bisporus* mycelium could colonise the compost. The trays were then covered with casing to a depth of 55 mm and incubated for approximately 7 days at the same environmental conditions until the mycelium was visible on top of the casing layer. The mushroom growth trays were then divided over 3 experiments. The start point of the experiments was identical and defined as "airing" (0 h).

2.2.2.1 Standard growth conditions

For this experiment, *A. bisporus* was grown under standard, commercial cultivation conditions designed to optimally stimulate the phase change. When mycelium was observed on the surface of the casing layer, the temperature was reduced from 25 °C to 18 °C and the growth room was ventilated to reduce levels of CO_2 and mushroom volatiles. These changes were applied simultaneously. Carbon dioxide levels were monitored daily using a CO_2 controller unit (Fuji Electric) to ensure that they were below 1,000 p.p.m.

2.2.2.2 High temperature but reduced levels of CO_2 and mushroom volatiles

For this experiment, temperature was maintained at 25°C while the gaseous headspace was altered by ventilating the mushroom growth rooms to reduce levels of CO_2 and mushroom volatiles. Carbon dioxide levels were monitored daily using a CO_2 controller unit (Fuji Electric) to ensure that they were below 1,000 p.p.m.

2.2.2.3 High levels of CO_2 and mushroom volatiles but reduced temperature

For this experiment, the mushroom growth rooms were not ventilated and the growth trays were covered with paper sheets while the temperature was reduced to 18 °C. This resulted in increasing levels of CO_2 and mushroom volatiles. Carbon dioxide levels were monitored daily using a CO_2 controller unit (Fuji Electric) to ensure that they did not drop below 3,000 p.p.m.

2.2.2.4 Collection and storage of fungal samples

Approximately 250 g colonised casing was collected in triplicate from randomly selected growth trays at airing (0 h), 24 h, 72 h and 168 h after airing for each of the experiments described in section 2.2.2. In addition to this, 12 g smooth undifferentiated primordia, 12 g elongated differentiating primordia, five stage 2 and five stage 4 fruit bodies were also collected from mushroom trays during the standard cultivation conditions experiment, when first observed. All samples were frozen immediately under liquid nitrogen. The casing samples were then freeze-dried (Edwards Modulyo 4K, Crawley, UK) until completely dry and stored at -70 °C prior to total RNA extraction as described in section 2.3.

2.2.3 **A. bisporus** ***grown under standard and experimental growth conditions in a flask-based system with a controlled gas supply***

Noble *et al.* (2009) designed a flask-based growth system that can be used to investigate the effects of individual components of the gaseous headspace (e.g. CO_2 or mushroom volatiles) on the phase change. Eight 500 ml glass microcosms (Kilner jars) were each filled with 100 g standard phase III compost (section 2.2.1.3) which was inoculated with *A. bisporus* spawn. The compost was then covered with 80 g standard casing material (section 2.2.1.4). These Kilner jars were then enclosed in a 20 L multi-adapter flask as illustrated in Figure 2-1. The flasks were sealed with bacterial air vents and incubated at 25 °C without ventilation until mycelium had colonised the casing layer of each jar. An air-supply (24 L h^{-1}) can be connected to this system to control the gaseous headspace composition. This growth system is capable of reducing mushroom volatiles below phase change inhibitory levels, even under axenic growth conditions. This system was used for four experiments whereby *A. bisporus* was grown under specific experimental conditions. These experiments were designed to investigate the effect of various levels of CO_2 and mushroom volatiles on reproductive development. The start point of these experiments was defined as “airing” (t= 0 hours).

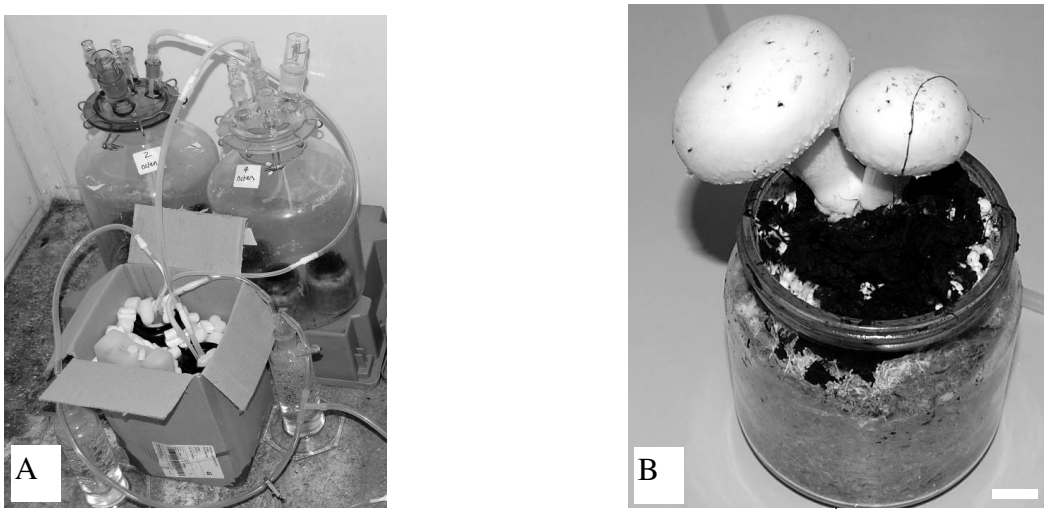


Figure 2-1 Set-up of the (A) flask-based growth system with controlled air-supply used to investigate the effect of individual components of the gaseous headspace such as CO_2 and 1-octen-3-ol on the phase change. (B) Fruit bodies developed normally under standard growth conditions. Scale bar = 10 mm.

2.2.3.1 Standard growth conditions

For this experiment, flasks were prepared as described in section 2.2.3 and incubated at 18°C while a pump with metered flow rate (24 L h^{-1}) was used to ventilate the flasks with atmospheric air to reduce levels of CO_2 (between 600- 1,000 p.p.m) and mushroom volatiles. The air was passed through sealed 300 ml flasks containing water to humidify the air. This flow rate ensured that the air in the flask was changed completely every 54 minutes. Carbon dioxide levels were monitored daily using a handheld GM11A CO_2 meter (Vaisala).

2.2.3.2 High level of 1-octen-3-ol but reduced level of CO_2 and reduced temperature

For this experiment, flasks were prepared as described in section 2.2.3 and incubated at 18 °C while a pump with metered flow rate was used to ventilate the flasks, similar to standard growth conditions (section 2.2.3.1). The air-supply was first passed through sealed 300 ml flasks containing water to humidify the air. The air was then passed through a sealed 300 ml flask containing 3-5 ml 1-octen-3-ol. The level of volatile was replenished at 4-5 d intervals and the flask containing the mushroom volatile was placed in ice to reduce volatilization. Approximate 2.1 ml 1-

octen-3-ol L-1 air (24 L h-1) was used during this experiment, based on the volume of air passing through the system per hour (measured using a flow meter) and the hourly 1-octen-3-ol volume decrease due to evaporation. The 300 ml flask containing the volatile was removed from the air-supply at 192 h after airing and the experiment was continued as described in section 2.2.3.3

2.2.3.3 Reduction of the level of 1-octen-3-ol by removing the 1-octen-3-ol solution

The volatile source was removed from the experiment described in section 2.2.3.2 at 192 h after airing to investigate the reversibility of the effects caused by high levels of 1-octen-3-ol.

2.2.3.4 High level of CO_2 but reduced level of mushroom volatiles and reduced temperature

For this experiment, flasks were prepared as described in section 2.2.3 and held at 18 °C and connected to a filtered air-supply as described for the control experiment in section 2.2.3.1. A gaseous CO_2 supply line was added to the air-supply in a mixer flask to create CO_2 levels of ~ 5,000 p.p.m. CO_2 levels were monitored using a handheld GM11A CO_2 meter (Vaisala).

2.2.3.5 Collection and storage of fungal samples

Approximately 80 g colonised casing material was collected from three randomly selected Kilner jars at airing (0 h), 24 h, 72 h and 192 h after airing for each of the experiments described in section 2.2.3. All samples were frozen immediately under liquid nitrogen. The casing samples were then freeze-dried (Edwards Modulyo 4K, Crawley, UK) until completely dry and stored at -70 °C prior to total RNA extraction as described in section 2.3

2.2.4 Imaging of **A. bisporus** *development*

An Olympus SZX7 stereo microscope was used in combination with an Olympus CAMEDIA C-5050 digital camera to document changes in mycelial morphology during growth in compost and casing substrate in 24-hour intervals until smooth undifferentiated primordia developed. A time-lapse photographic record was made of each standard growth conditions experiment using a Nikon D40 digital SLR

camera in combination with a micro-Nikkor 105 mm 1:2.8 lens to document changes in mushroom initiation and development. Nikon Camera Control PRO 2 software was used to control the Nikon D40 camera during the time-lapse experiments.

2.3 *Total RNA from fungal samples: extraction and analysis*

During this project, total RNA was extracted from a variety of mushroom developmental stages. Different methods were used to extract RNA from fruit body tissue and colonised casing samples. Extracted RNA was quantified and analyzed to confirm the integrity before it was processed to create fluorescently labelled cRNA, used for microarray hybridisation experiments.

2.3.1 Total RNA extraction from soil-free primordia and fruit bodies

A phenol:chloroform-based method was used to extract total RNA from frozen and/or freeze-dried primordia and fruit bodies (stage 1-7). Prior to the extraction, all glassware and relevant plastic-ware were DEPC treated by placing them in a 0.1% (v/v) DEPC (Sigma) Milli-Q ultrapure water solution and incubated overnight at room temperature. The equipment was then autoclaved to disassociate DEPC into ethanol and CO_2.Throughout the protocol, samples containing RNA were kept either on ice or at 65 °C to reduce degradation caused by RNases. 1 volume of extraction buffer (200 mM Tris, 25 mM NaCl, 25 mM EDTA and 0.5% SDS, pH 8.5) and 1 volume of acid phenol:chloroform pH 4.5 with isoamylalcohol (125:24:1) (Ambion) were placed in two separate plastic tubes with caps, in a 65°C water bath. 3 volumes of each buffer are required for 1 volume of sample. Frozen fruit body tissue was placed in a mortar containing liquid nitrogen and it was ground with a pestle until finely powdered. Without allowing the sample to thaw, approximately 5 g of the ground tissue was transferred to a 30 ml centrifuge tube containing extraction buffer. This centrifuge tube was mixed rigorously and the cap loosened frequently to prevent pressure build-up. The phenol:chloroform mixture was then transferred to this tube, which was mixed again, and placed for 15 minutes at 65 °C. The solution was mixed on a vortex mixer and subsequently placed on ice, mixed with sodium chloride, to promote rapid cooling. The tube was centrifuged at 15,000 r.p.m. (Beckman L5-65 Ultra Centrifuge) for 35 minutes at 4°C and transferred back to ice. The top layer was removed and transferred to a new chilled tube containing an

equal volume of chilled 24:1 chloroform:isoamyl-alcohol solution. The sample was centrifuged at 15,000 r.p.m. (Beckman L5-65 Ultra Centrifuge) for 35 minutes at 4 °C and transferred back to ice. The top layer was removed and transferred to a new tube, containing chilled 12 M lithium chloride in a final concentration of 2 M lithium chloride. The sample was left at 4 °C overnight to allow RNA precipitation. The following day the sample was centrifuged at 15,000 r.p.m. (Beckman L5-65 Ultra Centrifuge) for 20 minutes at 4 °C, and placed back on ice. The supernatant was discarded and the pellet was washed with 5 ml of 3 M sodium acetate pH 5.0. The sample was centrifuged at 15,000 r.p.m. (Beckman L5-65 Ultra Centrifuge) for 35 minutes, at 4 °C, and replaced immediately on ice. The supernatant was discarded and the pellet washed immediately with 20 ml of chilled ethanol and centrifuged at 15,000 r.p.m. (Beckman L5-65 Ultra Centrifuge) for 20 minutes at 4 °C. This step was performed twice and the pellet was then quickly air-dried in an incubator at 37 °C. The pellet was re-suspended in DEPC water. The RNA samples were stored at -70°C.

The RNeasy plant mini kit (Qiagen) was used to clean-up and concentrate the RNA as described by the RNA clean-up manual. Buffer RLT, provided with the kit, was used to suspend the RNA extracted by the phenol:chloroform method described above. The sample was treated with DNAse to remove any genomic DNA contamination and eluted in 30 µL DEPC water. RNA samples were stored at -70°C. This method was based on Sreenivasaprasad (2000).

2.3.2 Total RNA extraction from colonised casing samples

Extraction of total RNA from colonised peat is a complex procedure due to the presence of RNA degrading enzymes and humic acids. Humic acids are known to inhibit reverse transcriptase and polymerase activity, enzymes used in the preparation of cRNA for microarray hybridisation. During this project, various methods were tested for their ability to produce un-degraded, inhibitor free total RNA and one method was optimised to achieve this. Details of these experiments will be discussed in chapter 4, section 4.1.

Total RNA from peat colonised with *A. bisporus* used during this project was extracted using the modified FastRNA Pro Soil–direct method. The original method described in the Fast RNA pro soil-direct kit protocol

(http://www.mpbio.com/includes/technical/FastRNA%20pro%20Soil%20Direct.pdf, MP Biomedicals), was modified to increase integrity and concentration of the extracted total RNA. The modifications of this kit were as follows: 1) 10 g freeze-dried sample of peat or soil was mixed with an equal volume of dry-ice and ground in a coffee-grinder (Delonghi, UK). This mixture was transferred to a loosely closed 50 ml centrifuge tube and placed at -70 °C overnight to allow the evaporation of the dry ice (CO_2). 100 mg of ground sample was added to the provided purple-cap sample tube containing lysing matrix E. 1.4 ml of provided RNA pro Soil Lysis solution was added and the tube was vortexed to re-suspend the soil and lysing matrix. 2) The tube was then homogenised in the FastPrep (MP Biomedicals) instrument for 20 seconds at a setting of 4.0 after which the sample was immediately transferred to ice. The standard protocol was then followed, including the optional centrifugation through a quick-clean spin filter. 3) The extracted total RNA was then processed using the RNeasy MinElute cleanup kit (Qiagen) to remove residual salts and ethanol and to concentrate the total RNA as described in the manual (http://www1.qiagen.com/literature/render.aspx?id=351). A DNase treatment can be included during the RNeasy MinElute purification procedure if the sample has to be completely free of residual DNA (e.g. for quantitative RT-PCR. This step is optional for the Low Input Linear Amplification PLUS kit (Agilent Technologies) used for the production of fluorescently labelled cRNA as this is a poly-A based amplification method.

2.3.3 Qualitative and quantitative analysis of extracted total RNA

Total RNA was quantified using the Nanodrop-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington DE, USA). Total RNA concentration, 260 nm / 280 nm absorbance ratio and 260 nm / 230 nm absorbance ratio are given as output of a 1.5 µl sample to document the concentration and purity of the RNA sample. The 260 nm / 280 nm absorbance ratio was used to assess the purity of the measured sample as a ratio of ~ 2.0 indicates pure RNA. A lower ratio might indicate presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. The 260 nm /230 nm ratio is used as a secondary measure of nucleic acid purity and a ratio below 2 can indicate ethanol or salt contamination (http://www.nanodrop.com/Library/T009-NanoDrop%201000-&-NanoDrop%208000-Nucleic-Acid-Purity-Ratios.pdf).

The Bioanalyzer (Agilent Technologies) in combination with the RNA 6000 Nano kit (Agilent Technologies), standard protocol, was used to analyse the integrity of the RNA samples by comparing the large /small subunit rRNA ratio in combination with an assessment of the baseline where a flat baseline indicates absence of RNA degradation (http://www.chem.agilent.com/Library/usermanuals/Public/G2938-90035_QuickRNA6000Nano.pdf).

2.3.4 Preparation of fluorescently labelled cRNA

Approximately 300 ng (one-colour experiments) or 350 ng (two-colour experiments) of extracted total RNA was mixed with a known concentration of a spike-ins mixture (Agilent Technologies), consisting of 10 *in vitro* synthesized and poly-adenylated transcripts derived from the Adenovirus E1A transcriptome that are premixed at various ratios and will serve as a control for the labelling and hybridisation procedure as described in the One- or Two-Colour Microarray-Based Gene Expression Analysis protocol version 5.5 or 4.0 respective (Low RNA Input Linear Amplification PLUS kit, Agilent Technologies). This mixture was then processed following the standard protocol, to create cRNA that was labelled with Cyanine-3 (Cy-3), which is excited by a 532 nm laser or Cyanine-5 (Cy-5), which is excited by a 633 nm laser. Cyanine-3 is preferred for one-colour experiments as it is less prone to ozone degradation than Cyanine-5 (Branham *et al.*, 2007). The produced cRNA is then processed using the RNA plant mini kit (Qiagen), following the modified protocol described in the relevant One- or Two-Colour Microarray-Based gene expression protocol (Agilent Technologies) in order remove unincorporated nucleotides, salts, enzymes and reagents. Fluorescently labelled cRNA was analysed using the Nanodrop as described in section 2.3.3 to measure cRNA concentration, dye absorbance ratio and dye concentration. These values were used to calculate the cRNA yield and incorporation rate as described in the linear amplification PLUS kit (Agilent Technologies) manual. A cRNA yield over 0.75 µg and incorporation rate over 8.0 pmol per µg cRNA is recommended by Agilent Technologies. A copy of the One- and Two-Color Microarray-Based Gene Expression Analysis protocol can be found on the DVD at the back of this thesis.

2.4 **Agaricus bisporus** ***microarray design***

2.4.1 Collation of sequences related to **A. bisporus**

A. bisporus was not genome sequenced at the time of writing. The haploid genome of *A. bisporus*, as determined by re-association kinetics (Arthur *et al.*, 1983) and gel karyotyping (Kerrigan *et al.*, 1993; Sonnenberg *et al.*, 1996), is 34.2 Mb spread across 13 chromosomes. The *A. bisporus* GC content is 43.5% (Arthur *et al.*, 1983). Based on DNA:DNA re-association kinetics, ca. 16% of the genome is repetitive, which is consistent with the 10-20% observed in many fungi (e.g. *Neurospora, Rhizopus, Mucor spp.*) and is similar to the repetitive DNA content of *Coprinus spp*. (ca. 15%) (Dutta, 1974; Dutta *et al.*, 1972). The average gene size in fungi is between 1-2 kb as reported by Ospina-Giraldo *et al*. (2000), indicating that there should be around 8600 genes. In order to construct a microarray it was therefore necessary to collect and collate available sequences related to *A. bisporus*.

These data sources are:

1) The suppression subtractive hybridisation (SSH) project by Dr. Dan Eastwood (Warwick HRI, University of Warwick);

2) The EMBL-EBI nucleotide database. This publicly available database was queried on October 2005 for sequences related to *A. bisporus*;

3) Dr. Emilie Combet (Warwick HRI, University of Warwick) investigated genes involved in lipid metabolism and flavour biogenesis in *A. bisporus*. During this project, 254 *A. bisporus* cDNA sequences were sequenced;

4) A further non-published 95 sequences identified during a project by Dr. J. Thomas and Dr. M. Challen (Warwick HRI, University of Warwick) who investigated the interactions of *A. bisporus* and *Verticillium fungicola*.

2.4.2 Identification of unique sequences

A local nucleotide Basic Local Alignment Search Tool (BLAST) described by Altschul *et al.* (1990) was performed on the sequences in *A. bisporus* database to identify unique sequences, allelic variants and duplicate sequences. The BLAST

(blastn algorithm) results were analysed and a number of selection steps were performed to identify duplicate sequences. These steps were:

1) Duplicate sequences with stringent E-values (< 1.0 E-50) and a small amount of non-consecutive nucleotide base mismatches were merged if overlapping to generate a single sequence in order to extend the sequence length.

2) Matches with multiple consecutive mismatches (e.g. mismatches of 2 or more constitutive nucleotides) in sequences with high E-values (<1.0 E-30) were retained in the database as this number of mismatches could be caused by allelic variability

3) Matches with overlaps larger than 80 nucleotides were processed as described in step 1, while matches with shorter overlaps were retained in the database as independent sequences to avoid the merger of non-related sequences.

4) Sequences smaller than 50 nucleotides were excluded from further use. These sequences were too short to design stringent microarray oligonucleotide probes.

2.4.3 In silico *functional annotation of* A. bisporus *sequences*

The standard approach to functional annotation of sequences is to perform an *in silico* sequence comparison to previously characterised genes or proteins from related organisms. BLAST (Altschul *et al.*, 1990) was created for this purpose. Matches to the input sequences with sequences in user-specified nucleotide or protein databases are presented in an output which includes the putative functional description, public database ID and a statistical significance value of the match. A disadvantage of BLAST outputs is the need for manual vetting of the results to eliminate insignificant hits and to retrieve additional information. Depending on the characterisation of a matching sequence, protein domain information (InterPro database, http://www.ebi.ac.uk/interpro/), Gene Ontology (GO) terms (Ashburner *et al.*, 2000) or Enzyme Commission (EC) numbers (Webb, 1992) can be retrieved, providing more information on the putative function of the input sequence. The GO database comprises three vocabularies that describe gene products in terms of their associated biological processes, cellular components and molecular functions in a species- independent matter. GO terms are also structured so that they can be queried at different levels and they are a valuable tool for the classification of sequences by

(putative) function. Similarly, EC numbers are a numerical classification scheme for enzymes based on the chemical reactions they catalyze.

Recently, annotation pipelines have become available which are designed to annotate a large number of sequences in a semi-automated way. Each of these programs incorporates one or more of the previously described pieces of information, but the majority of these programs fail to present the large amount of data in a usable format.

Examples of these programs are ESTPASS (Lee *et al.*, 2007), EST explorer (Nagaraj *et al.*, 2007) and Blast2GO (Conesa *et al.*, 2005). These pipelines clean the sequences for vector contamination, cluster the sequences to remove duplicate sequences, BLAST the subject sequences to a number of (publicly) available databases and search for well characterised protein domains (InterPro). Blast2GO was selected for the *in silico* annotation of the *A. bisporus* sequences. This program is written using the JAVA computer language, which simplifies the installation and transfer of data to different computers. All databases used for the annotation procedure are remotely accessed, eliminating the need for local installation of large datasets. Blast2GO initially loads the sequences, presented in FASTA-format, into a local database. The sequences are then BLAST-compared to a remote database and the top 20 matches for each sequence, including statistical significance, length and similarity of each hit, are stored inside the database. The system then analyses the results and presents the best description, if known, for each subject sequence. The results are then processed to retrieve relevant functional information from the matching sequences: 1) BLAST result accessions are used to retrieve gene names, 2) BLAST result gene identifiers are used to retrieve UniPROT IDs from various protein databases and 3) BLAST result accessions are searched directly in the DBXRef and Gene_product table of the GO-database. Simultaneously, the sequences are assessed for known protein domains using the InterProScan function. Finally the program retrieves EC numbers based on the matching gene identifiers and all the acquired information is presented in a tab delimited text file.

2.4.4 Microarray oligonucleotide probe design

The most crucial part of microarray design is the selection of highly specific short oligonucleotide sequences (probes) representing genes of interest. The length of the probes depends on the chosen microarray platform. The Agilent Technologies custom expression platform selected for this project uses 60-mer oligonucleotide probes representing target RNAs. Agilent Technologies has provided a number of parameters to assist with probe design. These parameters are:

1) probes should be designed as a substring of the sense/coding strand and be represented in a 5' to 3' orientation and biased towards the 3' as reverse transcriptase tends to attenuate inherently as it progresses to the 5' end;

2) the GC-content and the melting temperature (Tm) should be respectively ~ 40% and ~ 80°C;

3) no base should be in excess (>60%) and homomeric runs greater than 6 nucleotides should be avoided.

Two software programs were used for probe design. The first, OLIGOPICKER (Wang & Seed, 2003), allowed the user to input probe length, Tm range, cross-reactivity (the threshold for rejection of contiguous matches) and BLAST-score (specificity of the probe in relation to other probes in the database). Multiple parameters were tested to find a balance between the amount of designed probes and the specificity of each probe, mainly influenced by the Tm and GC%. A second program, still experimental at the time, used for the design of microarray probes was OLIGOSTAR (Conesa *et al.*, 2005) which allowed the user to select the probe length, optimum Tm and GC%-range. The main advantage of OLIGOPICKER was that an optimal Tm could be specified, rather than a range so the probe melting temperature could be designed closer to the Agilent recommended parameter. OLIGOSTAR also allows the user to specify the GC%-content. In contrast to OLIGOPICKER, this program does not allow the modification of the Tm range, therefore only one set of probes, matching optimally to the parameters provided, will be presented. The number of probes designed is expected to be lower than the number of probes designed with OLIGOPICKER as the specificity cannot be

modified. Finally, the candidate probes designed by both programs were collated and analysed using local BLAST to eliminate overlapping probes.

The candidate probes were uploaded to the eARRAY platform. This online software package is provided by Agilent Technologies and is the start-point for any Agilent microarray design. At this point in the project, the eARRAY system was used to assess quality of the probes in relation to Agilent oligonucleotide probe design parameters and for the design of a custom microarray. The eARRAY platform can be found at the following location: https://earray.chem.agilent.com/earray/.

2.4.5 Microarray design

The eARRAY web-portal (Agilent technologies), as described in section 2.4.4, was used to organise (a selection of) the *A. bisporus* probes into three microarray designs.

2.4.5.1 The custom 2 X 11,000 60-mer oligonucleotide microarray design:

This "low-density" microarray slide design consisted of a microarray design which is replicated twice on the slide. This means that two one-colour or four two-colour samples can be hybridised simultaneously on one microarray slide. Each array is identical and contains the candidate *A. bisporus* gene expression probes, their complements and internal control probes. The composition of the internal control grid is proprietary but it is designed to assist with the positioning of the grid during data extraction, spatial detrending and quality control on the labelling and microarray procedure (using randomly distributed spike-in features). This array design contains two probe sets: 1) a complete probe set of 5,416 probes representing 1,105 sequences (2,708 probes) and their complements (2,708 probes) and 2) a reduced probe set of 4,238 probes which serves as technical replicate as it was not possible to duplicate the entire probe set due to the limitation of available features. The features representing *A. bisporus* sequences are randomly distributed but replicate features are equally spread on the array to prevent the loss of information when a part of the array becomes unusable (e.g. dust particle). This array design was used for 16 two-colour hybridisations to validate the designed *A. bisporus* probes and microarray methodology.

2.4.5.2 The custom 8 X 15,000 60-mer oligonucleotide microarray designs:

This "high-density" microarray slide design contains 8 copies of one array design. This means that eight one-colour or sixteen two-colour samples can be hybridised onto one slide simultaneously. The array design contains up to 2 validated *A. bisporus* probes for each unique sequence.

Version 1:

The 8 X 15,000 60-mer oligonucleotide microarray design was constructed from a probe group of 1,617 probes, containing 1,448 validated probes representing 1,079 *A. bisporus* sequences, 123 complementary probes and 46 un-validated probes representing sequences identified in a separate project where J. Green (Warwick HRI, University of Warwick) investigated *A. bisporus* transcriptional responses to virus infection. This probe group was replicated 5 times on each array. The features were randomly distributed on the array but replicate features were equally spread on the array. This custom 8 X 15,000 60-mer oligonucleotide design version 1 was used for 64 one-colour microarray hybridisations with colonised casing samples collected during the experiments where *A. bisporus* was grown under standard (sections 2.2.2.1 and 2.2.3.1) and experimental growth conditions (sections 2.2.2.2, 2.2.2.3, 2.2.3.2, 2.2.3.3).

Version 2:

This 8 X 15,000 60-mer oligonucleotide microarray design was constructed from a probe group of 2,197 probes, containing 1,448 validated *A. bisporus* probes (representing 1,079 sequences), 123 complementary sequences and a probe group 626 un-validated probes (representing 208 sequences) identified from a separate project where J. Green (Warwick HRI, University of Warwick) investigated *A. bisporus* transcriptional responses to virus infection. This probe group was replicated 5 times on each array. The probes were randomly distributed on the array but replicate probes were equally spread on the array. This custom 8 X 15.000 60-mer oligonucleotide design version 2 was used for 21 one-colour microarray hybridisations with colonised casing samples collected during the experiments where *A. bisporus* was grown under standard (section 2.2.3.1) and experimental growth conditions (section 2.2.3.4).

2.5 *Microarray hybridisation and data acquisition*

Total RNA was processed using the Low RNA Input Linear Amplification kits Plus One or Two-Color (Agilent Technologies) as and prepared for hybridisation as described in the relevant One- or Two-Color Microarray-Based Gene Expression Analysis protocol as described in section 2.3.4.

2.5.1 Preparation of the cRNA sample for microarray hybridisation

A hybridisation mixture containing 350 ng Cy-3 and Cy-5 labelled cRNA for a two-colour hybridisation or 600-1000 ng Cy-3 labelled cRNA for a one-colour hybridisation, 10 X blocking agent and 25 X fragmentation buffer as specified in the relevant kit manuals was prepared for each sample-pair (two-colour) or sample (one-colour). This mixture was incubated at 60°C for exactly 30 minutes to fragment the RNA. 2x GEx Hybridisation Buffer HI-RPM (Agilent Technologies) was added to the mixture as outlined in the relevant protocol and carefully mixed by pipetting to stop the fragmentation reaction. The mixture was centrifuged at 13,000 r.p.m. for 1 minute in a Sorvall Biofuge pico to reduce the size and number of bubbles and placed on ice.

2.5.2 Hybridisation of the cRNA mixture on a microarray

A gasket slide (Agilent Technologies) with 2 or 8 rubber gaskets, for use with a 2 X 11,000 60-mer oligonucleotide microarray slide (two-colour hybridisation) or a 8 X 15,000 60-mer oligonucleotide microarray slide (one-colour hybridisation) respectively, was placed inside a microarray hybridisation chamber (Agilent Technologies). The cRNA hybridisation mixture (section 2.5.1) was slowly dispensed onto the gasket slide, inside the area contained by one of the rubber gaskets as described in the relevant product manual. Once all samples were dispensed onto assigned positions on the gasket slide, a microarray slide of the corresponding sample format was carefully placed on top of the gasket slide, with the active side containing the microarray probes facing the hybridisation mixture. The hybridisation chamber was then closed and transferred into a pre-warmed hybridisation oven (Agilent Technologies) and hybridised at 65 °C for 17 hours.

2.5.3 Procedure to wash non-specific bound cRNA from the microarray after hybridisation

The hybridisation chamber containing the gasket – microarray slide pair described in section 2.5.2 was removed from the hybridisation oven. The hybridisation chamber was opened and the gasket – microarray slide pair was transferred into a microarray wash dish containing Gene Expression Wash Buffer 1 (Agilent Technologies). The gasket – microarray slide pair was carefully broken apart and the microarray slide was transferred to a slide holder placed in another wash dish, positioned on a magnetic stirrer, containing Gene Expression Wash Buffer 1 (room temperature) and a magnetic flea. The solution was then stirred for 1 minute. The slide holder with the microarray was then transferred to a third wash dish containing Gene Expression Wash Buffer 2 (Agilent Technologies) preheated at 37 °C and a magnetic flea. The solution was stirred for 1 minute after which the slide holder with the microarray was transferred to a fourth wash dish containing acetonitrile and a magnetic flea. The solution was stirred for 1 minute after which the slide holder with the microarray was transferred to the fifth and final wash dish, containing the Stabilization and Drying Solution (Agilent Technologies). The solution was stirred for 30 seconds after which the slide holder was carefully removed from the solution, avoiding carry-over of liquid on the microarray. The stabilization solution is designed to protect Cy-dyes from ozone degradation. This is particularly important for two-colour experiments as Cy-5 is very sensitive to this kind of degradation (Branham *et al.*, 2007).

2.5.4 Scanning of a hybridised microarray

After hybridisation (section 2.5.2) and washing (section 2.5.3), the microarray was transferred into a microarray scan holder (Agilent Technologies) and scanned using an Agilent Technologies B-model scanner. Scans were performed at 5 µm resolution for a scan area of 61 x 21.6 mm. The red and green channel was measured for two-colour experiments while the green channel (Cy-3) was measured for one-colour experiments The scanning mode was single pass, eXtended Dynamic range selected (XDR high 100 %, XDR low 10%). The use of the eXtended Dynamic range feature improved the dynamic range of the data by scanning each pixel twice, once at full laser power (100% PMT power) and once at reduced laser power (10% PMT

power). The data was stored as two TIFF (Tagged Information File Format) images, one for each intensity scan. Signal data from both images is combined by the data processing software, Feature Extraction Software (Agilent Technologies) to give a more accurate result of features that have expression levels in sub-optimal detection areas (close to background or detector saturation).

2.5.5 Extraction of signal intensity data from a microarray scan image

The TIFF-images of a hybridised microarray slide as discussed in section 2.5.4 were processed using Feature Extraction Software (Agilent Technologies). This program places a grid onto the TIFF image for each of the sub grids (2 sub grids for a 2 X 11,000 60-mer oligonucleotide array design; 8 sub grids for a 8 X 15,000 60-mer oligonucleotide array design) in order to link signal intensity at a specific location to a specific probe. The fluorescence levels are then converted into numerical data. Spatial detrending and linear Lowess dye normalisation (for two-colour experiments only) was performed to accommodate for positional and dye-intensity effects and the data is organised into a tab-delimited text file containing multiple columns (e.g. probe position, probe identifier, probe signal intensities and local background intensity, p-value for two-colour experiments, saturation and surrogate value). The p-value indicates the significance of the difference between the green and the red channel for that probe and is based on a 2-sided T-test with default p-value of 0.01. A surrogate value is a fixed expression value given to a feature where the raw intensity is very close to the background. Features with inconsistent saturation values (e.g. in only one of the two intensity channels of a self versus self experiment) were flagged. No local background subtraction was performed as suggested by Zahurak *et al.* (2007). The Feature Extraction software also generates a quality control (QC) report based on general background and intensity levels and the hybridisation results of the control grid (Spike-In probes and positive and negative controls). The QC report also provides information on the spatial distribution of outliers, hybridisation errors (e.g. dust particle), spike-in intensities and average fluorescence intensities. Feature Extraction Software version 8.5.1.1 with the two-colour gene expression protocol GE2_11kx2_1205 with grid file 014217_D_F_20060216 was used to extract the data from the microarray validation experiments which will be described in section 2.7. Feature Extraction Software version 9.5.3.1 with the one-colour gene expression protocol GE1-v5_95_Feb07 and grid file 017055_D_F_20070613 was

used to extract the data from the gene expression analysis experiments described in section 2.8.

2.6 *Microarray data analysis*

2.6.1 Normalisation

Microarray data normalisation is essential when comparing the signal from the Cy-3 channel to the Cy-5 channel (two-colour experiments) and / or comparing data between arrays (one- and two-colour experiments). A normalisation procedure optimised for this project was developed at Warwick HRI by Andrew Mead. This procedure considered the fact that a large portion of the genes on the array could be differentially expressed. For this purpose, a script was written in R, a statistical programming language (http://www.r-project.org/) and contained the following steps:

1) All data sets of hybridisations performed with the same microarray design were combined creating a file containing raw (non-normalised, etc.) data. Feature means and medians both with and without background correction and / or dark corner corrections were stored in separate data sheets. It was decided to initially analyse the spot medians without any corrections, strengthened by the uniformity of the data and the conclusions stated by Zahurak *et al.* (2007) who found that background is very uniform on printed Agilent Technologies microarrays and introducing this correction would introduce more error compared to corrective effects. The raw data (without any averaging across replicate features for each probe) was initially log (base 2) transformed. Separate information was included about the experimental design factor levels (array ID, treatment, time and replicate) associated with each sample.

2) The data was split up and two new data sets were created, one for the Agilent spike-in features and another for the features representing *A. bisporus* genes.

3) Identification of hybridisation artefacts. The means, medians, maxima and minima were calculated for each probe-set (5 replicate probes). This data was then

analysed for patterns in order to identify hybridisation issues such as dust particles or leakage during hybridisation. These areas were flagged and visualised in an image.

4) Identification of outliers. Probe-sets where the range of log-transformed expression values is greater than 2 (i.e. more than a 4-fold difference across replicates) are identified and the feature where the difference between the maximum or minimum and the median for the probe-set was greater than 0.95 was marked as an outlier and omitted from the data set and further analysis.

5) Normalisation of the dataset. This procedure used the medians and interquartile ranges - medians and quartiles calculated for each array. Adjustments were made to the dataset so that each array has the same median and the same interquartile range. Despite arrays only including probes for selected genes (rather than the whole genome) it was assumed that the median and interquartile ranges should be relatively consistent across arrays with the "central" proportion of probes showing little variation in expression response.

6) The means and ranges (maxima and minima) were calculated for each probe (across up to 5 replicates) using the cleaned, normalised data. This data was then used for gene expression analysis, using the calculated medians across the replicates for each probe, for each sample.

7) An additional per gene normalisation step was performed using Microsoft Excel to make the data conform to the time course analysis package used for the identification of genes that were significantly differentially expressed over the course of an experiment as described in 2.6.2.2. For each cleaned and normalised probe, the mean signal over all microarray experiments with the same array design was calculated. This value was subtracted from the probe signal intensity in each experiment. This biased the signal intensity for the majority of genes around 0 while an intensity deviating from 0 indicates differential expression in that experiment.

A copy of this R-script can be found on the DVD attached to the back cover of this thesis.

2.6.2 Identification of genes that are significantly differentially expressed between two or multiple one-colour microarray samples

Two analysis methods to identify genes that were significantly differentially expressed in one or multiple growth experiments.

2.6.2.1 Microarray analysis of variance

The Microarray analysis of variance (MAANOVA) R-package was one of the two methods used for a formal analysis of the normalised gene expression data generated during this project (Wu *et al.*, 2003). The mixed model analysis allowed for variation between slides and arrays (random model) and was used to test for variation due to treatments (fixed model). This analysis method can identify specific contrasts, e.g. genes that are differentially expressed over the course of a specific treatment. The analysis provided an overall test of variation in gene expression as well as specific contrasts associated with these comparisons of conditions. Calculated p-values associated with each of these tests were adjusted for false discovery rate to accommodate analysis of large gene numbers. Probes with p-values for the F-statistic (calculated using the 4 time-points of an experiment) ≤ 0.01 were retained for further analysis (section 2.6.2.3) as this p-value indicated that they were significantly differentially expressed between at least two time-points in the tested experiment.

2.6.2.2 Time course microarray analysis

The Bayesian Analysis of Time Series program, BATS (Angelini *et al.*, 2008) was the second analysis method used during this project and is, in contrast to the MAANOVA method described in section 2.6.2.1, specifically designed to analyze time course microarray experiments. This program was used to identify genes that are differentially expressed over the course of a growth experiment. The per-gene normalised data sets, as described in section 2.6.1 (normalisation of microarray data), were used as input. Default settings were used to process the microarray data. The outputs, lists of significantly differentially expressed genes for the analysed experiments, were used for further analysis as described in section 2.6.2.3.

2.6.2.3 Identification of differentially expressed genes based on the results from MAANOVA and BATS

Only a small part of the predicted gene number of *A. bisporus* (< 12%) was available at the start of this project. A significant fraction of these sequences were selected because of differential expression during mushroom development. The samples collected during the various experiments were colonised casing samples which meant that a change of environmental condition is perceived at different times throughout the casing layer and mycelium might respond to these changes at different moments in time throughout the casing. This sample collection method was expected to increase the "noise" of the gene expression profiles but was essential for the construction of a gene expression profile that represented the status of the entire, active fungal biomass present in the casing layer at a certain time point. Changes in gene expression linked to developing fruit bodies might be subtle, compared to the expression levels in the supporting mycelium. To increase the statistical strength of the data, it was decided to only consider the genes identified to be differentially expressed in both the MAANOVA method (described in section 2.6.2.1) and the BATS package (described in section 2.6.2.2) for further analysis.

2.7 ***Experimental microarray probe validation***

Three hybridisation series were performed to assess the reproducibility of the probes designed for the *A. bisporus* sequences described in section 2.4.4 and the microarray hybridisation methodology described in section 2.3.4 and section 2.5. These three experiments are described below.

2.7.1 Self versus self validation experiment

This first validation experiment was designed to assess reproducibility of each probe and the consistency of dye-incorporation of Cy-3 and Cy-5 using self versus self hybridisations. Under these conditions, after correction for dye-bias and spatial detrending to correct for positional effects by Feature Extraction software (Agilent Technologies) as described in section 2.5.5, it was expected to get a 1:1 signal intensity ratio for each probe unless a probe is binding non-specifically. For this experiment, *A. bisporus* was grown under phase change stimulating conditions in

mushroom trays as described in section 2.2.2.1. A colonised casing sample (24 hours after airing) and elongated differentiating primordia were collected as described in section 2.2.2.4 and total RNA was extracted with the methods described in section 2.3.2 for the colonised casing sample and section 2.3.1 for the elongated differentiating primordia sample. Approximately 350 ng total RNA of each sample was labelled once with Cy-3 and once with Cy-5 as described in section 2.3.4. The labelling reactions were performed in triplicate to provide technical replicates. Each labelling reaction passed the minimum recommended guidelines for yield and dye-incorporation described in section 2.3.4. Dye-pairs were hybridised onto three *A. bisporus* 2 X 11,000 60-mer oligonucleotides oligonucleotide probes microarrays (section 2.4.5.1) and microarray data was acquired following the procedure described in section 2.5. Table 2-1 illustrates the set-up of these hybridisations.

Table 2-1 Experimental set-up of the self versus self microarray hybridisations performed to validate the *A. bisporus* probes, cRNA preparation and hybridisation procedures.

slide	sub-array	Cy-3	Cy-5
1	1	colonised casing at 24 h after airing	colonised casing at 24 h after airing
	2	elongated differentiating primordia	elongated differentiating primordia
2	1	colonised casing at 24 h after airing	colonised casing at 24 h after airing
	2	elongated differentiating primordia	elongated differentiating primordia
3	1	colonised casing at 24 h after airing	colonised casing at 24 h after airing
	2	elongated differentiating primordia	elongated differentiating primordia

2.7.2 Dye-swap validation experiment

The second experiment was designed to assess the incorporation efficiency of Cy-3 and Cy-5 using a dye-swap experiment. In this setup, it was expected to get inverted Cy-3:Cy-5 signal ratios unless there is a different incorporation rate for each dye. Non-stringent probes will also be detected with this method as the signal intensities for these probes will be inconsistent, after correction for any dye incorporation bias. For this experiment, *A. bisporus* was grown under phase change stimulating conditions in mushroom trays (section 2.2.2.1) and stage 2 and stage 4 fruit-bodies (veil-break) were collected. Half of these fruit bodies were frozen under liquid nitrogen and stored at -70°C while the other half of the fruit bodies was stored at room temperature for 2 days prior to freezing under liquid nitrogen and storage at -

70°C. Total RNA was extracted from these samples using the method described in section 2.3.1 and each sample was labelled once with Cy-3 and once with Cy-5 as described in section 2.3.4. The cRNA was then hybridised, in duplicate, onto four *A. bisporus* 2 x 11.000 60-mer oligonucleotide microarrays (section 2.4.5.1) and processed as described in section 2.5. Table 2.2 illustrates the set-up of these hybridisations.

Table 2-2 Experimental set-up of the dye-swap microarray hybridisations performed to validate the *A. bisporus* probes, cRNA preparation and hybridisation procedures.

slide	sub-array	Cy-3	Cy-5
4	1	stage 2 fruit body	stage 4 fruit body
	2	stage 4 fruit body	stage 2 fruit body
5	1	stage 2 fruit body	stage 4 fruit body
	2	stage 4 fruit body	stage 2 fruit body

2.7.3 Hybridisation with samples from a range of developmental stages

The third experiment was designed to test a variety of developmental stages to ensure that each probe is hybridised at least once (e.g. if a gene is only expressed in a specific stage). For this experiment, *A. bisporus* was grown under phase change stimulating conditions in mushroom trays. Various developmental stages were collected: colonised casing at airing (0 h), colonised casing 24 h after airing, smooth undifferentiated primordia, elongated differentiating primordia, stage 2 fruit bodies and stage 4 fruit bodies of samples collected from *A. bisporus* grown under phase change stimulating conditions described in section 2.2.2.1. Total RNA was extracted for each of these samples using the methods described in section 2.3.1 (soil-free primordia and fruit bodies) and section 2.3.2 (colonised casing samples). The total RNA from each sample was fluorescently labelled with either Cy-3 or Cy-5 as described in section 2.3.4 and hybridised in pairs onto *A. bisporus* 2 x 11.000 60-mer oligonucleotide microarrays (section 2.4.5.1) following the procedure outlined in section 2.5. Table 2.3 illustrates the experimental set-up of these hybridisations.

Table 2-3 Experimental set-up of the third series of microarray hybridisations performed to validate the *A. bisporus* probes, cRNA preparation and hybridisation procedures. For this hybridisation series, samples of 6 developmental stages were hybridised in pairs.

slide	sub-array	Cy-3	Cy-5
6	1	smooth undifferentiated primordia	elongated differentiating primordia
	2	stage 2 fruit body	stage 4 fruit body
7	1	smooth undifferentiated primordia	elongated differentiating primordia
	2	stage 2 fruit body	stage 4 fruit body
8	1	colonised casing at airing	colonised casing 24 h after airing
	2	colonised casing at airing	colonised casing 24 h after airing

2.7.4 Validation of normalised microarray expression data using quantitative real time reverse transcription PCR

Transcript levels of 5 genes (sequences 318, 982, 1119, 1542 and 1550) thought to be differentially expressed in *A. bisporus* developing during the reproductive phase change from vegetative mycelium to elongated differentiating primordia based on validation microarray data were quantified by quantitative real time reverse transcription PCR (qRT-PCR). Colonised casing samples were collected in triplicate (random location in the mushroom house) at airing (0 h), 72 h and 168 h after airing, under standard cultivation conditions as described in section 2.2.2.1. Total RNA was extracted using the modified total RNA extraction method described in section 2.3.2, including treatment with RQ1 DNase enzyme (Promega) to remove contamination of remaining genomic DNA. Randomly primed first strand cDNA was produced for each sample using the Thermoscript™ RT-PCR system (Invitrogen, Life Technologies, Paisley). Each cDNA sample was then treated with RNase H (Invitrogen, Life Technologies) and diluted to 100 µl final volume.

An ABI Prism 7900HT sequence detector (TaqMan™, Applied Biosystems, Warrington Cheshire) was used for qRT-PCR transcript analysis and primers were designed using the ABI Primer Express® software version 2.0. The sequences of the forward and reverse primers used for this validation experiment are shown in Table 2-4.

Table 2-4 Sequences of oligonucleotides (primers) used for qRT-PCR

Sequence ID	forward primer (5'-3')	reverse primer (5'-3')
318	GCAATTGTCAACCATCGCATT	CCTTCAGGAGCCAGACGTATCTA
824	GGCCTTGGACTGGGTGTACTT	ACCAATTGCCGTGATAGGACTAC
982	GAAGCCTTCTGACGCATCCA	AATGAGTTGCTACCGACAGGTTTAC
1119	TACCGTTGCCTACACGATGATC	AGGTTAGGAAGTGCTGGAAGTTGT
1542	TCGAACTTGCCGGTATCATCT	AGGCTTGACGACAATGACCAA
1550	GCACCATTTGCTTCCGATTC	ATCGATTTGCTGGTCATTTGTG

After preparation of the cDNA, triplicate quantitative real time PCR reactions were performed for each sample in a 15 µl volume consisting of 1 µM of each primer, 20 ng cDNA sample and 7.5 µl of 2 × SYBR Green PCR mix (Applied Biosystems). Cycling parameters comprised 50 °C for 2 min; 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. A dissociation step (95 °C for 15 s, 60 °C for 15 s, and increase to 95 °C with a 2 % ramp rate) was used to detect primer dimers and non-specific products. The *A. bisporus* 18S rRNA gene was used as an internal standard for determining mRNA levels of the target genes as described previously (Goidin *et al.*, 2001). Control treatments to detect contaminating DNA included replacing the cDNA sample with DEPC-treated water and using the DNase-treated RNA (which had not been through a reverse transcription reaction) for each sample. Inactivation of the Thermoscript™ reverse transcriptase was tested by including a sample where the RT reaction was carried out in the absence of random hexamers. Transcript levels were quantified using the ΔCt equation ($2^{(Ct\ 18S\ rRNA-Ct\ gene)}$) and averaged for each time point. Outlier replicates were removed from further analysis. The standard deviation on the ΔCt for the replicates of each time point was calculated.

2.8 *Gene expression profiling of A. bisporus grown under standard and experimental environmental conditions*

A series of experiments were performed where *A. bisporus* was grown under standard and experimental growth conditions. Samples collected during these experiments were used to investigate changes in gene expression in *A. bisporus* as a response to specific environmental conditions.

Colonised casing samples collected from the growth experiments described in the following sub-sections were processed similarly. Total RNA was extracted using the modified Fast RNA pro soil protocol described in section 2.3.2 and used to create Cy-3 labelled cRNA as described in section 2.3.4. Each labelled cRNA sample that passed the minimum recommended yield and dye-incorporation rate (section 2.3.4) was prepared for hybridisation as described in section 2.5.1 and onto a 8 X 15.000 60-mer oligonucleotide microarray (section 2.4.5.2). Variable amounts of labelled cRNA were used for the microarray hybridisation to compensate for different balances of fungal to bacterial total RNA in the input material as samples collected at airing had a lower fungal / bacterial RNA balance compared to samples collected at 168 h after airing due to increase of fungal biomass over time. For this reason, 1000 ng cRNA was used for colonised casing samples at airing and 24 h after airing, 800 ng cRNA was used for colonised casing samples collected at 72 h after airing and 600 ng cRNA was used for colonised casing samples collected at 168 or 192 h after airing. After hybridisation, arrays were processed as described in section 2.5.3. The microarray data was acquired, processed and analysed as described in sections 2.5.4, 2.5.5 and 2.6.

2.8.1 Gene expression profile of **A. bisporus** *grown under standard growth conditions*

Samples were collected in triplicate from *A. bisporus* grown under phase change stimulating conditions in a:

1) growth tray (section 2.2.2.1) at airing (0 h), 24 h, 72 h and 168 h after airing;

2) flask-based system (section 2.2.3.1) at airing, 24 h, 72 h and 192 h after airing. This experiment was performed in duplicate (separated in time) and gene expression results were averaged prior to analysis.

The colonised casing samples collected from a growth tray were processed and hybridised onto a 8 X 15,000 60-mer oligonucleotide microarray version 1 (section 2.4.5.1). Samples collected from the first flask-based growth system experiment were processed and hybridised onto a 8 X 15,000 60-mer oligonucleotide version 1 while the samples from the second flask-based growth experiment were processed and

hybridised onto a 8 X 15,000 60-mer oligonucleotide microarray version 2 (section 2.4.5.2). The microarray data was acquired and processed as described in section 2.8

2.8.2 Gene expression profile of **A. bisporus** *grown under high temperature but reduced levels of CO_2 and mushroom volatiles*

Samples were collected in triplicate from *A. bisporus* grown in mushroom trays under high temperature (25 °C) but reduced levels of CO_2 and mushroom volatiles (section 2.2.2.2) at 24 h, 72 h and 168 h after airing. The samples at airing (0 h) were common to standard growth conditions experiment (section 2.8.1). The samples were processed and hybridised on 8 X 15,000 60-mer oligonucleotide microarrays version 1 (2.4.5.2). The microarray data was acquired and processed as described in section 2.8.

2.8.3 Gene expression profile of **A. bisporus** *grown under high levels of CO_2 and mushroom volatiles but reduced temperature*

Samples were collected in triplicate from *A. bisporus* grown in mushroom trays under high levels of CO_2 and mushroom volatiles (section 2.2.2.3) at 24 h, 72 h and 168 h after airing. The samples at airing (0 h) were common to the standard growth conditions experiment (section 2.8.1). The samples were processed and hybridised on 8 X 15,000 60-mer oligonucleotide microarrays version 1 (section 2.4.5.2). The microarray data was acquired and processed as described in section 2.8.

2.8.4 Gene expression profile of **A. bisporus** *grown under high level of 1-octen-3-ol but reduced level of CO_2 and reduced temperature*

Samples were collected in triplicate from *A. bisporus* grown in the flask-based growth system under high level of 1-octen-3-ol, reduced CO_2 level and reduced temperature (section 2.2.3.2) at 24 h, 72 h and 192 h after airing. The sample at airing (0 h) was common to the standard growth conditions experiment (section 2.8.1). The samples were processed as hybridised on the *A. bisporus* 8 X 15,000 60-mer oligonucleotide microarray version 1 (described in section 2.4.5.2). The microarray data was acquired and processed as described in section 2.8.

2.8.5 Gene expression profile of **A. bisporus** *grown under reduced level of 1-octen-3-ol after removal of the 1-octen-3-ol solution*

This experiment was the continuation of the experiment described in section 2.8.4 and the volatile source was removed at 192 h after airing. Samples were collected in triplicate at 24 h, 72 h and 168 h after removal of the volatile source from the air-supply. The samples were processed and hybridised on the *A. bisporus* 8 X 15,000 60-mer oligonucleotide microarray version 1 (described in section 2.4.5.2). The microarray data was acquired and processed as described in section 2.8.

2.8.6 Gene expression profile of **A. bisporus** *grown under high level of CO_2 but reduced level of mushroom volatiles and reduced temperature*

Samples were collected in triplicate from *A. bisporus* grown in the flask-based growth system under high level of CO_2 but reduced level of mushroom volatiles and reduced temperature (section 2.2.3.4) at 24 h, 72 h and 192 h after airing. The sample at airing (0 h) was common to the standard growth conditions (section 2.8.1). The samples were processed and hybridised on the *A. bisporus* 8 X 15,000 60-mer oligonucleotide microarray version 2 (described in section 2.4.5.2). The microarray data was acquired and processed as described section 2.8.

Chapter 3. Defining reproductive morphogenesis in A. bisporus *and characterisation of the responses to specific environmental conditions*

3.1 *Introduction*

This project aimed to investigate the molecular mechanism of the phase change from vegetative to reproductive growth in *A. bisporus*. Before an in depth analysis of the gene expression changes during this process could be performed, it was essential to get an understanding of how the fungus responds to changes in its environment on a morphological level and to identify "key" stages of differentiation and development. This chapter gives the results of the first major study in over 30 years of the morphological development of *A. bisporus* during the phase change from vegetative to reproductive growth, using time-lapse photography. For this purpose, *A. bisporus* was grown under phase change stimulating conditions and under experimental conditions where the effects of individual environmental parameters on morphological development were assessed. Observations made during these experiments were used to:

1) define various key morphological stages of the phase change;

2) investigate the effects of individual environmental factors (e.g. temperature, CO_2 or volatile levels) on this process;

3) organise a sampling regime to collect samples for gene expression profiling.

3.2 *Identification of key morphological stages during the phase change in* A. bisporus

In order to identify key morphological stages of the phase change from vegetative to reproductive growth in *A. bisporus*, a number of identical growth

experiments were performed. Detailed time-lapse recordings of *A. bisporus* mushroom initiation were made from two independent growth experiments (2 further growth experiments had been observed previously to gain and understanding of the system, but were not recorded). In one experiment, the camera was positioned above the growth tray. During the other experiment, the camera was positioned on the side of a growth pot. Images were taken at 1 hour intervals. *A. bisporus* was grown in mushroom trays or growth pots, similar to commercial cultivation conditions (section 2.2.2.1). Similarly, two growth experiments where *A. bisporus* was grown under phase change stimulating conditions in the flask-based system (section 2.2.3.1) were observed visually. *A. bisporus* was grown in mushroom trays or growth pots, similar to commercial cultivation conditions (section 2.2.2.1). Detailed time-lapse recordings of *A. bisporus* mushroom initiation were made from two independent growth experiments (2 further growth experiments had been observed previously to gain and understanding of the system, but were not recorded). In one experiment, the camera was positioned above the growth tray. During the other experiment, the camera was positioned on the side of a growth pot. Images were taken at 1 hour intervals. Similarly, two growth experiments where *A. bisporus* was grown under phase change stimulating conditions in the flask-based system (section 2.2.3.1) were observed visually.For these growth experiments, *A. bisporus* was inoculated in compost (Figure 3.1 A) which was subsequently covered with casing material and incubated at 25 °C in the absence of ventilation as described in sections 2.2.1 and 2.2.2.1. These conditions promoted vegetative growth and the substrate was gradually colonised by a fine web of **vegetative hypae**. Figure 3-1 (B) shows a section of this mycelial web found on the surface of the casing layer. At what appears to be random positions in the casing layer, loosely bound aggregations of hypae, **mycelial cords**, can be observed as illustrated in Figure 3.1 (C & D). The diameter of mycelial cords depends on the number of individual hyphae comprised in this structure but measurements showed an average diameter of approximately 60 µm. Both individual mycelial cords and clusters of mycelial cords were observed. These cords were only found in the casing layer and appeared to be absent in the compost layer.

After colonisation of the casing layer by vegetative hyphae, a change in environmental conditions was applied to stimulate the phase change from vegetative to reproductive growth. This change involved 1) a reduction in temperature of the

growth system from 25 °C to 18 °C and 2) ventilation of the growth system to reduce levels of carbon dioxide levels (< 1,000 p.p.m) and mushroom volatiles as described in section 2.2.2.1 (mushroom house) and section 2.2.3.1 (flask-based growth system). This process is defined as "airing" and is adopted by the mushroom industry to optimally "initiate" fruit body production.

Approximately 32 h after airing, subtle but significant changes of the mycelium could be detected at localised areas on the casing layer. The initially "bare" hypae (i.e. with low density of branching) underwent a change in growth and exhibited intense local branching. These localised branches did not extend much and gave the hyphae and cords a "fluffy" appearance. This was most obvious on mycelial cords. Further localised branching of a single or multiple neighbouring mycelial cords resulted in a two-dimensional aggregation of hyphae around 48 h after airing.

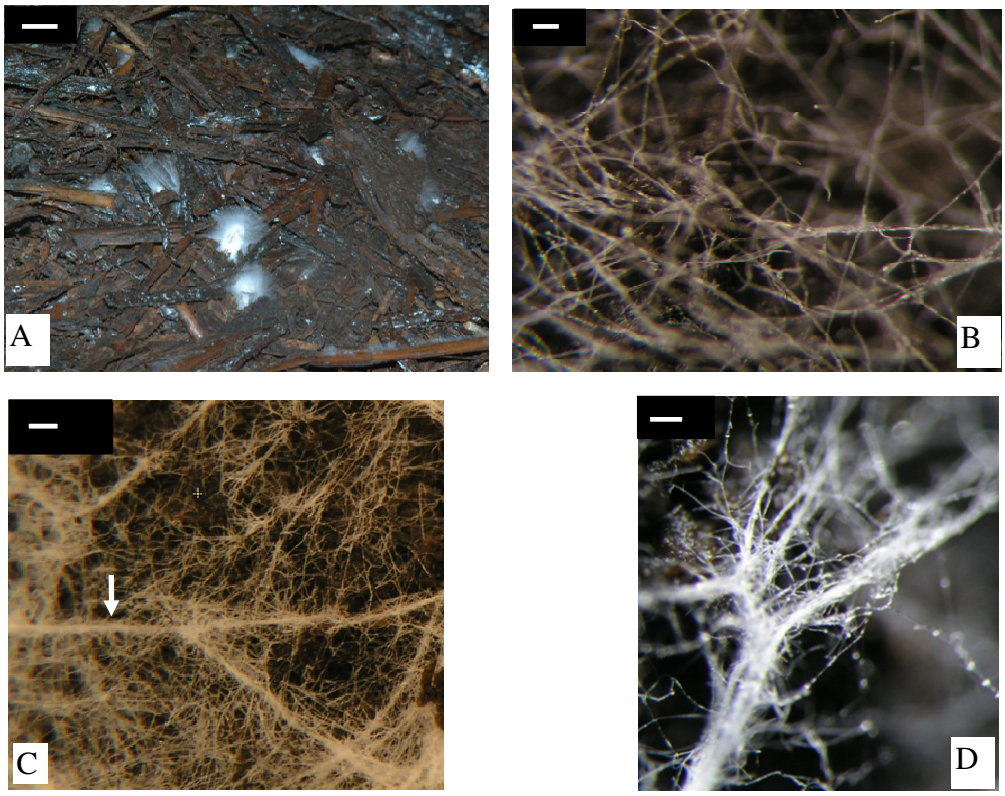


Figure 3-1 Microscopic observations of vegetative *A. bisporus* mycelium. (A) *A. bisporus* inoculate colonising wheat straw compost. Scale bar = 2 cm. (B) Individual hypae on the surface of the casing layer. Scale bar = 10 µm. (C) Loose aggregation of hyphae into a mycelial cord on the surface of the casing layer. Scale bar = 250 µm. An example of an individual mycelial cord has been marked with a white arrow. (D) Mycelial cord. Scale bar = 50 µm .

These two-dimensional structures of approximately 0.5-1 mm in diameter will be referred to as **fluffy hyphal knots** and are illustrated in Figure 3-2 (A & B). Gradually, more fluffy hyphal knots developed and at 96 h after airing, numerous of these structures could be distinguished on the surface of the casing layer as illustrated in Figure 3-2 (C). During the following 48 hours, subsequent development resulted in a gradual, three-dimensional increase in size of the fluffy hyphal knots as illustrated in Figure 3.3 (A). This three-dimensional structure (~ 1-2 mm diameter) initially had a fluffy appearance and will be referred to as a **fluffy undifferentiated primordium**. The fluffy undifferentiated primordia developed further and gradually lost their fluffy appearance. By 160 h after airing, the casing layer was covered by numerous smooth, spherical structures referred to as **smooth undifferentiated primordia** (~ 2-4 mm diameter) illustrated in Figure 3.3 (B).

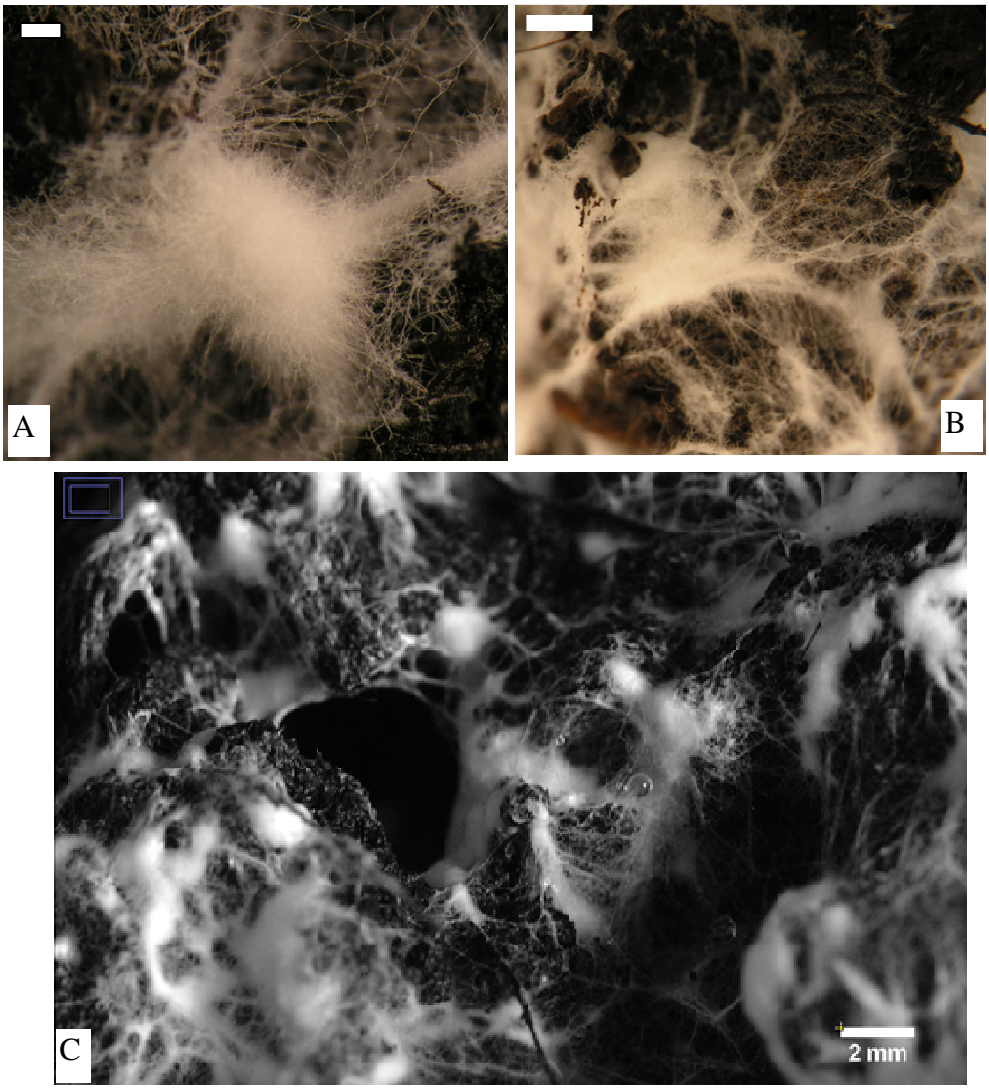
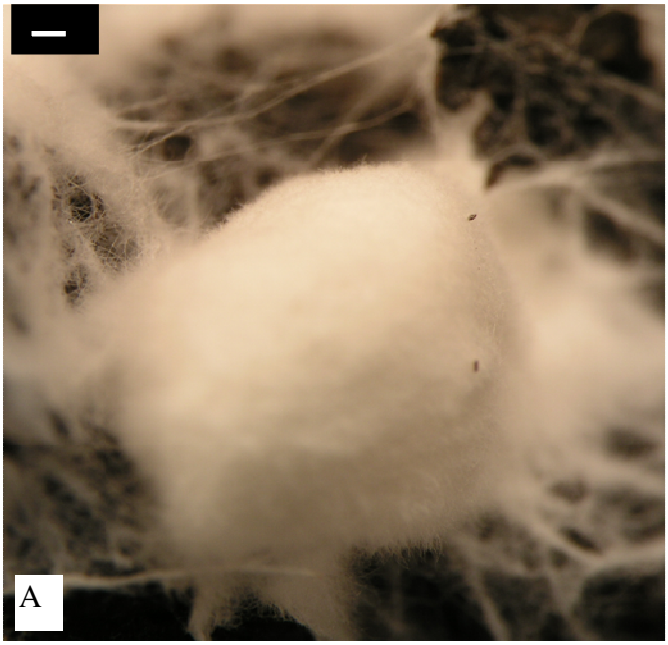


Figure 3-2 ***A. bisporus*** **mycelium developing under phase change stimulating environmental conditions. (A) Mycelial hyphae and cords are showing intense localised branching, giving the hypae a "fluffy" appearance (48 h after airing). Scale bar = 100 µm. (B) Localised branching of one or multiple overlapping mycelial cords resulted in the aggregation of a two-dimensional hyphal knot. Scale bar = 1 mm. (C) Numerous fluffy hyphal knots have developed on the surface of the casing layer at 96 h after airing. Scale bar = 2 mm.**



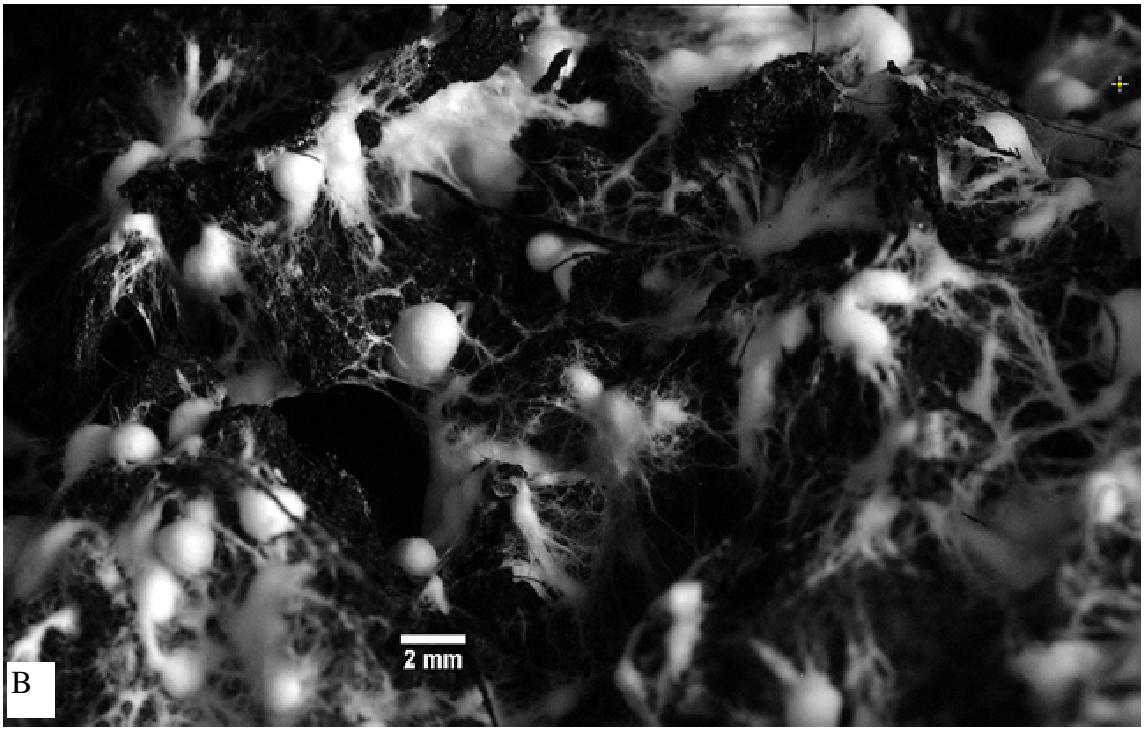


Figure 3-3 *A. bisporus* mycelium developing under phase change stimulating conditions. (A) A fluffy undifferentiated primordium. Scale bar = 200 µm. (B) At 160 hours after airing, multiple fluffy hyphal knots developed into smooth undifferentiated primordia of approximately 2 mm in diameter.

Time-lapse photography revealed that smooth undifferentiated primordia broke through the casing layer from 140 h after airing and the top part of these structures exhibited an upwards elongation. This elongated structure was originally referred to as a stage 1 fruit body by Hammond & Nichols (1976) but will be referred to as an **elongated differentiating primordium** in this document. The base part (~ 4-10 mm diameter) of this structure is wider than the top part. Further differentiation and development of the top part of the elongated differentiating primordium meant that the top/base diameter ratio gradually increased. The sizes of the base and top parts are approximately equal when the elongated differentiating primordium is approximately 10 mm in diameter and a region of reduced diameter has developed between top and base of the primordium, referred to as a waist. This structure is referred to as a **differentiating primordium with a distinct waist** and gradually increased in size to form a **stage 2 fruit body** at which point, differentiation of the gill tissue is observed. If not harvested, the stage 2 fruit body will develop further as described by Hammond & Nichols (1976). The development from elongated differentiating primordia to differentiating primordia with a distinct waist is illustrated in Figure 3-4.

Figure 3-5 gives a schematic representation of the morphological stages defined and discussed in this section. The suggested size for each stage is only an indication and some structures can be slightly larger or smaller.

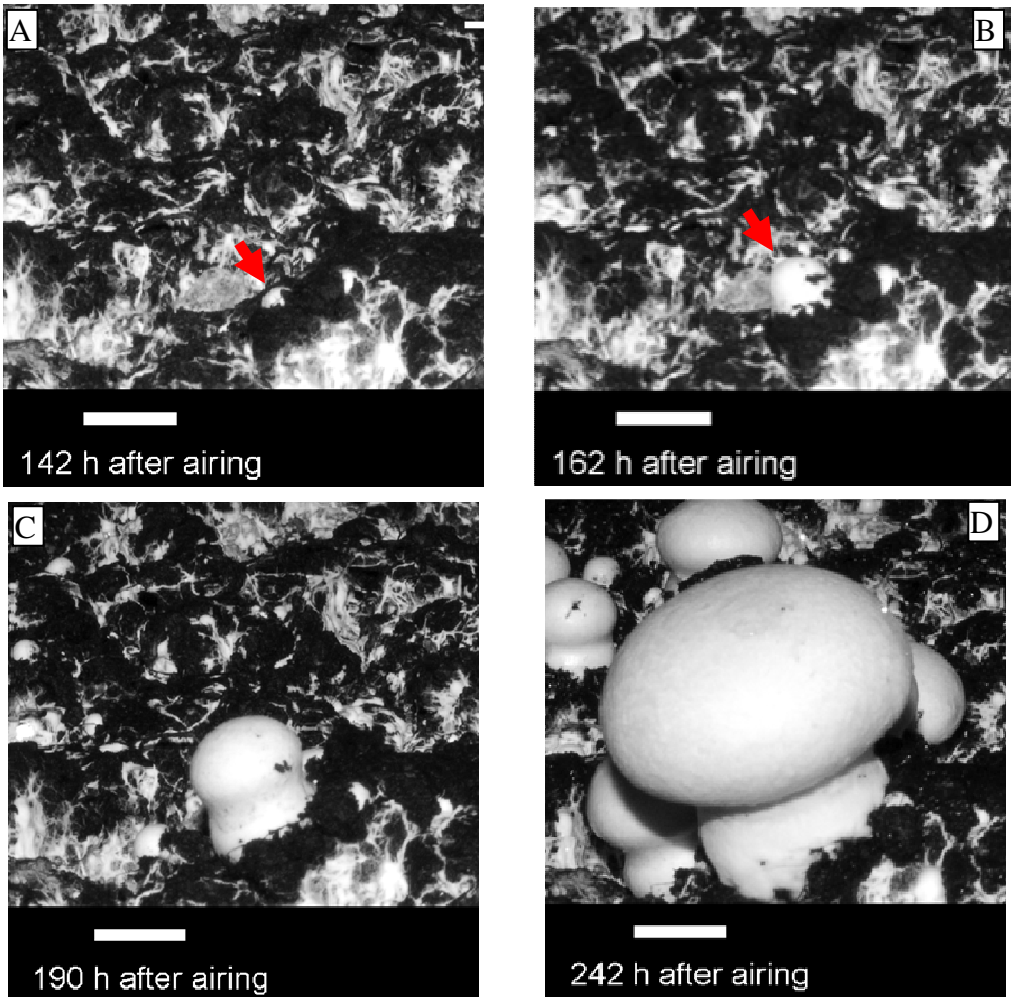


Figure 3-4 *A. bisporus* developing under phase change stimulating conditions. (A) An elongated differentiating primordium (red arrow) broke through the casing layer at 142 h after airing. (B) At 162 h after airing, the top region of the elongated differentiating primordium expanded further. (C) At 190 h after airing, the diameter from the top part of this primordium with a distinct waist has increased further and is now equal to the diameter of the base region. (D) At 242 h after airing, the primordium with a distinct waist developed into a stage 2 fruit body with differentiated gill tissue. Multiple primordia of different stages have also developed. Scale bar = 10 mm.

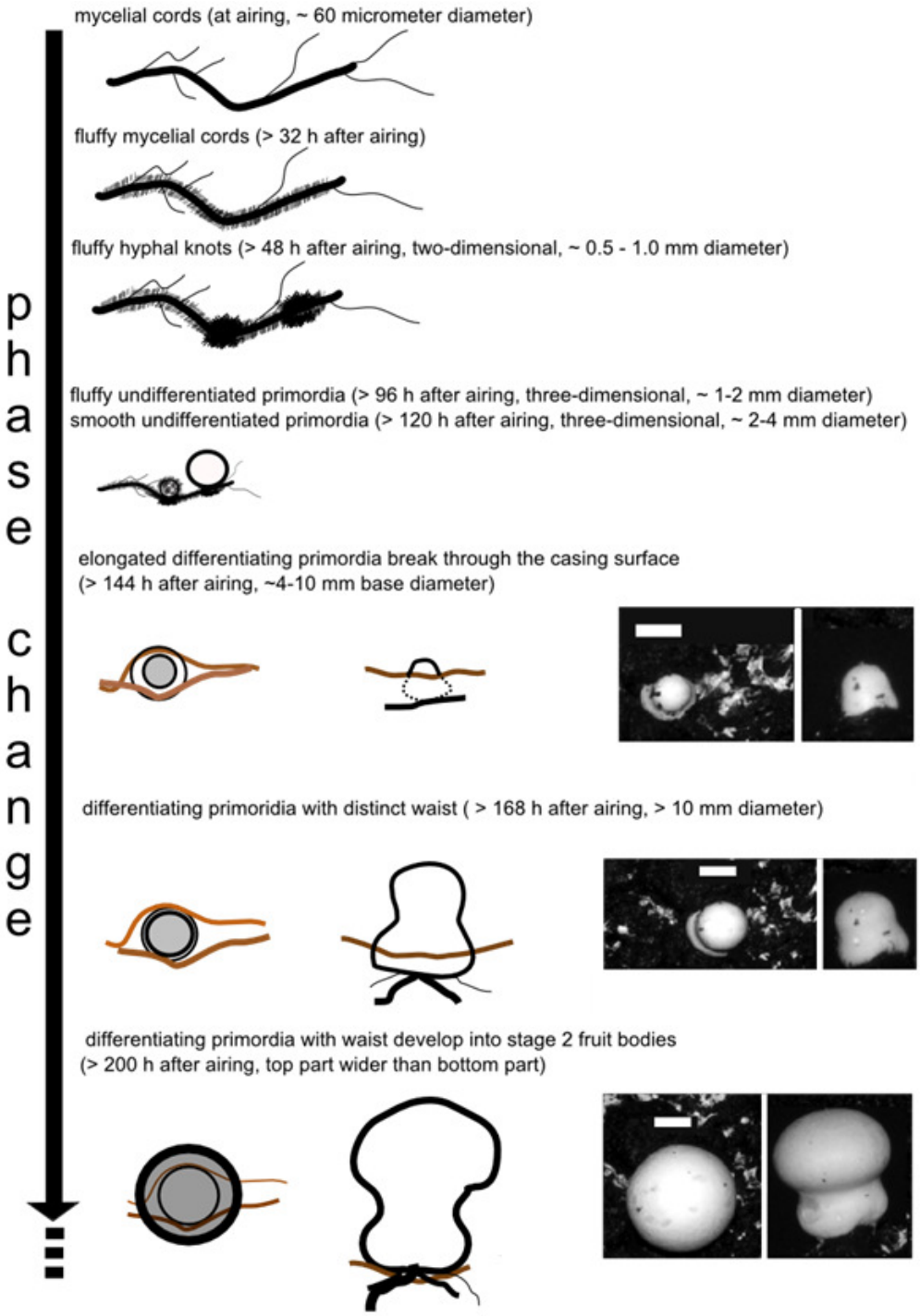


Figure 3-5 Schematic representation of key morphological stages during the reproductive phase change in *A. bisporus* from vegetative mycelium to stage 2 fruit bodies. (Scale bar in photographs = 10 mm).

3.3 *Examining the distribution and location of developing reproductive structures and their fate*

Section 3.2 focussed mainly on the identification of subsequent developmental stages observed during reproductive morphogenesis of mycelium exposed to phase change stimulating conditions. This section will investigate the general location and distribution of (developing) reproductive structures in a mushroom growth tray or growth pot and their fate. For this purpose, two time-lapse observation experiments as described in section 2.2.4 were performed on *A. bisporus* developing under phase change stimulating conditions as described in sections 2.2.2.1 and 2.2.3.1. For the first time-lapse observation, the camera was placed above a growth tray. For the second time-lapse observation, the camera was placed on the side of the growth pot as it was more convenient to use a pot compared to a tray for this set-up. The time-lapse images have been combined into media files which can be found on the DVD attached to the back cover of this document. The media file constructed from the top-view experiment is titled: "Top-view time-lapse recording of *Agaricus bisporus* development"; the media file constructed from the side-view experiment is titled: "Side-view time-lapse recording of *Agaricus bisporus* development".

3.3.1 Top-view observations of **A. bisporus** *during the reproductive phase change*

It was not possible to distinguish morphological development related to reproductive growth up to 100 h after airing based on the images from this experiment. During this period, the mycelium continued to colonise the casing layer (in an uneven distribution) as shown in Figure 3-6.

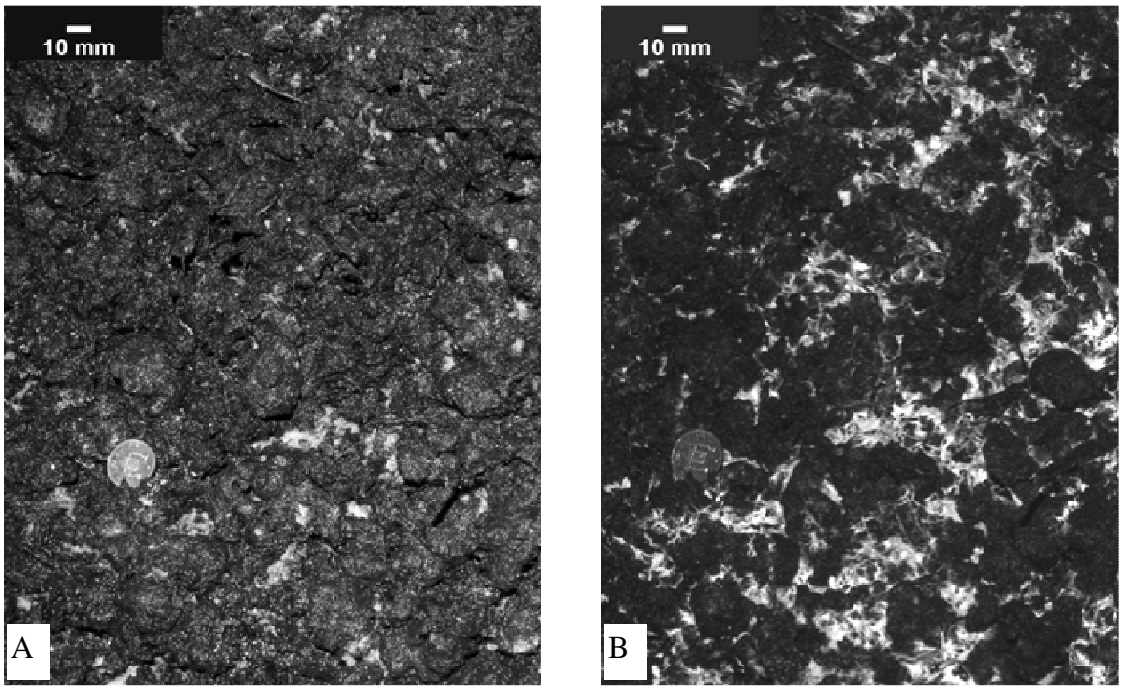


Figure 3-6 Top-view observation of *A. bisporus* mycelium developing under phase change stimulating conditions (A) at airing and (B) 100 h after airing.

Around 115 h after airing, the first **smooth undifferentiated primordium** had developed on the surface of the casing layer. Numerous other smooth undifferentiated primordia appeared shortly after and started to elongate. At 168 h after airing, numerous **elongated differentiating primordia** could be distinguished. A smooth undifferentiated primordium had developed subsequently into **differentiating primordium with distinct waist** as illustrated in Figure 3-7. Approximately 100 hours later (268 h after airing) multiple fruit bodies of stages 2- 4 as described by Hammond & Nichols (1976) had developed as illustrated in Figure 3-8. Similar observations were made of *A. bisporus* grown in a flask-based system with controlled air-supply as described in section 2.2.3.1 although occurrence of elongated differentiated primordia was seen around 192 h after airing (images not shown).

Detailed analysis of the time-lapse images revealed that numerous **elongated differentiating primordia** that originally developed below the surface of the casing layer broke through cracks in the casing at 144 h after airing. This meant that primordial structures can develop on mycelial strands on the surface layer and on mycelial strands below the surface of the casing layer. The time-lapse photography

showed that growth of primordial structures that initially developed below the surface of the casing is mostly synchronised. When these **elongated differentiating primordia** are fully exposed on the surface, this synchronised growth ends and a selection of these structures will develop further into mature fruit bodies. The remaining primordia either 1) do not develop further (held-back) until the point where developing fruit bodies are removed after which they continue their development into fruit bodies or 2) are held-back for over 100 h, even when developing fruit bodies are removed, after which they start to discolour and rapidly decay. This decay process was previously described as "abortion of pins" and observations have shown that even stage 2 fruit bodies can undergo this process. As illustrated in Figure 3-9, it appears that primordia that are held-back while the other primordia develop into mature fruit bodies are positioned in a seemingly random way, with no obvious preference in terms of position or proximity to other developing primordia.

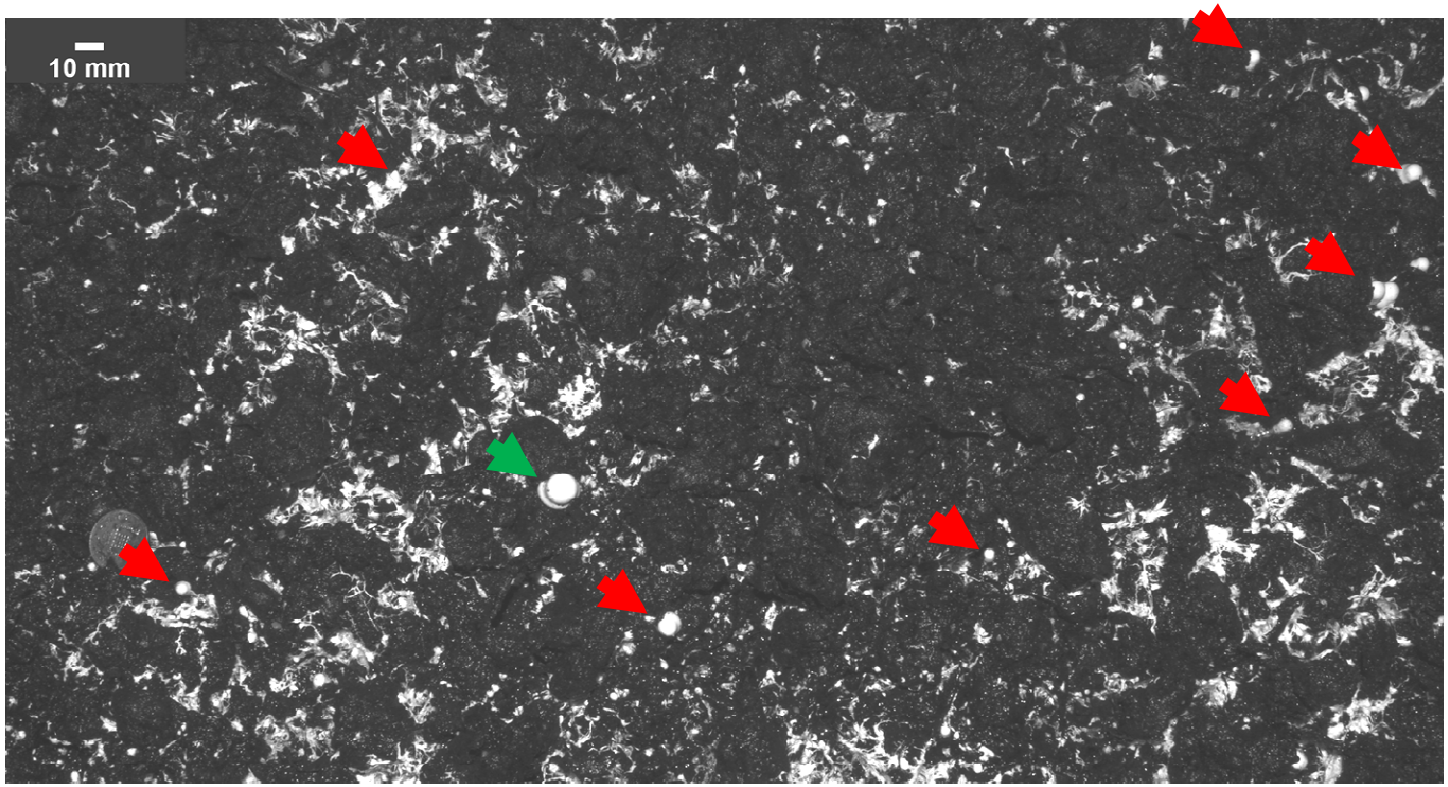


Figure 3-7 Top-view of *A. bisporus* grown under phase change stimulating conditions, 168 h after airing. A selection of elongated differentiating primordia have been marked with a red arrow, a differentiating primordium with distinct waist was marked with a green arrow.

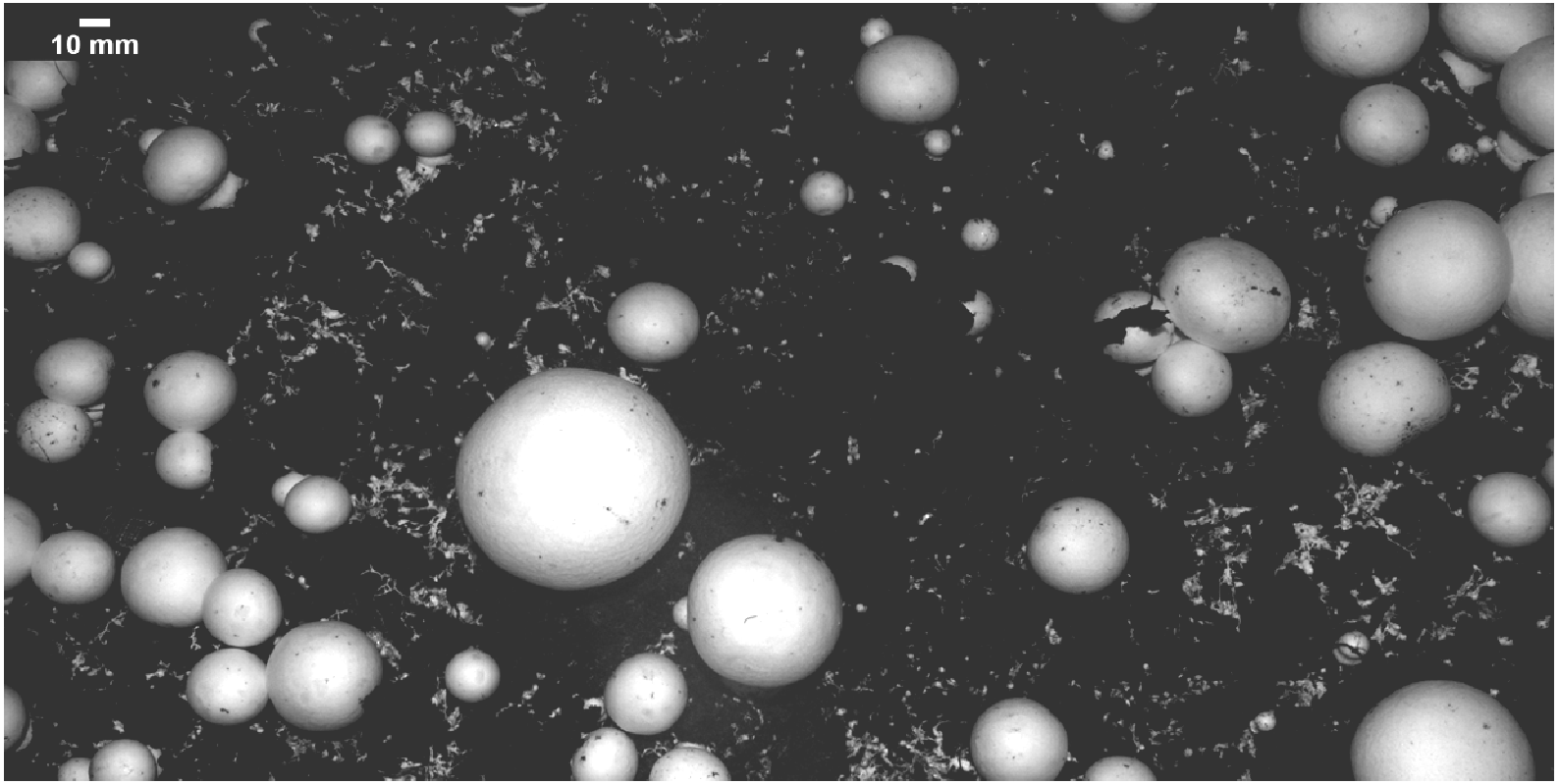


Figure 3-8 Top-view of *A. bisporus* grown under phase change stimulating conditions, 268 h after airing. Various fruit bodies (stages 2-4) have developed in less than 300 h after the change in environmental conditions.

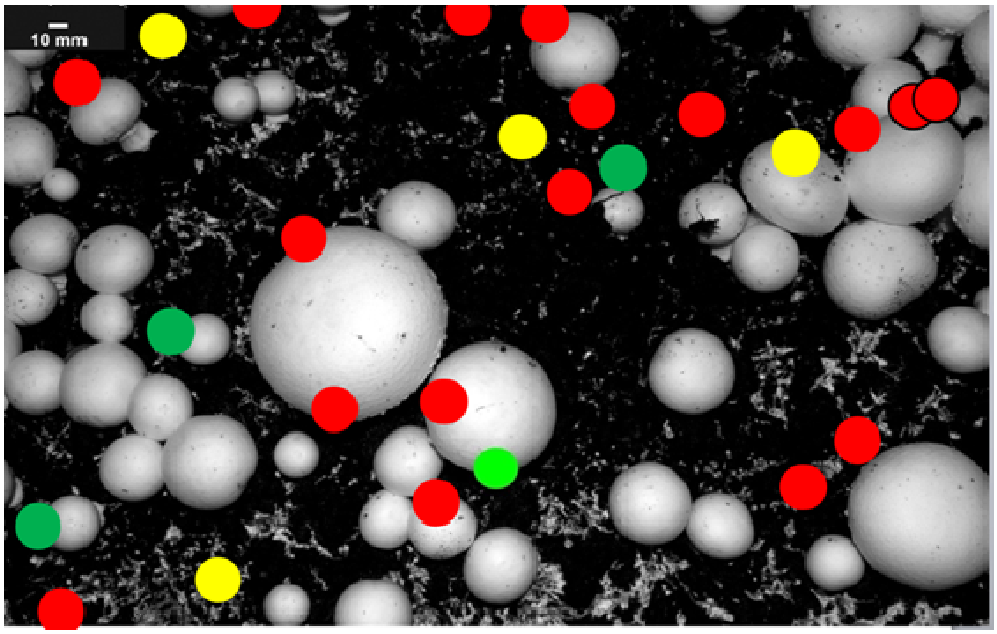


Figure 3-9 Image illustrating the apparently random distribution of undifferentiated and elongated differentiating primordia that are being held-back in their development while other elongated differentiating primordia developed further into fruit bodies. Held-back primordia either continued development at a later time point (green), remained held-back for the duration of the time-lapse recording (red) or aborted (yellow).

At 286 h after airing, the majority of developing fruit bodies had reached stage 3 or further and were removed from the growth tray. This procedure is referred to as the first "flush". Remarkably, shortly after the first flush, a significant number of spherical, smooth undifferentiated primordia broke through cracks in the casing and started to expand as illustrated in Figure 3-10 (A & B). This expansion was also observed for smooth undifferentiated primordia already present on the surface of the casing layer. After this initial expansion around 303 h after airing, the smooth undifferentiated primordia stopped expanding and did not develop further. At this point (335 h after airing) an elongated differentiating primordium broke through the casing layer and continued to develop into a differentiating primordium with distinct waist as illustrated in Figure 3-10 (C).

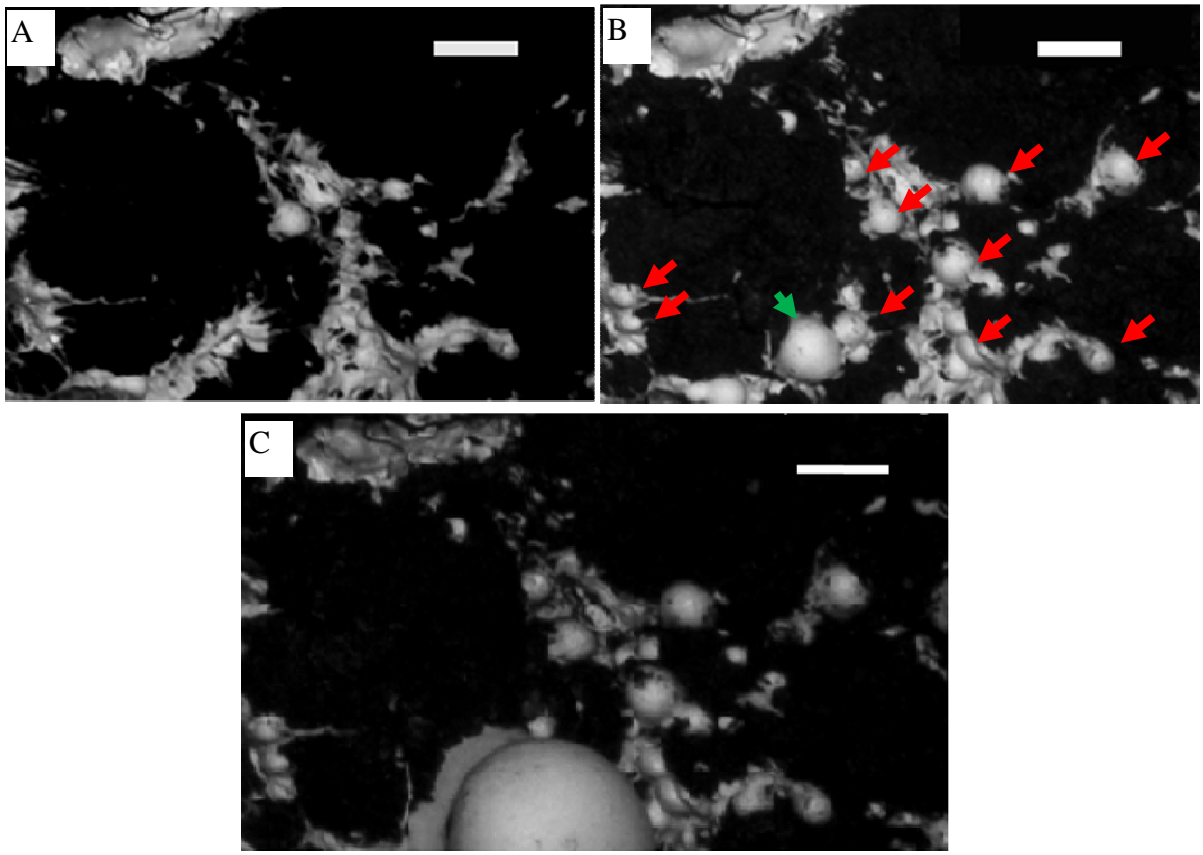


Figure 3-10 Top-view of *A. bisporus* grown under phase change stimulating conditions. Images were taken at (A) 291 h after airing, 5 h after the first flush where fruit bodies were removed, (B) 335 h after airing and (C) 381 h after airing. (B) Multiple smooth undifferentiated primordia (red arrow) originating from both below and on the surface of the casing layer started to expand after the first flush. This expansion stopped while an elongated differentiating primordium (green arrow) was breaking through a crack in the casing layer. Scale bar = 5 mm.

At approximately 350 h after airing, a stage 2 fruit body that was held-back for over 100 h showed initial signs of abortion, noticeable by discoloration and reduction in volume. Figure 3-11 illustrates this process. Two time lapse recording media files illustrating this process are accessible from the DVD attached to the back cover of this thesis and are titled “abortion of a differentiating primordium (top-view) 1 & 2”.

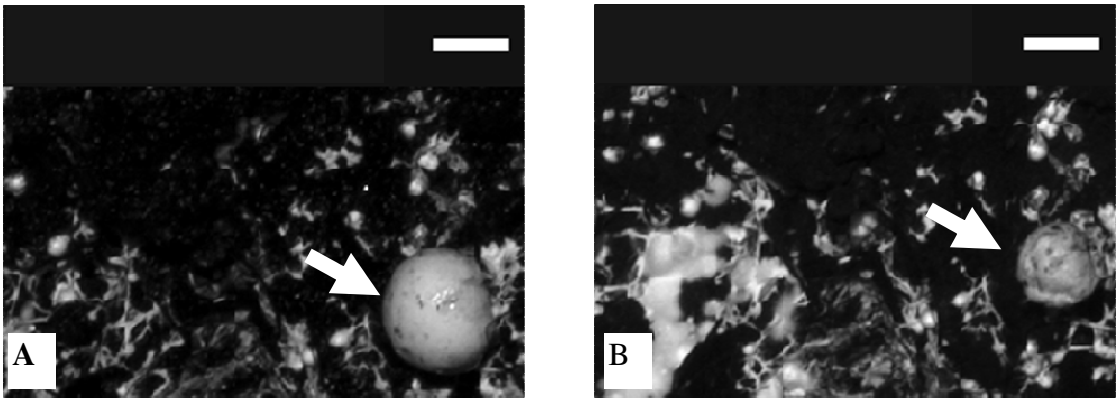


Figure 3-11 Top-view of *A. bisporus* grown under phase change stimulating conditions. At 339 h after airing (A), a small differentiating primordium with distinct waist (red arrow) had developed but its development was held-back at this point. At 447 h after airing (B), the differentiating primordium showed signs of abortion and decay. Scale bar = 10 mm.

3.3.2 Side-view observations of A. bisporus *during the reproductive phase change*

The observations reported in section 3.3.1 were based on a top-view time-lapse. These observations were further confirmed using a side-view time-lapse photography with a mushroom growth experiment in pot-based system. At 140 h after airing, the first three elongated differentiating primordia could be clearly distinguished. Over 25 elongated differentiating primordia broke through cracks of the casing layer during the next 70 hours. At 292 h after airing, various stage 2- 5 fruit bodies covered the surface of the growth pot. At this point, the majority of these fruit bodies were removed (partial first flush) and at 336 hours after airing, the remainder of mature fruit bodies were removed. During the following 50 hours, increased growth activity was observed and multiple smooth undifferentiated primordia and elongated differentiating primordia could be distinguished, originating mostly from within cracks of the casing layer. This activity gradually slowed down and around 384 hours after airing, only a selection of the newly appeared elongated differentiating primordia continued to develop into mature fruit bodies. Similar to the top-view observations, examples of structures that were held-back in their development and ultimately decayed could be found, e.g. a (small) stage 3 fruit body (Hammond & Nichols, 1976) and a differentiated primordium. These

structures ultimately discoloured and decayed as indicated by the red arrows in Figure 3-12.

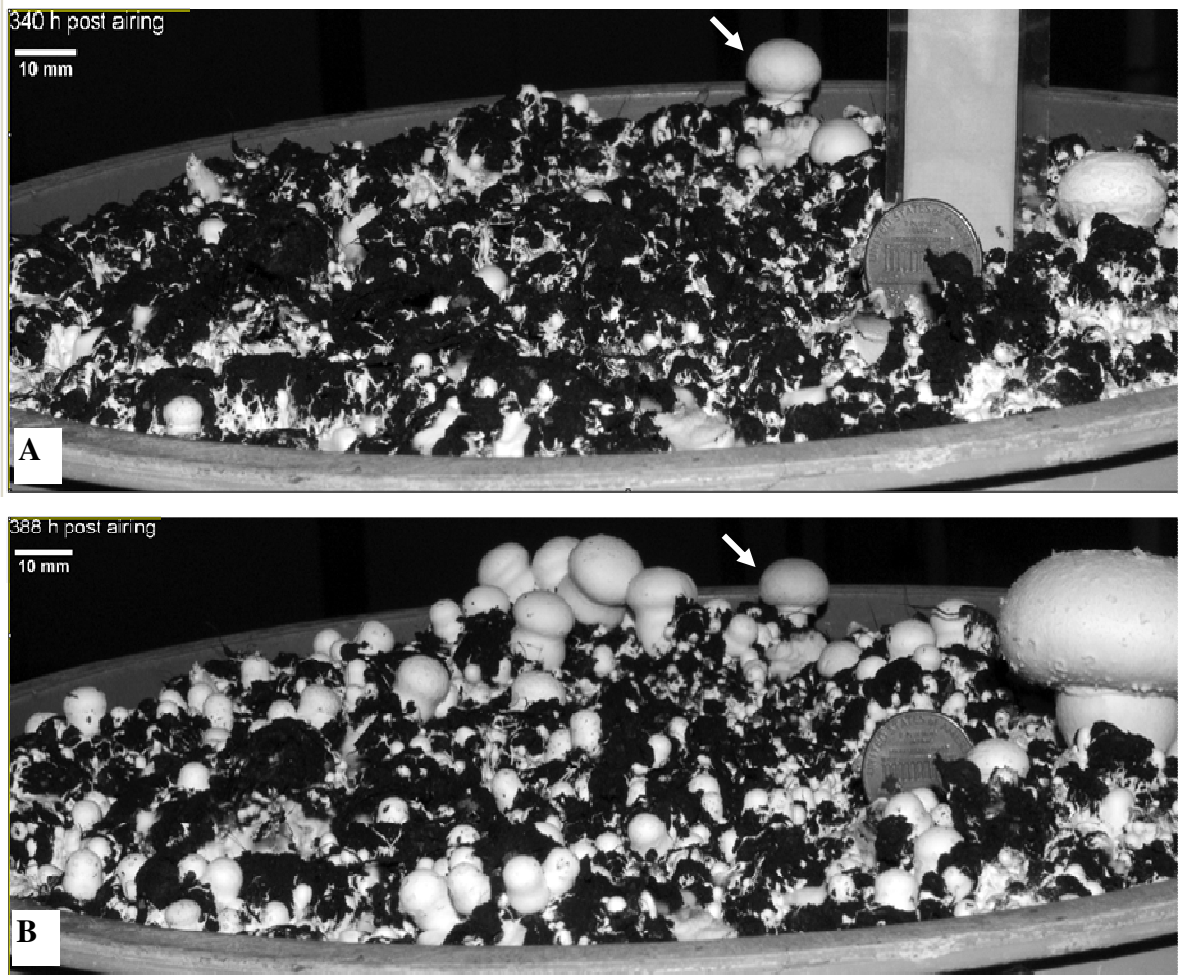


Figure 3-12 Side-view of *A. bisporus* developing under phase change stimulating conditions at (A) 340 h after airing, immediately after removal of mature fruit bodies (flush) and (B) 388 h after airing. In approximately 48 h after the first flush, multiple undifferentiated primordia and elongated differentiating primordia that originally developed beneath the surface of the casing layer, had broken through cracks in the casing layer. A selection of these structures developed into differentiating primordia with a distinct waist. A stage 3 fruit body started to discolour at 340 h after airing and subsequently decayed (red arrows).

3.3.3 Measurements of reproductive morphogenesis

The software ImageJ (http://rsbweb.nih.gov/ij/) was used to measure the diameter and the height of the stipe and the cap during the development of elongated differentiating primordia up to stage 7 fruit bodies at 4 hour intervals. This has proven to be a difficult task as other developing primordia blocked the view after a certain time of the measurements. Figure 3-13 (A) shows the increase in cap (top part) diameter of 7 elongated differentiating primordia while developing into mature fruit bodies (top-view observations). Primordia # 4 and # 5 became distinguishable at the same time as primordia # 1, # 2 and #6 but their development was halted while primordia # 1, # 2 and #6 developed undisturbed to ultimately form stage 2-4 fruit bodies. Primordia #4 and #5 resumed their development in less than 4 h and 8 h respectively after the first flush as illustrated in more detail in Figure 3-13 (B). Remarkably, all these primordia showed similar development patterns, illustrating that primordia #3, #4 and #5 were not negatively affected by being temporarily held-back. Primordium #7 broke through the casing after the first flush and its development was halted until the second flush where primordia #3, #4 and #5 (stage 5-7 fruit bodies at this point) were removed. An indication of renewed development of primordium #7 could be seen at 454 h after airing but could not be measured further as this was the end of the time-lapse observation. A polynomial trend line of the third order with formula $y = 3E\text{-}05x^3 - 0.0186x^2 + 3.6691x - 247.18$ and $R^2 = 0.9992$ showed a good fit to the development curve in Figure 3-13 (A) for primordia that developed immediately after airing while the formula $y = 2E\text{-}05x^3 - 0.0168x^2 + 4.0648x - 317.01$ and $R^2 = 0.9952$ showed a good fit to the development curve for primordia that were temporarily halted in their development.

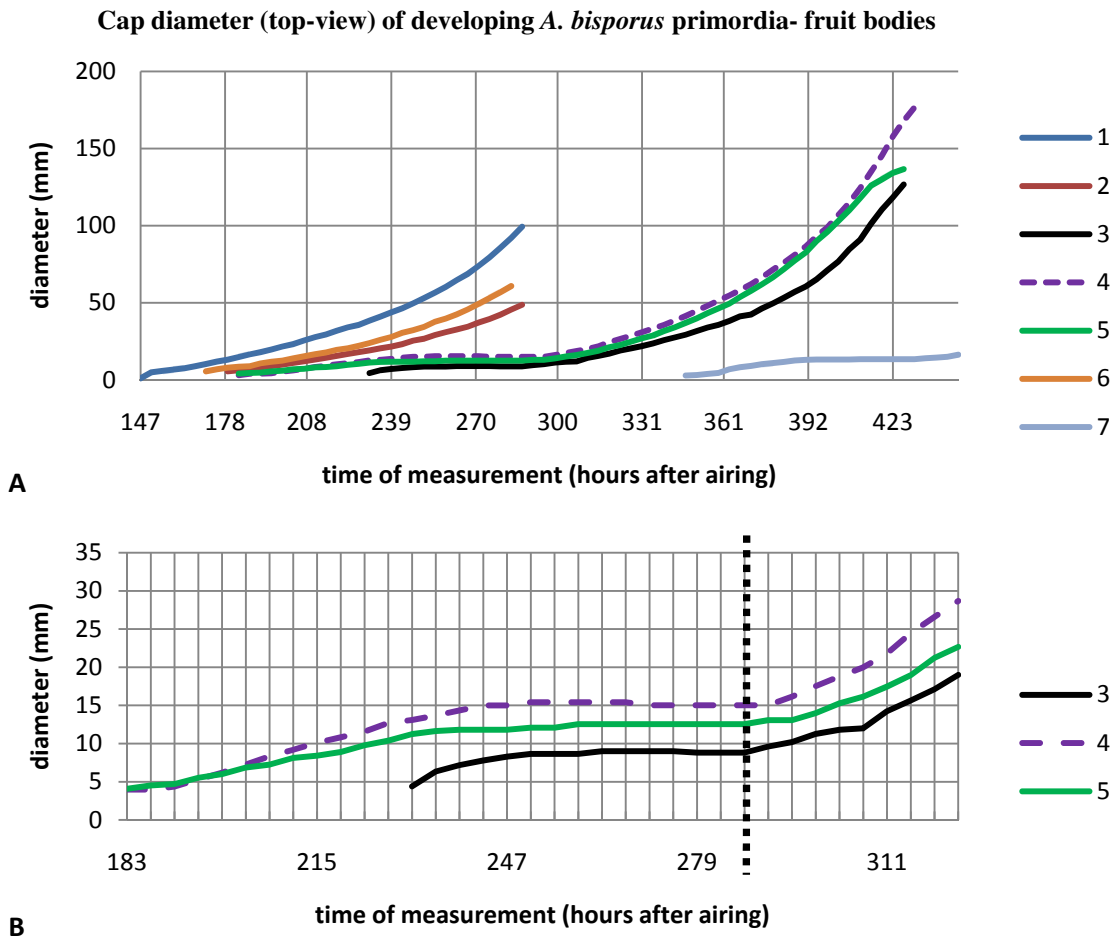


Figure 3-13 Measurements made on (A) 7 developing primordia during the top-view observation series. (B) Detail from graph (A) shows that development of primordia #3 and #4 restarted less than 4 hour after the first flush (287 h after airing, black dotted line) while the development of primordium #5 restarted less than 8 h after the first flush.

The graph in Figure 3-14 shows measurements made of an elongated differentiating primordium developing under phase change stimulation conditions during the side-view observation series. At approximately 10 mm, the stipe and top part (cap) had the same diameter and a waist had developed. The stipe of this differentiating primordium with a distinct waist did not expand much further while the cap underwent a significant growth, similar to the expansions measured in the top-view observation. The resulting fruit body was allowed to mature up to stage 6-7 at which point the veil break

was complete and the cap had flattened to allow release of the spores. The height of the cap showed a less dramatic increase compared to the increase of height of the stipe and the height of the cap even reduced during the flattening observed in stage 7 fruit bodies.

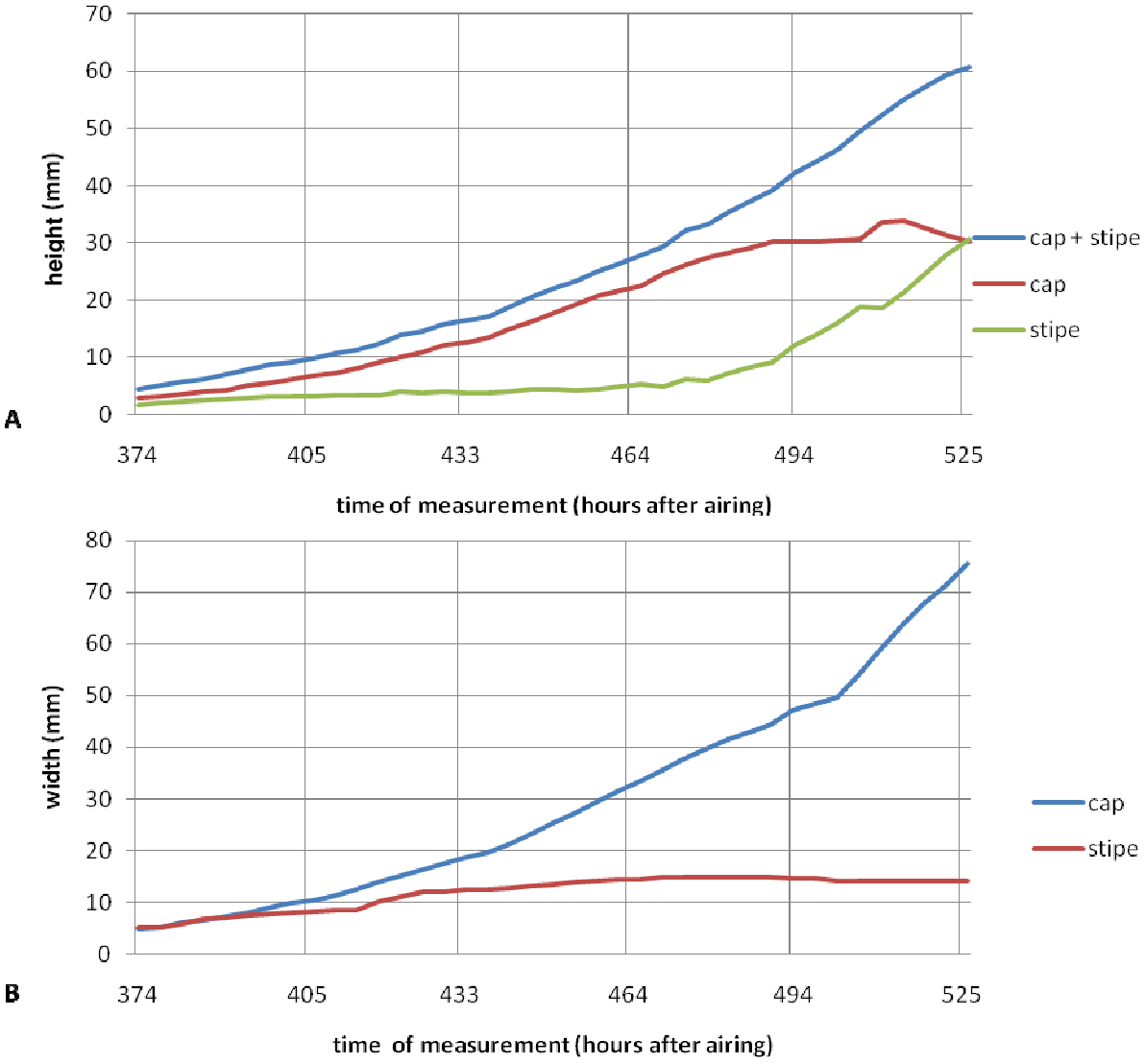


Figure 3-14 Measurements made on the (A) height and (B) width of an elongated differentiating primordium under phase change stimulating conditions. At 525 h after airing, the primordium had developed into a stage 7 fruit body.

3.4 *Investigating the effects of individual environmental parameters on reproductive morphogenesis*

The results of observations made of *A. bisporus* developing under phase change stimulating conditions, a combination of a reduction in temperature and ventilation of the growth room, were discussed in sections 3.2 and 3.3. A further 5 mushroom growth experiments were performed to investigate the effect of individual environmental parameters (CO_2, volatiles and temperature) on reproductive morphogenesis. A photographic record was made of *A. bisporus* response to maintained levels of the three environmental parameters: CO_2 (sections 2.2.2.3 and 2.2.3.4), volatiles (sections 2.2.2.3, 2.2.3.2 and 2.2.3.3) and/ or temperature (section 2.2.2.2), which were compared with a standard treatment control as described in section (sections 2.2.2.1 and 2.2.3.1). Two individual growth experiments were conducted for each treatment, the first was observed to gain an understanding of the system, but was not recorded. Observations made for each of these experiments are discussed in this paragraph. Samples collected during these experiments as described in the sections 2.2.2.4 and 2.2.3.5 were used for gene expression analysis, discussed in Chapter 5.

3.4.1 Effect of high temperature but reduced levels of CO_2 and mushroom volatiles

For this experiment, *A. bisporus* mycelium was allowed to colonise compost and casing at 25 °C in the absence of ventilation. When mycelium was visible on the surface of the casing layer, the growth room was ventilated to reduce levels of CO_2 and mushroom volatiles but the temperature was maintained at 25 °C, as described in section 2.2.2.2.

Observations made during this experiment are illustrated in Figure 3-15 A (72 h after airing), Figure 3-15 B (168 h after airing), Figure 3-16 A (268 h after airing) and Figure 3-16 B (304 h after airing). Around 72 h after airing, large parts of the casing layer were covered by a thick mat of mycelium, also referred to as “stroma”. A significant number of **fluffy hyphal knots** had developed on mycelium covering the surface of the casing layer. Around 168 h after airing, large numbers of **smooth undifferentiated primordia** developed. No **elongated differentiating primordia** were

seen throughout the experiment. Around 304 h after airing, a brown discoloration was observed indicating potential decay of the fungal biomass. Similar results were observed using the flask-based system with controlled gas-supply described in 2.2.3, using the same environmental conditions as illustrated in Figure 3-17. Five mushroom growth beds were at this point transferred to a room which was ventilated and at 18 °C. Fruit bodies developed in the following 200 hours (image not shown).

The outcome of this experiment is of significant value to our understanding of the phase change process and subsequent reproductive growth. The reduction of levels of CO_2 and mushroom volatiles by ventilation of the growth room or flask-based system was sufficient to initiate the phase change and **smooth undifferentiated primordia** developed. However, maintaining the temperature at 25 °C prevented the differentiation of these structures and no **elongated differentiating primordia** developed. Five mushroom growth trays were then transferred into a growth room which was ventilated and at 18 °C. Around 200 h later, normal fruit bodies developed on these trays.

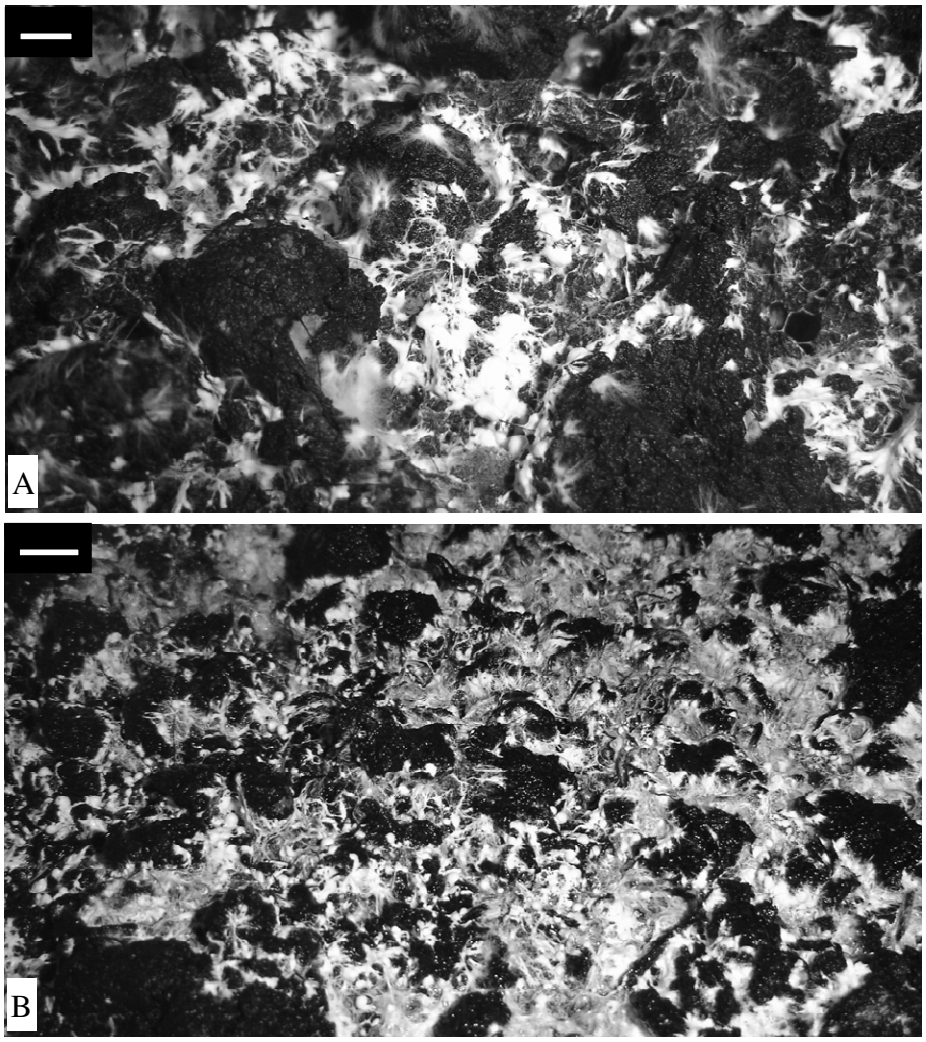


Figure 3-15 *A. bisporus* grown in a mushroom growth tray under high temperature while the room was ventilated to reduce levels of CO_2 and mushroom volatiles. (A) 72 h after airing. Scale bar = 5 mm. (B) 168h after airing. No elongated differentiating primordia had developed, in contrast to *A. bisporus* grown under phase change stimulating conditions. Scale bar = 10 mm.

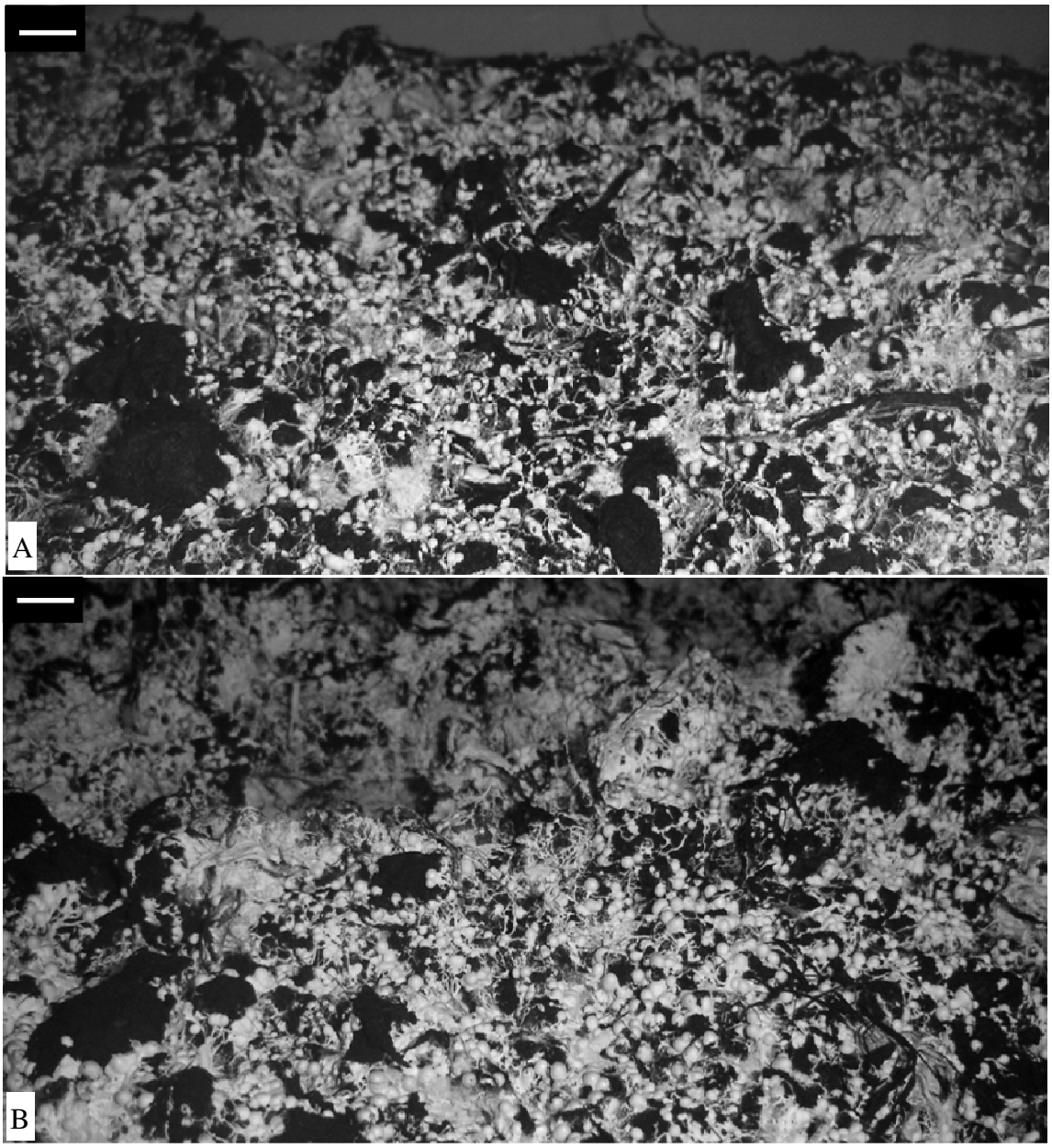


Figure 3-16 *A. bisporus* grown in a mushroom growth tray under high temperature while the room was ventilated to reduce levels of CO_2 mushroom volatiles. (A) 268 h after airing. Scale bar = 10 mm. (B) 304 h after airing. Scale bar = 10 mm. At 304 h after airing, a brown discoloration of the mycelium covering the casing layer was observed. No elongated differentiating primordia had developed.

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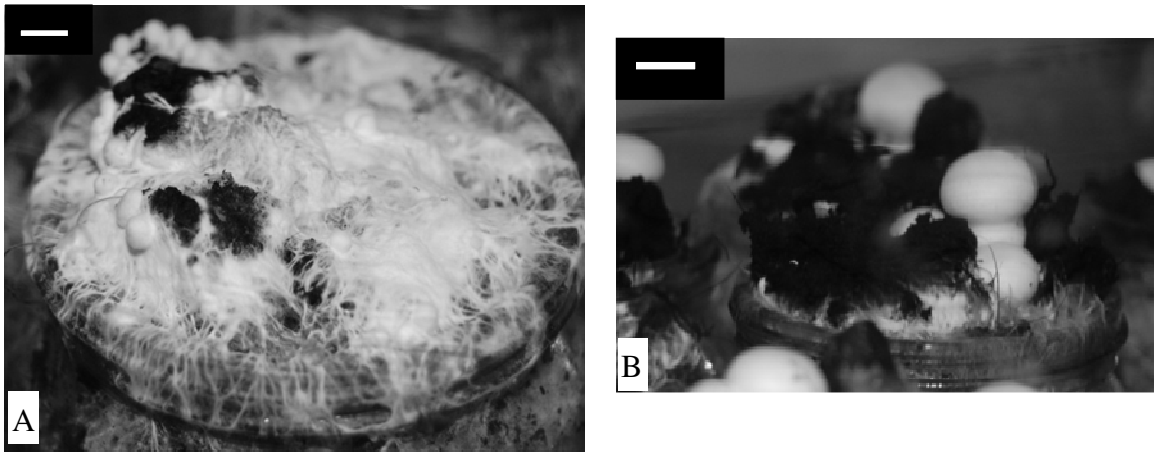


Figure 3-17 ***A. bisporus*** **grown in a flask-based system with controlled air-supply under high temperature but reduced levels of CO_2 and mushroom volatiles. (A) At 268 h after airing, the casing layer of the Kilner jars was covered by a thick mat of mycelium and multiple smooth undifferentiated primordia had developed. Scale bar = 5 mm. This was in contrast to standard growth conditions experiment (B) where the temperature was reduced and the growth system was ventilated as multiple stage 2 fruit bodies had developed. Scale bar = 10 mm.**

3.4.2 Effect of high levels of CO_2 and mushroom volatiles but reduced temperature

A. bisporus mycelium was allowed to colonise compost and casing while grown at 25°C in the absence of ventilation. When mycelium was visible on the surface of the casing layer in the growth trays, the temperature was reduced to 18°C but the growth room was not ventilated, as described in section 2.2.2.3. Paper sheets were used to cover the growth trays in order to maintain high CO_2 and volatile levels inside the casing layer.

Observations made during this experiment are illustrated in Figure 3-18 (72 h after airing), Figure 3-18 A (168 h after airing) and Figure 3-19 B (304 h after airing). At 72 h after airing, the casing layer of the growth trays was covered by a fine web of mycelium and a few fluffy hyphal knots could be distinguished. This situation remained very similar up to 304 h after change in environmental conditions. Beyond this time

point a small number of **fluffy hyphal knots** could be distinguished and a few **stage 2 fruit bodies** had developed on the edges of the growth trays, which were not completely covered by paper sheets. The results of this experiment are a clear contrast to the control experiment as the centre of the growth trays did not show any distinct sign of the phase change, indicating that a reduction of the levels of CO_2 and mushroom volatiles is required to allow the development of **fluffy undifferentiated primordia**. In contrast to observations made in section 3.4.1 (maintained high temperature, reduced levels of CO_2 volatiles), the results of this experiment were not absolute and two experiments were designed to investigate the individual effects of CO_2 and volatile levels on the phase change and subsequent reproductive growth.

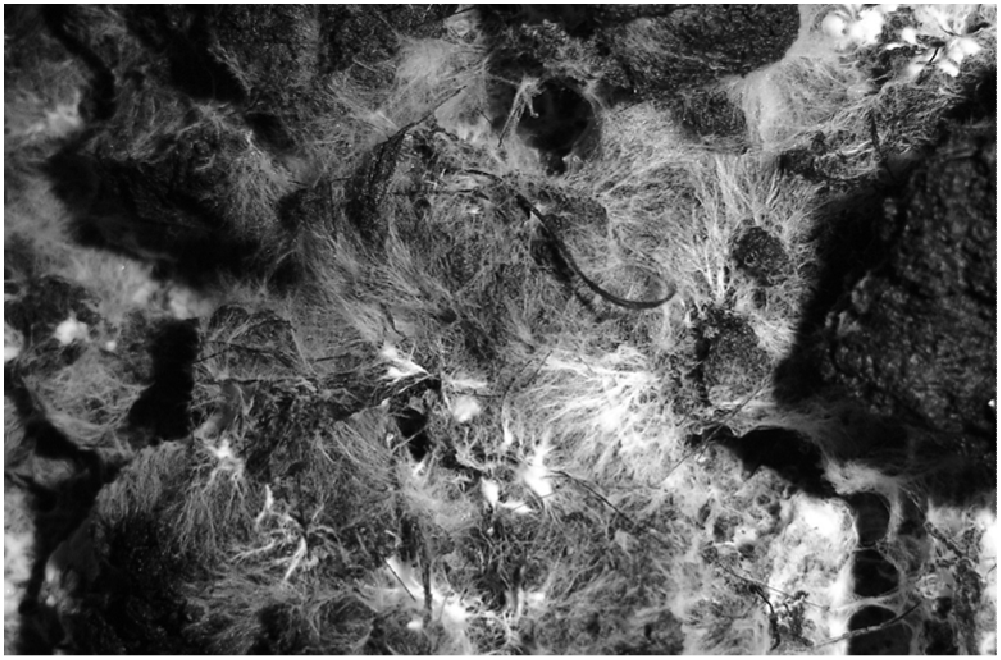


Figure 3-18 *A. bisporus* grown in a mushroom growth tray under high levels of CO_2 and mushroom volatiles while the temperature was reduced to 18 °C. Image was taken at 72 h after airing.

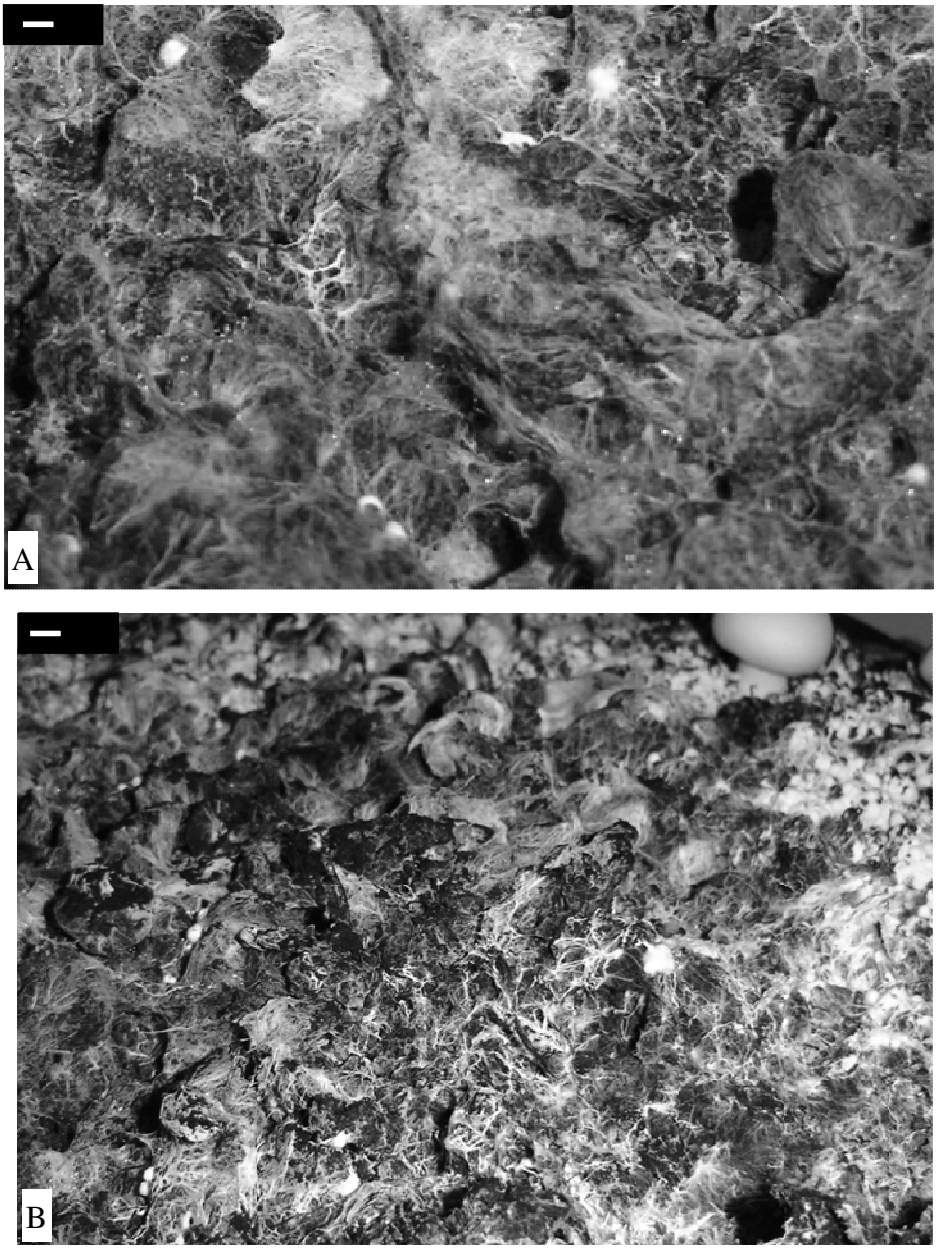


Figure 3-19 *A. bisporus* grown in a mushroom growth tray under high levels of CO_2 and mushroom volatiles while the temperature was reduced to 18 °C. (A) At 168 h after airing, a small number of fluffy hyphal knots or localised thickening of hyphae could be observed. Scale bar = 0.5 mm. (B) At 304 h airing, the mycelium in the centre of the growth tray had a fine stranded appearance with exception of the development of a few fluffy hyphal knots. Scale bar = 2 mm. A small number of smooth undifferentiated primordia and stage 2 fruit bodies had developed on the edges of the growth trays, which were not completely covered by paper sheets.

3.4.3 Effect of high level of 1-octen-3-ol but reduced level of CO_2 and reduced temperature

A. bisporus mycelium was allowed to colonise compost and casing in Kilner jars (flask-based growth system) at 25 °C in the absence of ventilation. When mycelium was visible on the surface of the casing layer in the Kilner jars, the temperature was reduced to 18 °C and the growth flasks were ventilated. A continuous supply of the mushroom volatile 1-octen-3-ol was added to the airflow as described in section 2.2.3.2. This eight carbon volatile, 1-octen-3-ol is known to influence fungal morphogenesis in various fungi, including *A. bisporus*, as described previously in section 1.4.2.2. The flask-based cultivation system used is suitable for controlled mushroom growth as described in 1.4.2.2.

The outcome of this experiment was very dramatic as reproductive growth appeared to be completely inhibited as illustrated in Figure 3-20. At 192 h after airing, there was no indication of reproductive morphogenesis while stage 3 fruit bodies had developed in the flask-based growth system under phase change stimulating conditions. The addition of 1-octen-3-ol in a concentration of 2.1 mg L^{-1} air was capable of completely inhibiting the phase change, even though the temperature and CO_2 level were reduced to phase change stimulating levels. The reversibility of this inhibition of development was investigated in section 3.4.4, where the volatile source was removed from the air-supply.

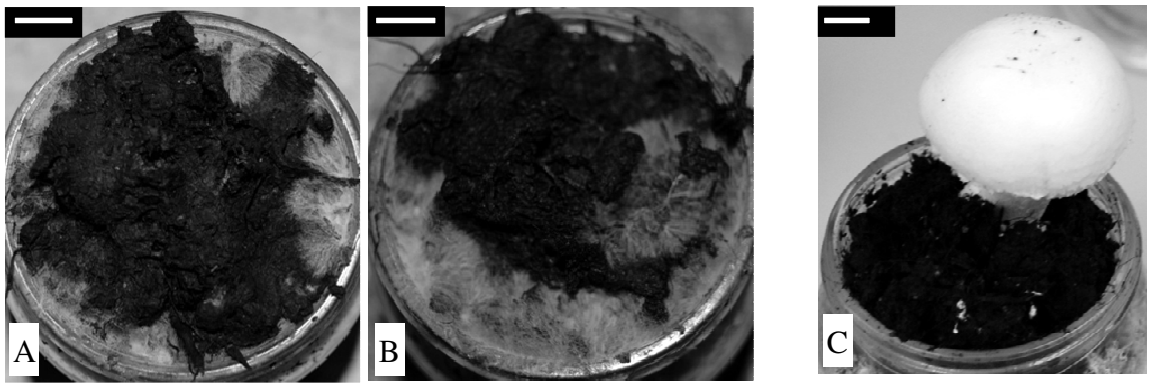


Figure 3-20 *A. bisporus* grown in a flask-based system with controlled gas supply under high level of 1-octen-3-ol but reduced level of CO_2 and reduced temperature. (A) 24 h after airing. (B) 192 h after airing. No development related to reproductive growth could be observed, in contrast to (C) *A. bisporus* grown under phase change stimulating conditions (image taken at 240 h after airing). Scale bar = 10 mm.

3.4.4 Investigating the reversibility of 1-octen-3-ol-related inhibition of reproductive development

This experiment was the continuation of the experiment described in section 3.5 where 1-octen-3-ol was initially added to the air-supply while temperature and level of CO_2 were reduced. However, at 192 h after airing, the start of this experiment, the volatile source was removed from the air-supply as described in section 2.2.3.3. This reduction of the level of 1-octen-3-ol initiated reproductive growth as illustrated in Figure 3-21. Normal stage 2 fruit bodies developed after 168 h, illustrating the reversibility of the 1-octen-3-ol-related inhibition of reproductive development.

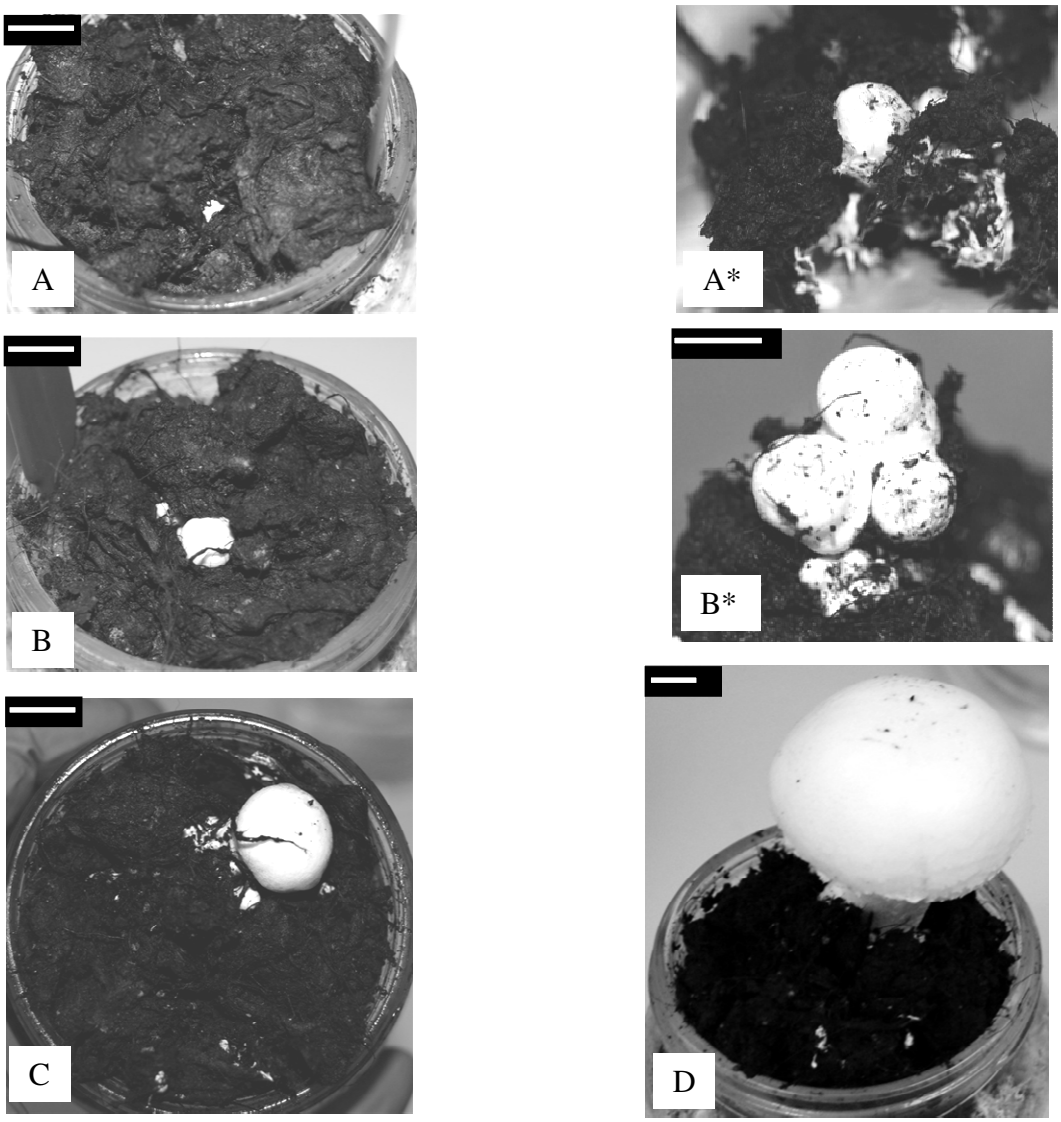


Figure 3-21 *A. bisporus* grown in a flask-based system with controlled air-supply after an initial exposure of 192 h to elevated 1-octen-3-ol levels but reduced temperature and reduced CO_2 level. The 1-octen-3-ol source was removed from the air-supply at 192 h after airing. (A) 72 h after removal of the volatile source. (B) 120 h after removal of the volatile source. (C) 168 h after removal of the volatile source. (D) 240 h after removal of the volatile source. "*" indicates where structures were removed from the casing to allow a more detailed observation. Scale bar = 10 mm.

3.4.5 Effect of high level of CO_2 but reduced level of mushroom volatiles and reduced temperature

A. bisporus mycelium was allowed to colonise compost and casing in Kilner jars (flask-based growth system) at 25 °C in the absence of ventilation. When the mycelium was visible on the surface of the casing layer, the temperature was reduced to 18 °C and the flask-based system was ventilated. In addition to this, a continuous supply of CO_2 was added to the airflow as described in section 2.2.3.4 to create a CO_2 concentration of ~ 5,000 p.p.m.

Observations made during this experiment are described in Figure 3-22 . The results of this experiment showed that normal fruit bodies developed when exposed to high level of CO_2 but at a significantly lower frequency, less than one third, compared to *A. bisporus* grown under phase change stimulating conditions in the flask-based system. The resulting fruit bodies were comparable in size and structure to fruit bodies from the control experiment.

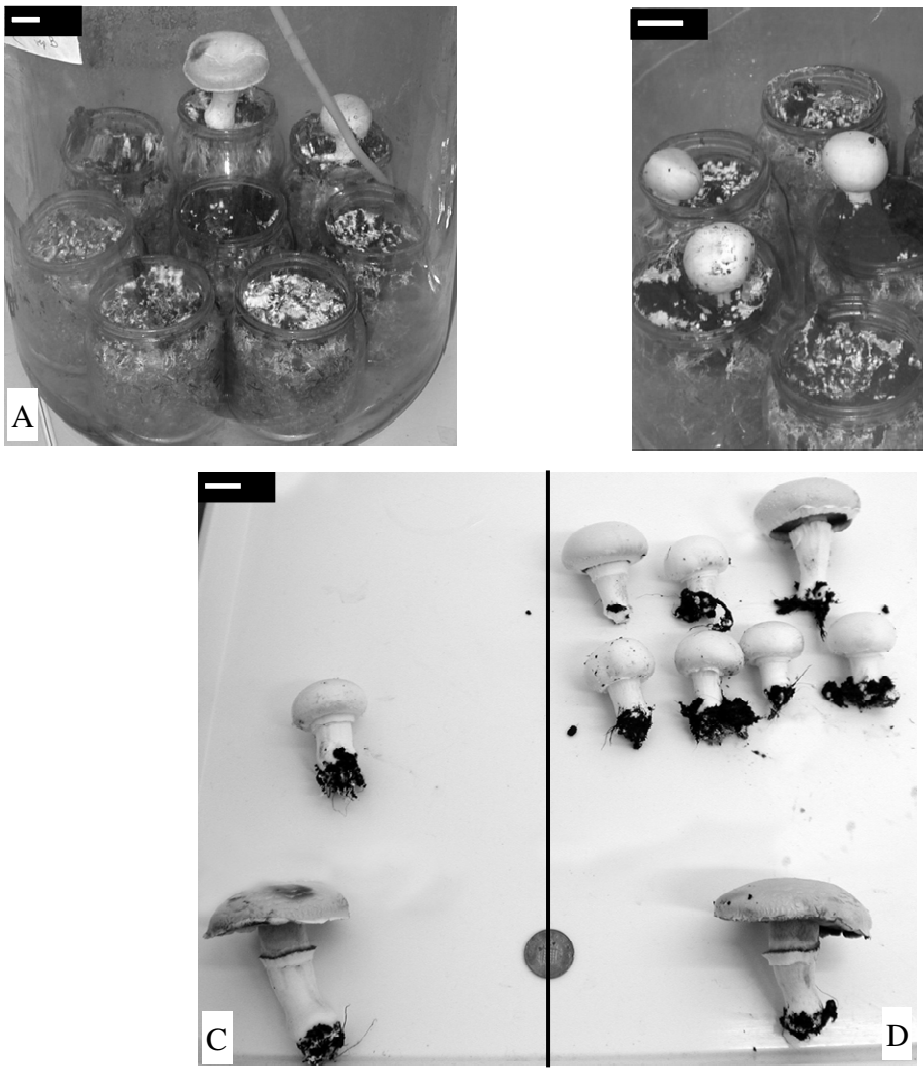


Figure 3-22 *A. bisporus* grown in a flask-based system with controlled air-supply under high level of CO_2 (≥ 5,000 p.p.m.) but reduced temperature and reduced level of mushroom volatiles. At 264 h after airing, (A & C) two fruit bodies (stage 3 and 5) had developed. This is 3-fold less compared to the results from (B & D) the standard cultivation conditions experiment where the growth system was incubated at 18 °C and ventilated to reduce the levels of CO_2 (< 1,000 p.p.m.) and mushroom volatiles. Scale bar = 20 mm.

3.5 *Discussion*

This is the first study of the phase change from vegetative to reproductive growth in *A. bisporus* in over 30 years. Modern technology such as controlled growth systems and automated time-lapse photography made it possible to analyse this process in a 4-dimensional way.

Microscopic and time-lapse observations of *A. bisporus* were used to identify and define key morphological stages that occur during the development from vegetative mycelium into mature, spore bearing fruit bodies. For this purpose, *A. bisporus* was inoculated in compost, covered by a peat casing layer. The growth trays were incubated at 25 °C and in the absence of ventilation to stimulate vegetative growth. Under these conditions, the mycelial hyphae rapidly colonised the growth substrate and casing layer. The lack of nutrients in the casing layer promoted the aggregation of individual hyphae into vegetative, **mycelial cords**. At this point, a change in environmental conditions was applied to stimulate the phase change. This procedure, referred to as "airing", involved 1) a reduction of the temperature to 18 °C and 2) ventilation of the growth trays to reduce the level of carbon dioxide below 1,000 p.p.m and to reduce the level of mushroom volatiles. The vegetative mycelium then underwent several stages of development:

- **fluffy mycelial cord** (from 32 h after airing, ~ 60 µ mm diameter): heavy localised branching of mycelial cords and hyphae gave them a fluffy appearance;
- **fluffy hyphal knot** (from 48 h after airing, ~ 0.5-1.0 mm diameter): further localised branching of a single or multiple neighbouring fluffy mycelial cords resulted in fluffy, two-dimensional aggregations of hyphae;
- **fluffy undifferentiated primordium** (from 95 h after airing, ~ 1-2 mm diameter): fluffy hyphal knots developed gradually to form fluffy, three-dimensional spherical structures;
- **smooth undifferentiated primordium** (from 120 h after airing, ~ 2-4 mm diameter): fluffy undifferentiated primordia increased in size and lost their fluffy appearance;

- **elongated differentiating primordium** (from 144 h after airing, base part is ~ 4-10 mm in diameter): the top part of smooth undifferentiated primordia started to elongate and as a result, elongated differentiating primordia that initially developed below the surface of the casing layer started to break through cracks on surface of the casing layer. The top part of the primordium has a smaller diameter than the base part at this stage. This structure was previously defined as a stage 1 fruit body by Hammond & Nichols (1976);
- **differentiating primordium with a distinct waist** (from 200 after airing, ≥ 10 mm diameter): elongated differentiating primordia gradually increased in size and the top (cap) part expanded faster than the base part. When the top part had the same diameter as the base part, a distinct region of reduced diameter, referred to as a waist, had developed between these two parts. Craig & Gull (1977) and Craig *et al*. (1979) described that internally, structures of this size have a final mushroom-like form where the hypae in the stipe are predominantly oriented vertically, and those of the cap radially. Gill tissue started to differentiate in the cap tissue at this stage;
- **stage 2-7 fruit bodies**: differentiating primordia with a distinct waist developed and expanded further into mature fruit bodies. Gill tissue was differentiated in stage 2 fruit bodies. The veil covering the gill tissue started to break in stage 4 fruit bodies. The maturation of the fruit bodies was complete at stage 7 as defined by Hammond & Nichols (1976).

These developmental stages only occur under specific environmental conditions, which consist of a change in temperature and gaseous headspace composition. Five growth experiments using a mushroom house and the flasks-based growth system with controlled air-supply were performed to separate and study the effects of individual environmental parameters on reproductive morphogenesis. These experiments revealed that primordia development is controlled by 3 environmental parameters which appear to work in different ways. Two of these parameters are on/off switches and one is a quantitative regulator:

- High level of mushroom volatiles inhibits localised branching of mycelial cords which meant that no **fluffy mycelial cords** or subsequent stages developed. This inhibition is "absolute" as the phase change was not initiated even though the temperature and CO_2 level were reduced to phase change stimulating levels. Removal of the volatile source resulted in heavy localised branching of the mycelium (**fluffy mycelial cords**) and subsequent development. It is therefore possible to state that mushroom volatiles are an on/off switch that affects the initiation of the phase change;
- The second switch is temperature. A reduction of the level of mushroom volatiles at airing stimulates the development of **fluffy mycelial cords**, followed by the development of **fluffy undifferentiated primordia** and later **smooth undifferentiated primordia**. When the temperature was maintained at 25 °C, no further differentiation could be observed as the development of **elongated differentiating primordia** and development of subsequent development of fruit bodies was inhibited. A subsequent reduction of the temperature to 18°C initiated development of **elongated differentiating primordia.** Temperature therefore acts as a second on/off switch as it controls the development from **smooth undifferentiated primordia** into **elongated differentiating primordia**.
- The third parameter is carbon dioxide. Low level of CO_2 (< 1,000 p.p.m) promotes the formation of high numbers of fruit bodies while high level of CO_2 (≥ 5,000 p.p.m) greatly reduces the number of developing fruit bodies. This indicates that carbon dioxide acts as a quantitative regulator.

These findings have been summarised in Figure 3-24. It is yet unknown whether these switches are involved with overcoming inhibition or stimulating further development. Based on the stages regulated by these switches, the phase change process could be divided into an "early phase change", initiated by a reduction in the level of mushroom volatiles and a "late phase change", initiated by a reduction in temperature (after the original reduction in volatile levels).

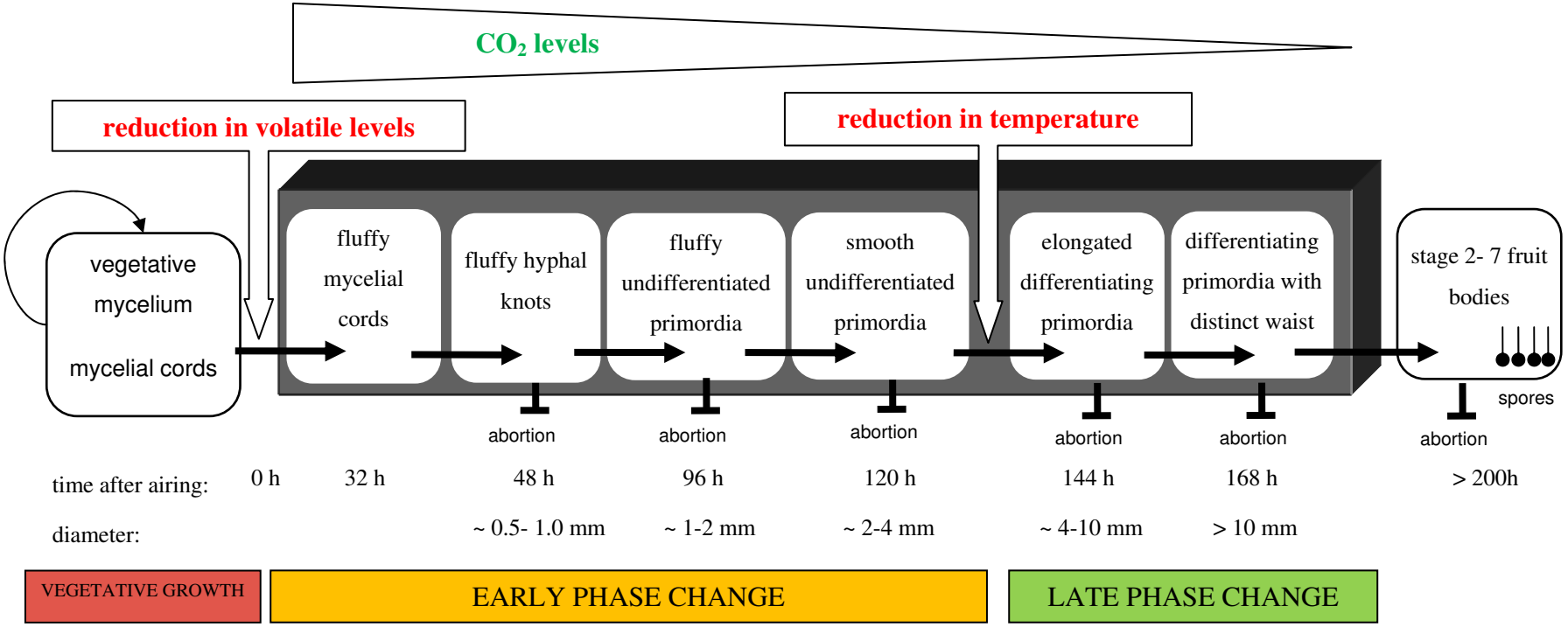


Figure 3-23 Updated "black box" representing the key morphological stages observed during the phase change from vegetative to reproductive growth in *A. bisporus*. This process is controlled by 2 on/off switches (red text) and one quantitative regulator (green text).

The top- and side-view observations of *A. bisporus* were further used to investigate the location and distribution of developing reproductive structures and their fate. A detailed analysis of these images revealed that elongated differentiating primordia that developed subsequently into mature fruit bodies originally developed below the surface of the casing layer. Cross-sections of the casing, as illustrated in Figure 3-24 confirmed the presence of mycelial cords, fluffy hyphal knots, fluffy and smooth undifferentiated primordia, and elongated differentiating primordia inside the casing layer.

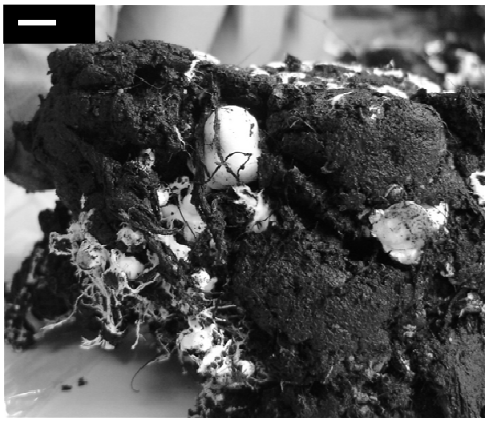


Figure 3-24 Cross-section of a colonised casing sample taken from a growth tray of *A. bisporus* developing under phase change stimulating conditions. This section confirms that various developmental stages can be found below the surface of the casing layer. Scale bar = 10 mm.

In addition to this, none of the smooth undifferentiated primordia that developed on mycelial cords present on the surface of the casing layer differentiated and developed further. The reason why primordia, originating from mycelium present on the surface of the casing, do not differentiate is yet unknown. This has not been reported previously for *A. bisporus* and has a significant impact on our investigation into reproductive morphogenesis of this fungus as primordia that develop directly on the surface of the casing layer are not submitted to high CO_2 and volatile levels prior to the application of phase change stimulating conditions, in contrast to mycelium inside cracks of the casing layer.

It could be argued that these three parameters were adopted by the fungus as a means of identifying the optimal environmental conditions for reproductive development. The breakdown of fatty acids might be to produce biologically important eight carbon volatiles (ECVs) as discussed in section 1.4.2.2. This work has shown that the most favourable place for early primordia development is located inside cracks in the casing layer. The level of ECVs produced by the mycelium is most likely lower in these cracks than around buried cells due to gas exchange with the surface atmosphere. It was also confirmed that an increased level of 1-octen-3-ol completely inhibited the early phase change (Noble *et al.*, 2009). A reduction of the level of this volatile by removal of the external 1-octen-3-ol source and ventilation was sufficient to initiate intense, localised branching followed by subsequent early phase change development. This could be a method of the fungus to detect the best route to the surface, along with the concentration of other volatiles such as CO_2. Geotaxis has not been demonstrated in *A. bisporus* so the concentration of volatiles could also be used to identify the direction of elongation of the primordium and subsequently, the stipe and cap. The mechanism of this regulation is not yet identified. It was previously suggested that *Pseudomonas putida*, found in the casing layer, reduces the level of mushroom volatiles and therefore stimulates the phase change (Flegg *et al.*, 1985). The role of this bacterium is currently under discussion (section 1.4.2.2). Their volatile metabolising properties can be replaced by ventilation or absorbents such as activated charcoal as demonstrated by Noble *et al.* (2003; 2009).

A reduction in temperature is required to initiate the late phase change (elongation and differentiation of primordia). Fungi such as *A. bisporus* might have associated this reduction in temperature with increased availability of nutrients (autumn) and used this environmental change to guide their development. *A. bisporus* was studied here in a well-controlled environment, similar to commercial cultivation, but recent work (Gange *et al.*, 2007) has shown that changes in climate, in particular temperature, have a dramatic effect on the fruiting of fungi inhabiting deciduous woods. Fungi that inhabit coniferous woods are not affected by this change.

The role of carbon dioxide on regulation of the phase change is less obvious. The Carbon dioxide is likely to accumulate in the soil of forests where microbial activity (initial degradation of leaf litter) increases and stimulates hypal growth and substrate colonisation. A reduction of the carbon dioxide was shown to stimulate the phase change. This reduction could occur closer to the surface of the soil and in places not covered by a thick layer of leaf litter, making spore dispersal more efficient, similar to the effect seen for other volatiles such as ECVs as described previously.

The time-lapse observations also revealed a synchronised development of elongated differentiating primordia breaking through the casing layer between 140 h and 168 h after airing. The mechanism of this synchronisation is not yet identified but it could be suggested that these structures detected the change of environmental conditions at similar time points and that the response of the fungal network to those changes and subsequent developmental processes might be co-ordinated.

This novel information has a significant impact on the validity of previous studies, made on the hypothesis that any "primordial structure" present on the surface of the casing layer would have the ability to develop into a fruit body. This hypothesis was followed e.g. by Flegg (1979; 1979), who counted the number of "primordial structures" on the surface of the casing layer to investigate the effect of temperature and competition on fruit body development. Umar and Van Griensven (1999) indicated that less than 5 % of the "primordial structures" present on the surface of the casing layer developed into mature fruit bodies. However, they did not identify that the primordia that developed below the surface layer are more likely to form mature fruit bodies then primordia that developed below the surface layer. It was also observed that elongated differentiating primordia, which originally developed below the surface of the casing layer, can be held-back in their development. These held-back primordia are positioned in a seemingly random way, with no apparent preference in terms of position or proximity to developing fruit bodies. Some of these held-back primordia resumed their development in less than 4 hours after the removal of the mature fruit bodies present on the growth tray (flush). The speed of this response is remarkable as development of these structures was halted for over 50 h in some cases. It could be suggested that the

flow of nutrients and water supporting the developing fruit bodies is re-routed after the flush to drive further development in these halted structures, rather than regulation of development at a molecular level. It was previously suggested that a movement of nutrients and liquids exists in a fungal colony and is dynamic (Tlalka *et al.*, 2008; Watkinson *et al.*, 2005). This suggestion can also help to explain the expansion of numerous smooth undifferentiated primordia immediately after a flush. The majority of undifferentiated primordia will not form elongated differentiating primordia.

Fruit body autolysis is not uncommon in fungi. The primordia of *Coprinus macrorhizus* autolyse when transferred from continuous light exposure to continuous darkness. In contrast to *Coprinus*, there is no obvious (external) stimulus in *A. bisporus* which could explain the abortion of certain primordia or fruit bodies. Analysis of the time-lapse observations also showed that the occurrence of aborting fruit bodies is minimal compared to the frequency of normal fruit body development and the occurrence of fruit bodies that are temporarily halted in their development. Burton *et al.* (1994) investigated serine protease activity during fruiting and found that protease levels in fruit bodies fluctuated during a crop but peaked when increased abortion activity of stage 1 fruit bodies was observed. They suggested that this activity could play a role in abortion and autolysis. The mechanism and conditions which trigger this process are unknown.

The time-lapse images created during this project were mainly used to identify and investigate developmental patterns on a spatial and temporal level. The contrast of the white mycelium versus the darker casing made it possible to distinguish hyphae, mycelial cords and morphological development on the surface of the casing layer. Manual measurements were made using the software ImageJ on a number of fruit bodies, at 1 h intervals, over the course of two growth experiments as described in this chapter. These measurements suggested that each fruit body exhibits a similar development and growth speed and that the (temporary) halt of development and growth seen in some fruit bodies can be lifted in a matter of hours, after the removal of other developing fruit bodies. An automated analysis of the time-lapse data would strengthen

this data further. A meeting with Professor Roland Wilson (University of Warwick, Image and Signal Processing Group) who has specialised in the development of algorithms to identify and quantify changes and patterns (e.g. elongation of an undifferentiated primordia) in time-lapse photography was therefore organised. During this meeting it was concluded that it would be possible to develop an algorithm to analyse time-lapse images of *A. bisporus* development on the surface of a growth tray. This would however take significant time and input from the Image and Signal Processing group. For this reason it was decided not to pursue this during the current project. The development and application of an automated image analysis algorithm could however be considered for a future project. Information from in-depth growth analysis of *A. bisporus* might identify developmental patterns that could indicate continuous development or a halt of growth / abortion. This would allow the researcher to identify individual, developing or aborting fruit bodies which could be used for a more targeted gene-expression or biochemical analysis.

Chapter 4. Development of resources and methodologies: RNA extraction from peat casing, microarray design and validation and bioinformatics

Simultaneous examination of the gene expression profiles of thousands of genes in a sample representing the fungus at a certain time point can be achieved thanks to the maturation of high-throughput gene expression profiling technologies such as microarrays. Microarray technology has been used to study e.g. metabolism, development, pathogenesis in a number of filamentous fungi including *Aspergillus nidulans, Phanerochaete chrysosporium, S. macrospora, Neurospora crassa* (Breakspear & Momany, 2007) and has shown to be a great tool to investigate these processes. The oligonucleotide gene expression microarray platform from Agilent Technologies was chosen to investigate changes in gene expression of *A. bisporus* grown under standard and experimental growth conditions. This technology eliminates the need for the construction of a cDNA library as short oligonucleotides (probes) representing sequences of interest are synthesised directly on the microarray. This technology also allows the use of 60-mer oligonucleotide probes which offer a much higher sensitivity-specificity balance than other platforms which use shorter oligonucleotides (Hughes *et al.*, 2001).

Comparison of the gene expression profiles of the growth experiments performed during this project was expected to give an insight into the molecular mechanism that controls and supports the phase change from vegetative to reproductive growth and the effects of various environmental parameters on this process. However, before this gene expression analysis could be performed, various resources and methodologies had to be developed. These resources and tools are:

1) optimisation of a method to extract un-degraded, inhibitor free total RNA from humic acid-rich colonised casing samples. The development of this resource is described in section 4.1;

2) development of an *A. bisporus* sequence database. At the time of writing, the genome of *A. bisporus* had not been sequenced. Therefore, sequences related to *A. bisporus* had to be collected and collated in a database. The development of this database is described in section 4.2;

3) selection of highly specific oligonucleotides (probes) representing the sequences in the *A. bisporus* database and organisation of these probes into a microarray design. This procedure has been described in section 4.3;

4) validation of the microarray sample preparation procedure, hybridisation procedure and specificity of the candidate microarray probes. These validation procedures have been described in section 4.4 (validation of microarray sample preparation and hybridisation procedure), section 4.5 (selection of high quality microarray probes) and section 4.6 (validation of microarray hybridisation data using quantitative real-time PCR);

5) design of a microarray data normalisation procedure to allow comparison of data between microarrays specific to this project (section 4.6) and selection of a method to identify genes that are differentially expressed over the course of an experiment (section 4.7);

6) i*n silico* analysis of the *A. bisporus* sequences collated in the *A. bisporus* database to identify a putative functional annotation, based on homology to other previously annotated sequence information from fungi and other pro- and eukaryotic organisms. This procedure has been described in section 4.8.

4.1 *Isolation of total RNA from peat colonised by* **A. bisporus** *mycelium*

4.1.1 Introduction

Analysing levels of nucleic acids from environmental samples of soil and other high humus-containing materials such as peat offers enormous opportunities to understand the impacts of environmental management and perturbation on biodiversity and active processes such as gene expression. However, extraction and purification of RNA and DNA from soils and humus-containing material is problematic as humic substances are co-extracted with nucleic acids and interfere with the subsequent processing reactions used for analysis (Jacobsen & Rasmussen, 1992; Tebbe & Vahjen, 1993). In addition, RNA is highly susceptible to heat and enzymatic degradation. Therefore, methods chosen to optimise extraction must also be aimed at minimising any degradation. Auer *et al.* (2003) have investigated the effects of RNA degradation on microarray based gene expression analysis and found that up to three quarters of genes showed a difference in expression level between intact total RNA and partially degraded total RNA samples. As a result, the full application of molecular techniques to the microbial communities of soil and high humus containing materials is limited due to the lack of a purification technique which can yield high levels of un-degraded, inhibitor-free RNA. Therefore, a robust, reproducible and quick RNA extraction method is required.

Current technologies investigating differences in gene expression levels require total RNA as input material. For microarray studies, isolated total RNA is reverse transcribed by the enzyme reverse transcriptase and the second strand of the resulting copy RNA (cRNA) is synthesised and amplified by the RNA polymerase enzyme with incorporation of fluorescently labelled nucleotides. Small scale gene expression analysis is routinely performed using quantitative real time reverse transcription PCR (qRT-PCR) while high-throughput analysis requires microarray technology or upcoming sequencing technology such as the single molecule Digital Gene Expression platform by Helicos Biosciences (Lipson *et al.*, 2009). PCR and microarray based technology requires intact

and inhibitor free starting material as the RNA molecules will be enzymatically modified before analysis and quantification is possible.

Inhibition of these reactions can be caused by poor quality of the input material or presence of inhibitory contaminants. Humic compounds are the most commonly reported group of inhibitors present in soil samples and have deleterious effects on several reaction components as described by Jacobsen & Rasmussen (1992). These substances are formed by the microbial degradation of plant and animal tissues and are a major component of peat, accounting for approximately 37% of the organic carbon content soil, and between 80-90% in peat (Rydin & Jeglum, 2006). The main functional groups of humic acids, carboxylic acids and hydroxyl phenolic groups, confer chemical properties which are broadly similar to nucleic acids. Tebbe & Vahjen (1993) found that DNA extracted from sandy soils using a phenol:chloroform based method contained 0.7 to 3.3 µg/ µl of humic acids. They also showed that a commonly used polymerase (*Taq polymerase*) was highly susceptible to inhibitory properties of humic acids. The minimal inhibitory concentration (MIC) of humic acids for this enzyme was 0.24-0.48 µg/ml of the co-extracted humic acids per ml. Reverse transcriptase (used to create the first cDNA strand) was also shown to be inhibited by humic acids (Schneider *et al.*, 1996). Tebbe and Vahjen (1993) reported that DNAse I was less susceptible (MIC of standard humic acids, 912 µg/ml) and RNAse could not be inhibited at all (MIC > 7.6 mg/ml.). This implies that the presence of humic acids in total RNA extractions from colonised peat samples can affect the activity of polymerase and reverse transcriptase activities, used to prepare the total RNA for microarray analysis, but will not affect the activity RNA-degrading enzymes. The mechanism of inhibition of enzymatic activity is not yet known, but it was suggested that it was unlikely to be due to chelation of Mg^{2+}, a cofactor of polymerase enzymes. Young et al (1993) suggested that phenolic groups of humic compounds denature biological molecules by bonding to amide or oxidize to form a quinine which covalently bonds to DNA, RNA or proteins. They also suggested that addition of polyvinylpolypyrrolidone (PVPP) or poly-1-vinylpyrrolidone-2 (PVP) overcame inhibition as these molecules bound to humic acids and allow separation from the total RNA. McGregor et al (1996) suggested that the inhibition is caused by an interference of the interaction of polymerase to the target molecule.

Our research into the environmental regulation of the phase change of the fungus *A. bisporus* (the cultivated mushroom) from vegetative mycelium to the development of fruiting bodies has created the need for the extraction of intact and inhibitor-free RNA from peat for gene expression analysis using microarrays. This paper is a thesis chapter-amend and describes an improved method to extract total RNA from small quantities of peat, a soil containing high humus levels, to a quality (integrity and purity) that allows the proportions of prokaryotic and eukaryotic rRNA to be quantified and any inhibitory products have been reduced below inhibiting levels.

A number of methods have been developed to extract DNA or RNA from soil samples (Hurt *et al.*, 2001; Jackson *et al.*, 1997; Miller *et al.*, 1999). However, most of these phenol:chloroform-based phase separation methods are optimised for DNA isolation and use sepharose filtration. These sepharose columns retain humic acids due to their complex structure but also remove low-molecular-weight RNA rendering them unusable for gene expression profiling as they modify the RNA pool (Jackson *et al.*, 1997). We found that a phenol:chloroform extraction of both DNA and RNA followed by a purification of the RNA using a Qiagen resin column based on the method described by Hurt *et al* (2001) did not remove humic acids from peat samples and yielded degraded RNA (data not shown). Therefore, various extraction methods were tested during the course of this project for their ability to remove inhibitory substances and reduce degradation of total RNA. McGrath *et al*. (2008) published a research project on the isolation and analysis of mRNA from environmental microbial communities using cDNA microarrays. They concluded that the PowerSoil RNA extraction kit (MoBio, USA) provided the best results. The agarose gel image used to illustrate the quality of the extracted total RNA indicates some degree of degradation as the 23 S rRNA fragment is less intense than the 16 S rRNA fragment and a smear can be seen below the 23 S rRNA fragment (Figure 4-1). This method was not tested for the extraction of total RNA from humic rich peat samples.

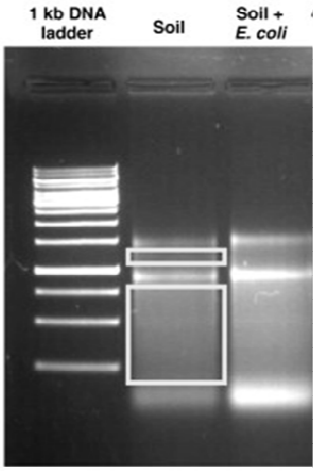


Figure 4-1 Agarose gel image used to assess the quality of the extracted total RNA from soil samples (soil) and soil supplemented with 25 µl of log-phase *E. coli* culture as reported by McGrath *et al.* (2008). The modified PowerSoil RNA (MoBio, USA) was reported to deliver total RNA with the lowest degree of degradation compared to other available commercial kits and methods using CTAB (hexadecyltrimethylammonium bromide) or LiCl.

4.1.2 Optimisation of an extraction method for the isolation of total RNA from A. bisporus *peat-based samples*

Agaricus bisporus mycelium was grown on compost and allowed to colonise an upper layer of peat containing 20 % lime for 10 days, as per commercial practice. Approximately 500 g portions of colonised peat and 100 g of fruit bodies were sampled in triplicate and frozen under liquid nitrogen. Half of each sample was stored at -70°C while the other half was freeze-dried prior to storage at -70°C. In an initial experiment, six different total RNA extraction methods were tested on their ability to deliver un-degraded and inhibitor-free total RNA.

4.1.2.1 Method 1: Phenol:chloroform-based phase separation

This method is based on Sreenivasaprasad (2000) who published a method for the extraction of total RNA from soil-free fungal samples. 5 g of the frozen colonised peat sample was ground under liquid nitrogen using a pestle and mortar and processed as

described in detail in section 2.3.1 (extraction of total RNA from fruit bodies). The ground sample was mixed with three volumes of lysis buffer (200 mM Tris, 25 mM NaCl, 25 mM EDTA and 0.5% sodium dodecyl sulphate, pH 8.5) and three volumes of phenol:chloroform:isoamylalcohol (125:24:1) pH 4.5 (Ambion) was added to the mixture and incubated at 65 °C to stimulate a separation of the RNA fraction from the protein, lipid and DNA fraction. The aqueous fraction containing the RNA was transferred to a new 30 ml centrifuge tube with screw cap and an equal volume of chilled chloroform:isoamyl alcohol (24:1) was added to further purify the sample. The aqueous fraction was transferred to a new 30 ml tube containing chilled 12 M lithium chloride (LiCl) to a final concentration of 2 M LiCl and incubated overnight at 4°C to allow precipitation of total RNA. The mixture was then centrifuged to pellet the total RNA. The supernatant was discarded and the pellet was washed with 5 ml of 3 M sodium acetate pH 5.0 followed by a wash with 20 ml of chilled ethanol. The total RNA pellet was then re-suspended in 500 µl DEPC treated water. The RNeasy plant mini kit (Qiagen) was then used to further purify and concentrate the total RNA using the standard RNA purification protocol, eluted in 30 µl DEPC-treated water and stored at -70°C prior to use.

4.1.2.2 Method 2: Modified phenol:chloroform-based phase separation

This method was a modification to the method described in section 4.1.2.1 as 2% CTAB (hexadecyltrimethylammonium bromide) and 2% PVP was added to the extraction buffer as described by Doyle & Doyle (1987). PVP was found to form complexes with polysaccharide and polyphenol compounds while CTAB was found to form complexes with various proteins and polysaccharides, theoretically improving purification of total RNA.

4.1.2.3 Method 3: the RNeasy plant mini kit (Qiagen)

For this method, the standard protocol for extraction of total RNA from filamentous fungi using the RNeasy plant mini kit (Qiagen) was used (manual: http://www1.qiagen.com/Products/RnaStabilizationPurification/RNeasySystem/RNeasyMini.aspx). This method is based on selective binding of RNA to a silica based

membrane under high salt conditions and in the presence of alcohol. Purified total RNA is eluted after addition of water or a low salt buffer.

4.1.2.4 Method 4: Trizol reagent (Invitrogen)

For this method, the standard protocol for extraction of total RNA from samples using the he Trizol reagent (Invitrogen) was used (manual: http://tools.invitrogen.com/content/sfs/manuals/15596018%20pps%20Trizol%20Reagent%20061207.pdf). The Trizol reagent (Invitrogen) contains guanidinium thiocyanate and phenol-chloroform. The guanidinium thiocyanate disrupts cell walls (lysis method) and prevents degradation of RNA by inhibiting activity or RNAse. The phenol:chloroform separates the RNA fraction from the lipid, protein and DNA fraction as described in the first method. The aqueous phase containing the nucleic acid fraction is separated and isopropanol is added to precipitate the nucleic acids.

4.1.2.5 Method 5: FastRNA pro soil-direct kit (MP Biomedicals)

For this method, the standard protocol for extraction of total RNA from soil samples using the FastRNA pro soil- direct kit (MP Biomedicals) was used (manual: http://www.mpbio.com/includes/technical/FastRNA%20pro%20Soil%20Direct.pdf).
This method is based on the principle of cell lysis through shearing of the cells, stabilisation of RNA by chaotropic reagents, phase separation of the total RNA fraction from the protein, lipid and DNA fraction using phenol:chloroform (1:1), precipitation of inhibitory substances using a proprietary solution, purification of the total RNA fraction by binding to small silica particles under proprietary conditions followed by precipitation of RNA with isopropanol.

4.1.2.6 Method 6: the modified FastRNA pro soil-direct kit (MP Biomedicals)

This method is a modification on the standard FastRNA pro soil- direct kit (MP Biomedicals described in method 5. The original method was modified to increase integrity and concentration of the extracted total RNA. The modifications of this kit were as follows:

1) 10 g of freeze dried colonised peat was mixed with an equal volume of dry-ice and ground in a coffee-grinder (Delonghi, UK). This mixture was transferred to a loosely closed 50 ml centrifuge tube (TRP) and placed at -70°C overnight to allow the evaporation of the dry ice as CO_2. 100 mg of ground sample (instead of 100 mg frozen soil sample) was added to the provided purple-cap microfuge sample tube containing lysing matrix E. 1.4 ml of RNApro Soil Lysis solution was added and the tube was vortexed to resuspend the soil and lysing matrix.

2) The sample was homogenised in the FastPrep instrument for 20 seconds at a setting of 4.0 (instead of 40 seconds at a setting of 6.0) after which the sample was immediately transferred to ice. The standard protocol was then followed, including the optional centrifugation through a quick-clean spin filter as described in detail in Chapter 2, section 2.3.2.

3) The extracted total RNA was then processed using the RNeasy MinElute cleanup kit (Qiagen), standard protocol to remove residual salts and ethanol and to concentrate the total RNA as described in the manual. A DNAse treatment can be included during the RNeasy MinElute step if the sample has to be completely free of residual DNA (e.g. for quantitative RT-PCR). This step is optional for processing of the extracted total RNA using the low input linear amplification kit (Agilent Technologies) used for the production of fluorescently labelled cRNA as this is a poly-A based amplification method.

4.1.3 Results

The quality of the total RNA extracted using the six methods was investigated for 1) integrity of the total RNA using the Bioanalyzer RNA 6000 Nano kit (Agilent Technologies), 2) presence of humic acids by a visual observation of the samples for brown discoloration and 3) inhibition of reverse transcriptase and RNA polymerase activity by spectrometric measurement of respectively cRNA creation and fluorescent dye incorporation rates using the Low Input Linear Amplification Kit (Agilent Technologies, Chapter 2, section 2.3.4).

4.1.3.1 Integrity of the extracted total RNA

The results of the analysis of the total RNA using the Bioanalyzer 6000 Nano kit on total RNA extracted with the six methods tested in this work are shown in Figure 4-2. Only the RNeasy plant mini kit (Figure 4.2, method 3) and modified Fast RNA Pro soil method (Figure 4-2, method 6) were able to extract un-degraded total RNA, based on distinct ribosomal peaks (18 S and 28 S), a 28/18 S rRNA ratio higher than 1 and a low baseline in the area under the 18 S rRNA peak as described in: http://www.ambion.com/techlib/tn/111/8.html. RNA degradation as indicated by reduced rRNA peaks and a raised base-line was observed with the other extraction methods. Moderate degradation was observed for total RNA extracted with the original Fast RNA Pro soil method (Figure 4-2, method 5) and heavy degradation was observed for total RNA extracted with the phenol/chloroform phase separation method (Figure 4.2, method 1), the modified phase separation method (Figure 4-2, method 2) and the Trizol reagent method (Figure 4-2, method 4).

The RNA extracted by the RNeasy plant mini (Figure 4.2, method 3) and Fast RNA Pro soil methods (Figure 4-2, method 5 and method 6) showed four distinct peaks. It was hypothesised that the additional peaks would be from bacteria present in the substrate as *A. bisporus* cultures were not axenic and presence of *Pseudomonas putida* in the casing samples has been reported (Noble *et al.*, 2009). An experiment was designed to test whether a mixture of fungal and bacterial total RNA would give a peak pattern similar to that observed in previous extractions. Total RNA was extracted from 1) a mushroom fruit body containing no soil and 2) a liquid *P. putida* culture provided by A. Dobrovin-Pennington (Warwick HRI, University of Warwick) using the modified Fast RNA Pro soil method (method 6). Respectively 50 mg sample or 50 ml culture was mixed with 50 mg sterile silica and processed as described in Chapter 2, 2.3.2 (Total RNA extraction from colonised casing samples). The fruit body sample had distinct peaks corresponding to 18 S and 28 S rRNA while the *P. putida* sample had distinct peaks corresponding to 16 S and 23 S rRNA as visualised by using the Bioanalyzer 6000 Nano kit (Figure 4-3). A 50:50 fruit body:*P. putida* total RNA mixture had distinct peaks corresponding to 16 S, 18 S, 23 S and 28 S rRNA.

The observed peak pattern was comparable to total RNA extracted from peat inoculated with mushroom mycelium as described in this section, confirming that the additional peaks seen in total RNA from colonised casing samples originate from the bacterial community present in the casing samples and are not the product from RNA degradation.

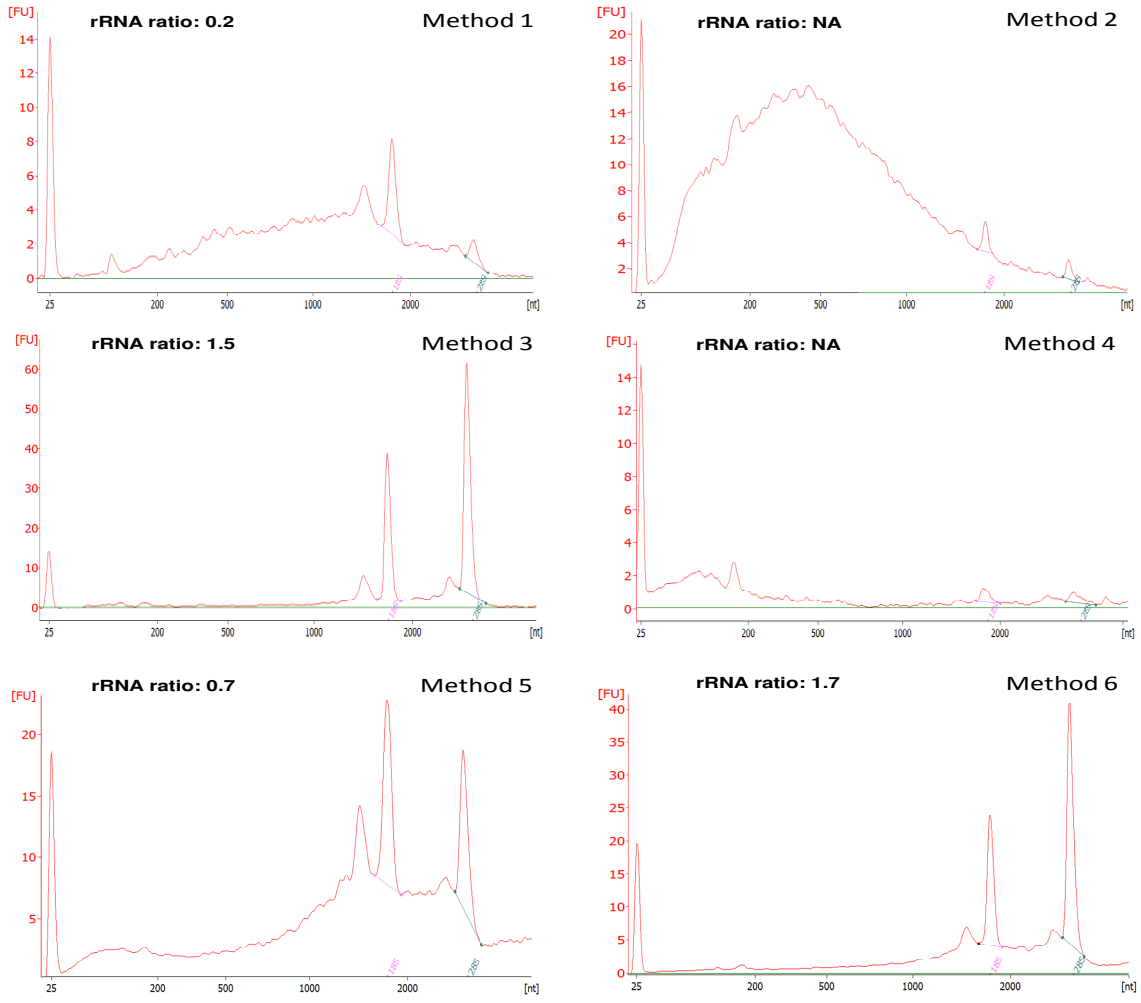


Figure 4-2 Electropherograms from total RNA samples analysed using the Agilent Bioanalyzer 6000 Nano kit. The methods tested to extract total RNA from a humic acid-rich peat sample, colonised with *A. bisporus*, were: Method 1) phenol:chloroform-based phase separation, Method 2) the modified phase separation method, Method 3) RNeasy plant mini kit, Method 4) the Trizol reagent method, Method 5) the FastRNA Pro Soil-direct kit and Method 6) the modified FastRNA Pro Soil-direct kit.

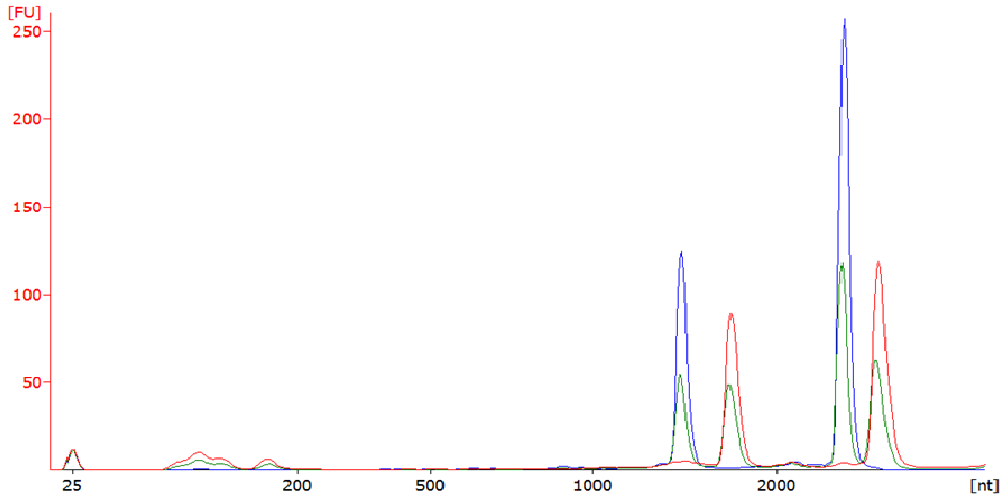


Figure 4-3 Electropherogram from total RNA samples analysed using the Agilent Bioanalyzer 6000 Nano kit. A sample isolated from (red) an *A. bisporus* fruit body sample, (blue) a *Pseudomonas putida* bacterial culture and (green) a 50:50 mixture of these two samples. The 50:50 mixture sample has 4 distinct ribosomal peaks, similar to total RNA extracted from peat colonised with *A. bisporus* shown in Figure 4.2.

4.1.3.2 Presence of humic compounds

Total RNA extracted using the phase separation method (method 1), the modified phase separation method (method 2), The RNeasy plant mini kit (method 3) and the Trizol reagent method (method 4) had a brown discoloration indicative of co-extraction of humic acids. Total RNA extracted using the FastRNA Pro Soil-direct method (method 5) and the modified FastRNA Pro Soil-direct method (method 6) had no observable discoloration.

4.1.3.3 Effect of extraction method on the inhibition of reverse transcriptase and fluorescent dye incorporation

Total RNA extracted using each method was processed to generate labelled cRNA using the low input linear amplification kit as described in section 2.3.4. The Nanodrop spectrophotometer was used to measure the cRNA yield and Cy-3

incorporation rate. Only the sample extracted using the modified FastRNA Pro Soil direct-method generated cRNA with a yield and dye incorporation rate that passed the minimum recommendations provided by Agilent Technologies, which indicates that it is usable for microarray gene expression analysis. This also indicated that any inhibitory substance was reduced below the MIC of the used enzymes. Total RNA extracted with the other methods failed to generate a measureable amount cRNA indicating that the total RNA was too degraded to be used or that inhibitory substances were present above the MIC of the enzymes used for the preparation of the cRNA.

4.1.3.4 Assessment of the efficiency and reproducibility of the modified FastRNA Pro Soil direct method to extract un-degraded and inhibitor free total RNA from peat samples with various degrees of fungal colonisation.

In order to verify the efficiency and reliability of the modified FastRNA Pro Soil-direct method to extract un-degraded, inhibitor free total RNA from colonised peat samples suitable for microarray analysis, a further 21 peat samples with various degrees of *A. bisporus* colonisation (at airing, 24 h after airing, 72 h after airing and 168 after airing) were collected as described in section 4.1.2. Total RNA was extracted using the modified FastRNA Pro Soil-direct method as described in section 4.1.2.6. The samples had an average total RNA concentration of 130.6 ng/ µl (SD ± 78.55) in 12 µl elution volume and an average 260 nm /280 nm absorbance ratio of 1.97 (SD ± 0.11). The integrity of the total RNA was assessed using Bioanalyzer 6000 Nano kit and all samples had 28 S/ 18 S rRNA ratios above 1 and a low baseline below the 18 S rRNA fragment, indicating that the total RNA was un-degraded. Each total RNA sample was then processed as described in section 2.3.4 (preparation of fluorescently labelled cRNA). The yield and Cy-3 dye incorporation rates of the resulting cRNA samples were checked using the Nanodrop spectrophotometer as described in section 2.3.4 for each sample. The samples yielded on average 3.55 µg (SD ± 1.30) cRNA, > 1.65 µg is recommended by Agilent Technologies after amplification. The average dye-incorporation rate was 9.60 pmol Cy3 per µg cRNA (SD ± 1.48), > 8 pmol Cy-dye per µg is recommended by Agilent Technologies. This indicates that any inhibitory substances were below the MIC of the reverse transcriptase and polymerase enzymes

used for the cRNA preparation. These samples were used for gene expression analysis as discussed in Chapter 5.

4.1.4 Conclusion

Here we report an optimised method which allows routine extraction of intact and inhibitor free prokaryotic and eukaryotic total RNA from peat samples. The extracted total RNA can be used for enzymatic processing such as reverse transcription. It is also possible to extract total RNA from soil free samples for comparison purposes. The extracted total RNA is suitable for microarray and qRT-PCR experiments. It is our intention to publish this optimised extraction method in a method paper.

4.2 *Generation of an* **A. bisporus** *sequence database*

4.2.1 Collection of sequences related to **A. bisporus**

At the start of this project, the *A. bisporus* genome sequence was not available. A number of databases were therefore queried to retrieve sequences related to this fungus, as described in section 2.4.1 (collation of *A. bisporus* related sequences). A significant contribution of sequences were generated as part of this project by Dr. Dan Eastwood (University of Warwick) who identified 447 expressed sequence tags (ESTs) that were differentially expressed between colonised casing at airing, colonised casing 24 h after airing, smooth undifferentiated primordia and elongated differentiating primordia using suppression subtractive hybridisation (SSH) as described in Table 4.1. The smooth undifferentiated primordia and elongated differentiating primordia were collected from the surface of the casing layer and are therefore a mixture of structures that developed both on mycelial cords beneath the surface of the casing layer and on the surface of the casing layer.

Table 4-1 Experimental set-up of the SSH subtractions from 4 stages of *A. bisporus* fruit body development by Dr. Dan Eastwood. The experiment identified genes that are abundantly expressed in the tester sample when compared to the driver sample.

Tester	Driver	# Sequences
airing	24 h after airing	68
24 h after airing	airing	22
24 h after airing	fluffy undifferentiated primordia	33
fluffy undifferentiated primordia	24 h after airing	103
fluffy undifferentiated primordia	elongated differentiating primordia	148
elongated differentiating primordia	fluffy undifferentiated primordia	73

This SSH comparison yielded genes that were differentially expressed in at least one developmental stage. Microarray normalisation tools assume that the majority of genes represented on the microarray are not differentially expressed during the course of an experiment so that these genes can be readily identified and used for the normalisation of the microarray data. This is not an issue for whole genome experiments where a whole transcriptome is represented on the microarray. It is however a problem when working with non-genome sequenced organisms such as *A. bisporus* and especially if the majority of the available sequences are thought to be differentially expressed in the planned experiments. Various public and private databases were therefore queried for *A. bisporus* related sequences in order to collect genes that were not identified by SSH as being differentially expressed during initiation. These sources were:

1) The EMBL-EBI nucleotide database: this publicly available database was queried on October 2005 and yielded 813 sequences related to *A. bisporus*. The majority of these sequences were also biased for expression at specific developmental stages and originate from a project by Ospina-Giraldo *et al.* (2000) who collected primordia and mature fruit bodies at respectively 15 and 21 days after casing. Total RNA was extracted and cDNA libraries were created. Plasmid DNA was prepared from randomly picked clones and sequenced resulting in identified 477 sequences.

2) Dr. Emilie Combet (Warwick HRI, University of Warwick) investigated genes involved in lipid metabolism and flavour biogenesis in *A. bisporus*. During this project, 254 *A. bisporus* cDNA sequences were sequenced.

3) A further non-published 95 sequences identified during a project by Dr. J. Thomas and Dr. M. Challen (University of Warwick, Warwick HRI) who investigated the interactions of *A. bisporus* and *Verticillium fungicola*.

The sequences related to *A. bisporus* collected from these various sources were then collated into an Excel file and each sequence was given a unique identifier. This file was then processed using local BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) as described in section 2.4.2 to identify duplicate sequences and allelic variants and sequences smaller than 50 nt were omitted from further analysis. 1,112 of the remaining 1,319 sequences were unique and 207 sequences with a high degree of similarity (blastn E-value < 100) to other sequences in the database. These 207 sequences were only different by one or two nucleotides to their matching sequence and differences in gene expression would therefore not be distinguishable using microarray technology. They were therefore omitted from further analysis. The average sequence length was 450 bp. This database was then used as the input for microarray probe design and *in silico* functional annotation.

4.2.2 Conclusion

Even though *A. bisporus* was not genome-sequenced at the time of writing, 1,319 sequences related to this fungus were collected and organised into a database. Of these sequences, at least 1,112 sequences were unique. This database, containing over 13 % of the predicted gene number as described in section 2.4.1 was the largest database of *A. bisporus* sequences available at the time of writing. Over 400 sequences were identified in a project that used SSH to generate sequence information of genes that show differential expression between 4 distinct reproductive stages of *A. bisporus.* This database was therefore a valuable tool to assist with gene expression profiling of *A. bisporus* grown under various environmental conditions using microarray technology. In

order to design a gene expression microarray, unique oligonucleotides representing each database sequence had to be identified, as described in the following section.

4.3 *Probe selection and microarray design*

The 1,317 unique *A. bisporus* sequences that were collated in the *A. bisporus* database described in section 4.2 were used as the input for two bioinformatics programs: OLIGOPICKER and OLIGOSTAR. These two programs, described in section 2.4.4, were designed to select multiple short sequences with a specific composition that are unique to a sequence in the input file. These short sequences are commonly referred to as "probes". Both programs use the recommended parameters provided by Agilent Technologies (section 2.4.4) for probe design in a slightly different way.

OLIGOPICKER allows the user to specify the desired probe length, end preference (3' end in the case of Agilent microarrays), the melting temperature range (Tm Range) and cross-reactivity value. It is important to keep the Tm range as low as possible to ensure equal specificity of each designed probe. The cross-reactivity value is a threshold for the rejection of contiguous matches (default value is 15 nt.). A short, contiguous match means a lower chance of cross-hybridisation as the presence of mismatches will affect the stability of such a hybridisation. Various conditions were tested to find a balance between stringency (small Tm range and long probe length) and the number of designed probes, based on the guidelines given by Agilent Technologies (section 2.4.4) by altering the probe length from 45 nt up to the recommended 60 nt, Tm from 5 °C up to 30 °C and cross-reactivity threshold from 11 nt to 20 nt.

Table 4-2 illustrates a selection of parameters used as input for OLIGOPICKER. Experiment 1 and 3 were designed to assess the maximum number probes that could be designed with relatively low-stringency conditions. The maximum number of sequences for which probes could be designed under these conditions was 1,045 sequences. Increasing the probe length to 60 nt, decreasing the Tm range to 10°C and decreasing the cross-reactivity threshold to 13 nt did not dramatically reduce the number of designed probes, as was seen in experiment 5, as probes could be designed for 1,012 sequences.

Table 4-2 Various settings of OLIGOPICKER were tested to find an optimal balance between stringency and the number of probes designed for the sequences in the *A. bisporus*. Parameters used in the fifth experiment were used to identify up to three unique oligonucleotide probes for each database sequence.

Experiment	**1**	**2**	**3**	**4**	**5**
Probe Length (nt)	45	50	60	60	**60**
Tm Range (°C)	12	12	30	5	**10**
Cross-reactivity (nt)	15	15	20	13	**13**
Sequences with probes:	1,045	1,038	1,039	766	**1,012**

The second program, OLIGOSTAR allowed for less flexibility of the parameters. Following the Agilent Technologies probe design parameters specified in section 2.4.4 yielded up to two candidate probes for 993 sequences. The probe length varied between 50-60 nucleotides.

Finally, the candidate probes designed by both programs were collated and analysed using local BLAST to eliminate overlapping probes. Up to three probes that met the recommended probe specifications most accurately for each *A. bisporus* sequence were selected with preference to probes designed by OLIGOPICKER as this program was well established while OLIGOSTAR was still experimental at the time. In total, 2,708 candidate probes were designed representing 1,105 *A. bisporus* sequences. The average melting temperature was 81.19 °C (SD ± 4.03). As the majority of sequences originated from publicly available sources it was difficult to confirm the orientation of each sequence. For that reason, the reverse complement of each probe was calculated and a probe group containing probes representing both sequence orientations was created.

This probe group containing 5,416 sequences was then used to design a custom 2 X 11,000 60-mer oligonucleotide Agilent microarray as described in section 2.4.5. As

the array design was not completely filled, a second reduced copy of this probe group was added to this design and served as a technical replicate. The reduced probe group contained 4,238 probes representing each of the 1,105 sequences in both orientations. The total probe number for this microarray design was 9,654 probes. At this point in the project, the eARRAY portal used to create the microarray design (section 2.4.5) was not capable of designing probes, but it provided a tool to check quality of designed probes in terms of uniformity and base composition. A base composition score (BC) is given to each probe indicating the predicted quality of the probe with a score of '1' given to probes with an optimal structure while a score 'poor' is given to probes with a potentially problematic structure (e.g. consecutive GC nucleotides and secondary structure formation). Scores of "2", "3" and "4" indicate decreasing but usable quality. Of the 9,654 probes, 31.5 % were given a BC score of "1", 49.9% got a score of "2", 15.2% got a score of "3", 2.7% got a score of "4" and only 0.7 % were given a score of "poor". This microarray design was then used in a number of microarray hybridisations designed to test the microarray hybridisation procedure (section 4.4) and to select stringent probes that give reproducible results (section 4.5).

4.4 *Experimental validation of the microarray hybridisation procedure*

Microarrays used during the microarray validation process (two-colour experiments, section 4.5) and gene expression profiling experiments (one-colour experiments, Chapter 5) were processed using Feature Extraction software (Agilent Technologies) as described in section 2.5.5 (extraction of signal intensity data from a microarray scan). This software converts analogue intensity data from the microarray TIFF file into digital data and creates 1) a tab delimited text file containing general information on the protocols used to extract the data, corrections used (e.g. spatial detrending and background subtraction) and information on each feature (e.g. position on the array, pixels used to calculate the signal and background intensity, processed signal intensity, error on the signal intensity) which is used for data analysis and 2) a quality control (QC)-report containing various metrics that provide information on the quality of the hybridisation. The QC-metrics used during this process to assess the quality of a microarray hybridisation are: 1) the "grid finding" metric (section 4.4.1), 2)

the “feature outlier” metric (section 4.4.2) and 3) the “spike-in hybridisation data” metric (section 4.4.3).

4.4.1 The spot (feature) finding metric

The first step in the data extraction software is the positioning of a grid over the TIFF image linking signal intensities at specific physical positions to feature information (e.g. probe ID). The Feature Extraction software uses complex, proprietary algorithms to find the corners of each array and to check if the middle of positive control features (also referred to as “spot”) is where the grid expects it to be, based on signal intensities. Damage to the arrays (handling during the array processing procedure), manufacturing issues, contamination of the wash dishes and absent or low signal intensities can interfere with the grid finding procedure and subsequent data analysis. The grid finding metric reported in the QC-report displays the four edges of the array and shows the overlay of the grid (white crosses on the expected feature positions) as illustrated in Figure 4-4. The metric can therefore be assessed visually. A parameter below the image reports if the grid could be placed and if the positive controls were detected at the expected positions. This parameter can be “grid normal” when no problems were detected or “check grid”. All of the arrays hybridised during the validation procedure (section 4.5) or the gene expression profiling experiment (Chapter 5) received a “grid normal” status.

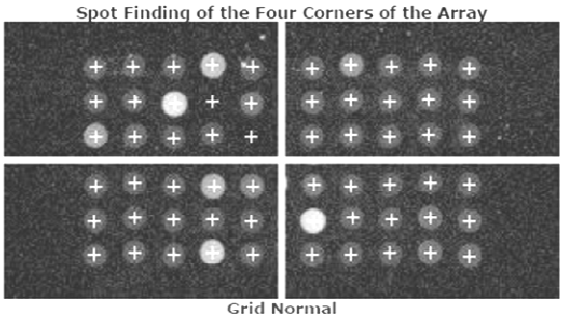


Figure 4-4 The spot finding metric found in the Feature Extraction (Agilent Technologies) QC-report was the first metric used to assess the quality of each microarray hybridisation performed during this project. This metric reports if the Feature Extraction software was able to place a grid of the expected position of each feature on top of each image (grid normal or check grid). The spot finding algorithm checks if the corners of the array were detected properly and if the positive controls are where they were expected to be.

4.4.2 The feature outlier metric

Once a grid is positioned over the array, the software calculates population and non-uniformity outliers for both the background and the red and green signal channels (for two-colour hybridisations) or green signal channel (one-colour hybridisation). A feature is a flagged as a population outlier if its signal is less than a lower threshold or exceeds an upper threshold determined using a multiplier of 1.42 times the interquartile range of the population of replicate features of a probe. Non-uniformity outlier flagging is based on statistical deviation from the expected noise in the Agilent microarray-based gene expression system based on internal validation experiments by Agilent. The outliers are visualised in a plot, as illustrated in Figure 4-5. In general, Agilent Technologies specifies that over 1% outliers (for each class) may be an indication of a hybridisation problem or microarray design issue. None of the arrays hybridised during the validation procedure (section 4.5) or gene expression profiling experiment (Chapter 5) had over 1 % outliers and therefore all arrays passed this QC-check. The spatial distribution of the outliers can help to identify problems with the hybridisation as a cluster of outliers can illustrate a microarray wash problem, contamination (e. g. dust or

hair) or leakage of the hybridisation mixture (outliers will be clustered in the middle of the array as the centre area will not be covered with hybridisation mixture).

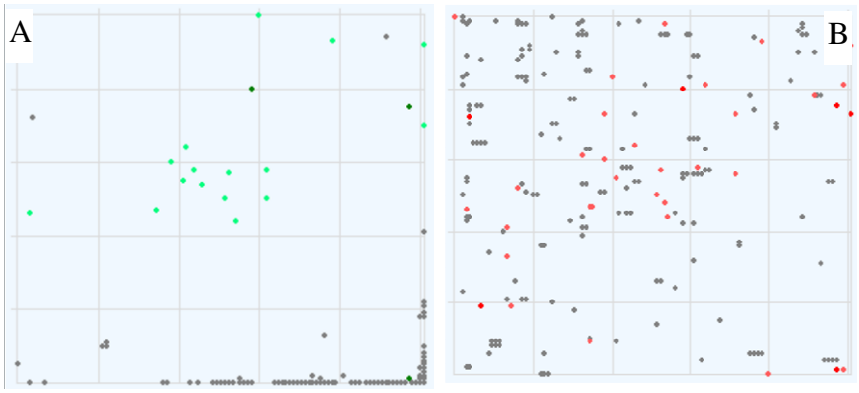
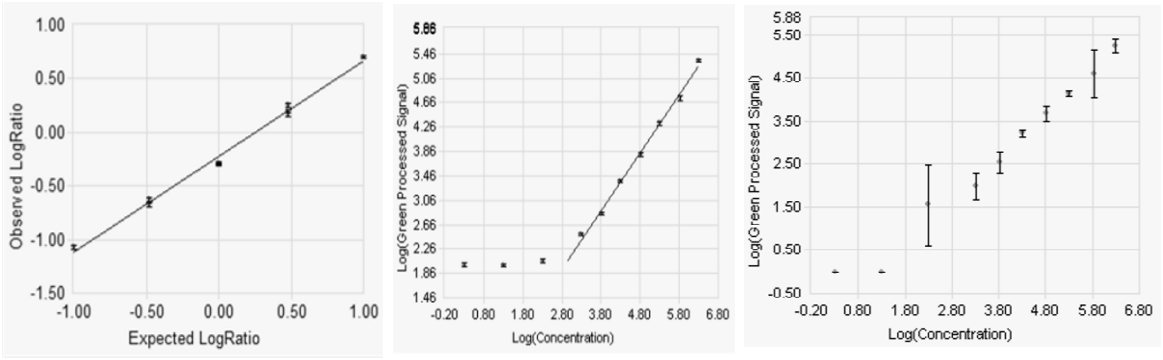


Figure 4-5 Spatial distribution plots of (A) background outliers and (B) feature outliers. Each graph represents the microarray and the outlier is represented as a dot and given a different colour depending on the type of outlier: (yellow) background non uniform outlier, (grey) background population outlier, (light red) red feature population outlier, (red) red feature non uniform outlier, (light green) green feature population outlier, (green) green feature non uniform outlier.

4.4.3 Spike-in hybridisation data metric

The one- or two-colour microarray-based Low Input Linear Amplification gene expression kit protocol version 5.5 from Agilent Technologies used during this project to create fluorescently labelled cRNA has the option to include an RNA spike-in mixture which will be added to the total RNA input material. This RNA spike-in mixture was developed to provide positive controls for monitoring the microarray workflow from sample amplification and labelling to microarray processing and contains 10 *in vitro* synthesized, poly-adenylated transcripts derived from the *Adenovirus* E1A transcriptome that are premixed at various ratios. The expected signal intensities can then be compared to observed signal intensities to assess the quality of the cRNA preparation and hybridisation procedure. This information is represented in a graph in the Feature Extraction QC-report for each array as illustrated in Figure 4-6. Two more metrics are calculated: 1) a trendline to check the linearity of the results and 2) error bars indicating

the standard deviation of the observed signal of the replicates of each spike-in. The spike-in hybridisation data metrics for the arrays hybridised during this project revealed that all of the 16 hybridisations performed during the validation experiment (section 4.5) and 78 out of 85 hybridisation performed during the gene expression profiling experiment (Chapter 5) were of excellent quality (trendline with $R^2 > 0.9$ and small error bars). Unfortunately 7 of the arrays hybridised during the gene expression profiling experiment experienced some degree of leakage during the hybridisation. Because of this leakage, less hybridisation mixture covered the middle area of the array. This caused the standard deviation of the spike-in replicate probes to increase (higher error bars as illustrated in Figure 4.6 C. The eight-pack array format is sensitive to this if the array slide is not positioned horizontally on the gasket slide (after dispersion of the samples). Even though the affected features were not flagged as outliers (as described in section 4.4.2., indicating that the software was able to compensate for the reduced signal intensity in the middle of the affected arrays), the respective samples were re-hybridised and the spike-in QC-metrics confirmed a good quality of the hybridisation as no leakage of hybridisation occurred that time.



A **B** **C**

Figure 4-6 Spike-in hybridisation QC-plot for a (A) two colour-experiment and (B) one-colour-experiment. (A) The observed logratio (after correction for dye incorporation bias and spatial effects) are compared to expected logratio of each spike-in. (B) The log of the green processed signal (after correction of spatial effects) was compared to the log of the concentration of each spike-in. A trendline was applied for both experiment types and should have a R^2-value > 0.9 and is expected to be linear for the entire signal range in two-colour experiments and to show linear range in the medium to high signal range in the one-colour experiment. The second metric visualised by error bars is the standard deviation of the observed signal (two-colour) and processed signal (one-colour) of the replicates of each spike-in. (C) Leakage of hybridisation solution increases the difference of signals of each spike-in replicate probe.

4.4.4 Conclusion

The QC-report generated by the Feature Extraction software provided valuable information to assess the quality of each microarray hybridisation. The spot finding metric, feature outlier metric and spike-in hybridisation data metric identified a potential problem with 7 of the 101 arrays hybridised due to leakage of the hybridisation solution. The respective samples were re-hybridised and passed the QC-checks.

4.5 *Experimental validation of the microarray probes*

4.5.1 Experimental set-up

Three series of microarray hybridisations were performed to validate the preparation of the cRNA, microarray hybridization procedure and specificity of the microarray probes. These experiments, described in detail in section 2.7 were:

1) a **self versus self** experiment. Total RNA extracted from a colonised casing sample collected at 24 after airing and elongated differentiating primordia was labelled once with Cy-3 and once with Cy-5 as described in section 2.7.1. This procedure was performed in triplicate with the same total RNA for each of the two samples (technical replicates). The samples had an average incorporation rate of 13.37 pmol dye per µg cRNA (SD ± 3.56) and average yield of 2.95 µg cRNA (SD ± 1.38). These values were above the minimum recommendation specified by Agilent Technologies (section 2.5.1) indicating successful labelling. The samples were then hybridised in a self versus self set-up as described in section 2.7.2. As this was a self versus self hybridisation, it was expected that the signal for each feature is 1:1 (Cy-3:Cy-5) after correction for dye-bias and spatial effects. Deviations from this ratio indicate non-specific binding to the probe, making it not reliable for microarray analysis.

2) a **dye-swap** experiment where total RNA extracted from stage 2 fruit bodies and stage 4 fruit bodies, two days after harvesting was isolated (section 2.7.2). Each sample was used to create Cy-3 and Cy-5 labelled cRNA. The samples had an average incorporation rate of 14.40 pmol dye per µg cRNA (SD ± 2.93) and average yield of 3.155 µg cRNA (SD ± 0.904). These values were above the minimum recommendation specified by Agilent Technologies (section 2.5.1) indicating successful labelling. The samples were then hybridised in a dye-swap set-up as described in section 2.7.2. It was expected that after correction for dye-bias and spatial effects, the results from the first hybridisations should compare to the inverse of the results from the second hybridisations (dye-swap).

3) hybridisations with samples from a range of developmental stages to ensure that each probe is hybridised at least once (section 2.7.3). Total RNA from colonised casing at airing, colonised casing 24 h after airing, smooth undifferentiated primordia, elongated differentiating primordia, stage 2 mushrooms and stage 4 mushrooms of samples collected from *A. bisporus* grown under phase change stimulating conditions were used to create Cy-3 or Cy-5 labelled cRNA. The samples had an average incorporation rate of 14.68 pmol dye per µg cRNA (SD ± 5.088) and average yield of 2.35 µg cRNA (SD ± 0.90). These values were above the minimum recommendation specified by Agilent Technologies (section 2.5.1) indicating successful labelling. The samples were then hybridised onto microarrays as described in section 2.7.3.

The slides that were hybridised during this validation experiment were processed as described in the sections 2.5.3 and 2.5.4. The microarray TIFF file from each hybridisation was processed using Feature Extraction version 8.5.1.1 as described in section 2.5.5 in order to extract gene expression data and to correct for dye-bias on a per-array basis and spatial effects. The corrected gene expression data, p-values indicating significant differential expression of a feature and surrogate values for each hybridisation were collated in a Microsoft Excel data file. This data was then analysed to identify probes that showed inconsistent results in relation to the type of experiment (e.g. differential expression in a self versus self experiment).

4.5.2 Identification of stringent microarray probes

The following procedure was followed to select consistent and specific probes from the 9,654 probes represented on the 2 X 11,000 60-mer oligonucleotide microarray:

1) Identification of probes that were significantly differentially expressed in the self versus self experiments. The p-value calculated by Feature Extraction (section 2.5.5) was used for this analysis and features with a p-value < 0.01 in at least 2 of the 6 self versus self hybridisations were identified as “not specific” and omitted from further use. 286 probes were identified by this procedure. Of these 286 probes, 54 probes were complementary probes while the other probes represented 135 unique sequences.

2) Probes with surrogate values were analysed in more detail. A surrogate value is an artificial expression value to a feature where the raw intensity is very close to the background. Features with inconsistent saturation values (e.g. in only one of the two intensity channels of a self versus self experiment or in the same channel of a dye-swap experiment) were omitted from further use. This corresponded to 5381 probes. Of these probes, 4475 were complementary probes while the other probes represented 340 sequences.

3) Identification of probes with inconsistent results in more than three hybridisations. This refers to probes that gave significant differences in dye-swap experiments or in technical replicate hybridisations. This corresponded to 234 probes which were omitted from further use. Of these 234 probes, 43 were complementary probes while the other probes represented 162 sequences. Identification of probes with inconsistent results in up to two hybridisations (from a total of 16 hybridisations). These probes were flagged and retained in a separate datasheet. This corresponded to 892 probes which were flagged. Of these 892 probes, 111 were complementary probes while the other probes represented 427 sequences.

Based on the information from this validation experiment, 2861 specific and consistent probes remained, including technical replicates and 100 complement probes. These 2761 probes represented 853 *A. bisporus* sequences. All sequences with a validated complementary probe had validated probes (representing the other strand) that showed expression. A second screen of probes that showed inconsistent results in up to two hybridisations was performed to try to have at least one probe for remaining *A. bisporus* sequences, with preference to two probes for each sequence to increase the strength of the results.

Ultimately, **1448 validated probes** representing **1079 *A. bisporus* sequences** and 123 complementary probes were selected. Of these 1079 sequences, 5 sequences are represented by 3 probes, 355 sequences are represented by 2 probes and 723 sequences are represented by 1 probe. These probes were used to create **the final custom *A. bisporus* 8 X 15,000 feature microarray design** (section 2.4.5.2) used to investigate the gene expression profiles of *A. bisporus* grown under various environmental conditions

(Chapter 5). The file "sequence and probe information *Agaricus bisporus* gene expression microarray v1.xls" present on the DVD attached to the back of this thesis contains the full length sequence and probe sequences of each of the *A. bisporus* sequences present on this microarray design.

4.6 *Validation of normalised microarray data using quantitative real-time RT-PCR*

Total RNA was extracted in triplicate (biological replicates) from colonised casing samples of *A. bisporus* grown under standard, phase change stimulating conditions in growth trays (section 2.2.2.1) collected at three time points (airing, 72 h after airing and 168 h after airing). Expression levels of 5 genes were assessed using quantitative real time reverse transcription PCR (qRT-PCR) and microarray hybridisations.

In order to generate qRT-PCR data for these genes, the samples were processed as described in section 2.7.4. Each reaction was performed in triplicate (technical replicate). Ct values of each sample were normalised to the 18S ribosomal RNA Ct value from the corresponding control sample using the ΔCt method. Replicates (biological and technical) for each time point were combined for every gene and the standard deviation on the resulting average ΔCt values was calculated. Melting curve analysis of the qRT-PCR data showed that in all cases a single product was obtained illustrating the high specificity of the reactions. The qRT-PCR non-template water control treatments showed no evidence of DNA contamination or active reverse transcription after the 85°C treatment (data not shown).

In order to generate microarray data for these genes, the samples were processed to create Cy-3 labelled cRNA as described in section 2.3.4. No technical replicates were performed, except for the 5 on-array replicates of each probe. Each cRNA was hybridised on a 8 x 15,000 feature microarray version 1 (section 2.4.5.2) as described in section 2.5 (microarray hybridisation and data acquisition). The extracted data was then normalised as described in section 2.6.1.

The results of this experiment are illustrated in Figure 4.7 and indicate that the 5 tested genes show differential expression between the three time points for both the qRT-PCR and microarray experiment. Moreover, the observed patterns show very similar trends.

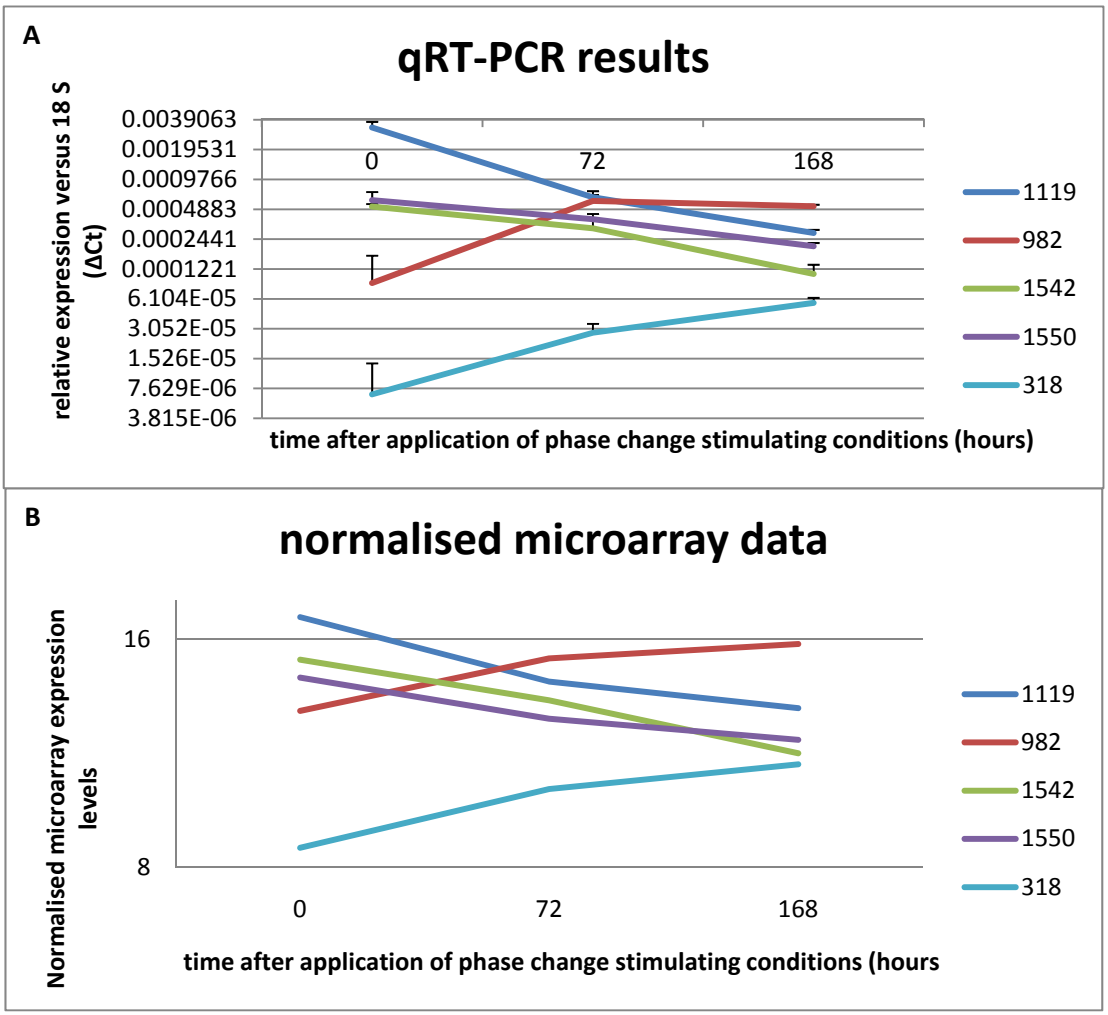


Figure 4-7 Validation of normalised microarray data using qRT-PCR. Gene expression patterns of 5 genes in colonised casing samples of *A. bisporus* grown under standard cultivation conditions and were collected at airing (0 h), 72 h after airing and 168 h after airing were investigated using (A) qRT-PCR and (B) microarray gene expression analysis. The error bars in the qRT-PCR results indicate the standard deviation of technical and biological replicates of the average ΔCt value, for each time point, for each gene.

Unequal quantities of starting RNA, differences in labelling or detection efficiencies between the fluorescent dyes used, and systematic biases in the measured expression levels imply the need of a normalisation procedure to allow inter- and intra-array comparisons. A data normalisation procedure was developed by Andrew Mead (Warwick HRI, University of Warwick) to assist with the analysis of time-course microarray experiments and is described in detail in section 2.6.1. This normalisation method accounts for feature outliers (e.g. replicate probes where the signal of one or more replicates deviates significantly from the mean signal of the total pool of replicates of that probe). Adjustments were made to the raw dataset of the gene expression profile experiments described in Chapter 5 so that each array has the same median and the same interquartile range. Despite arrays only including probes for selected genes (rather than the whole genome) it was assumed that the median and interquartile ranges should be relatively consistent across arrays for the majority of probes. The means and ranges (maxima and minima) were calculated for each probe (across up to 5 replicates) using the cleaned, normalised data. This data was then used for time-course based gene expression analysis, using the calculated medians across the replicates for each probe, for each sample. The correlation of qRT-PCR data, as described previously, with microarray was 0.931 (SD ± 0.037) for non-normalised data. This correlation coefficient increased to 0.976 (SD ± 0.025) after application of the normalisation procedure illustrating the positive effect of this procedure on the normalisation of the data to allow intra-sample comparisons. The data supporting this comparison is summarised in Appendix 15.

4.7 *Procedure to identify genes that are significantly differentially expressed over the course of a growth experiment*

Once a series of microarray data has been normalised to allow inter- and intra-array comparisons, gene expression levels can be compared in order to identify genes that are significantly differentially expressed over the course of an experiment (e.g. during development of *A. bisporus* from vegetative mycelium into elongated differentiating primordia).

4.7.1 Current approaches used to identify genes that are significantly differentially expressed in time course experiments

The time course approach is relatively new in microarray analysis. The MAANOVA (R-package) approach (Kerr *et al.*, 2000) could be used by treating the time variable as an experimental factor, as suggested by Mutarelli *et al*. (2008). Most classical time series algorithms (for non-microarray data) are quite rigid, but require a large number of time-points, uniform sampling intervals and missing data interferes with the analysis (Mutarelli *et al.*, 2008). Mutarelli *et al*. (2008) have reviewed three microarray time course analysis packages using artificial and experimental time course data and investigated the advantages and limits of each method in terms of usability, flexibility to microarray experimental designs, robustness to normalisations and overlap with other methods. These methods were:

1) Extraction of Differential Gene Expression (EDGE): This software developed by Leek *et al.* (2006) considered the time variable through a gene expression response curve which is expanded over the polynomial of B-spline basis with the coefficients estimated by the least squares method;

2) *timecourse*: this Bioconductor package uses a multivariate empirical Bayes approach to rank genes in the order of interest from longitudinal replicated microarray time course experiments (Tai & Speed, 2006). It does however not consider time curves from a functional point of view and does not provide any cut-off to select significantly differentially expressed genes;

3) Bayesian Analysis of Time Series (BATS): This method is a functional Bayesian approach in which each gene expression time profile is estimated globally by expanding it over an orthogonal basis, as described by Angelini *et al.* (2008)

Mutarelli *et al*. (2008) found that BATS delivered the most accurate results (for biological and artificially generated data), without the need for the end-user to select a "threshold" for differential expression, as was the case for EDGE. Moreover, they concluded that BATS can successfully handle various technical difficulties which arise in microarray time-course experiments such as a small number of observations available,

non-uniform sampling intervals, presence of missing data or multiple data as well as temporal dependence between observations for each gene, which are not completely addressed by the other two methods mentioned above. BATS was therefore one of the two methods selected to assist with the analysis of time course microarray data generated during this project as described in section 4.7.2.

4.7.2 Procedure to identify genes that are differentially expressed during an **A. bisporus** *growth experiment*

Multiple of the sequences present on the *A. bisporus* gene expression microarray (section 2.4.5.2) were expected to be differentially expressed during reproductive growth. The nature of the samples and growth method meant that a change of environmental condition is perceived at different times throughout the casing layer. The fungal mycelium might respond to these changes at different speeds throughout the casing. The samples are also a mixture of supporting, vegetative mycelium and developing (reproductive) mycelium. Changes in gene expression linked to development or response to a change of a specific environmental might therefore be subtle. To increase the statistical strength of the data, it was decided to consider only the genes identified to be differentially expressed in both the MAANOVA (R-package) method with p-value ≤ 0.01 (described in section 2.6.2.1) and the BATS package (described in section 2.6.2.2 and discussed in this section) for further analysis.

4.8 **In silico** *functional annotation of* **A. bisporus** *gene sequences*

A. bisporus sequences described in section 4.2 were assessed for matches to known and functionally characterised sequences of other organisms using the automated annotation pipeline tool BLAST2GO, as outlined in section 2.4.3. The final annotation on the sequences used for the *A. bisporus* 8 X 15,000 60-mer oligonucleotide microarray version 1 as described in section 2.4.5.2 was completed in June 2009. This array design consists of 1617 probes representing 1079 *A. bisporus* sequences. The result of this annotation process was:

- 226 (21%) sequences showed no significant similarity to publicly available sequences;
- 234 (22%) sequences showed significant similarity to previously submitted but not (putatively) annotated publicly available sequences;
- 619 (57%) sequences showed significant similarity to publicly available sequences that are associated with a putative function, based on homology to characterised sequences. It was possible to associate an EC number (as described in section 2.4.3) to 169 of these sequences.

Of the 619 sequences that demonstrated significant similarity to a publicly available sequence that are associated with a putative function, based on homology to characterised sequences:

- 55 sequences had a perfect match with E-value = 0;
- 42 sequences had similarity with E-value < 1.0E-100;
- 168 sequences had similarity with E-value < 1.0E-50 but > 1.0 E-100;
- 315 sequences had similarity with E-value <1.0E-10 but > 1.0 E-50;
- 39 sequences had similarity with E-value <1.0E-4 but > 1.0 E-10.

Even though a large part of the sequences in the *A. bisporus* originate from publicly available databases, only 55 sequences were a perfect match to a publicly available sequence that was associated with a putative function, based on homology to a characterised sequence. The main reason for this was that the majority of publicly available sequences collated in the *A. bisporus* database were not experimentally annotated and did not show a significant match to previously annotated publicly available sequences. Detailed results of this *in silico* annotation procedure are given in the file "*in silico* annotation results of *A. bisporus* genes.xls" attached to the DVD at the back cover of the thesis.

A representation of the putative biological processes and molecular functions of the genes represented on the *A. bisporus* microarray (section 2.4.5.2) is given in respectively Table 4-3 and Table 4-4. This information is based on the GO-ontology level 3 terms associated to the genes during the *in silico* annotation.

Table 4-3 Distribution of biological processes (GO-ontology level 3) of genes represented on the *A. bisporus* microarray. Only processes represented by 2 or more sequences were considered.

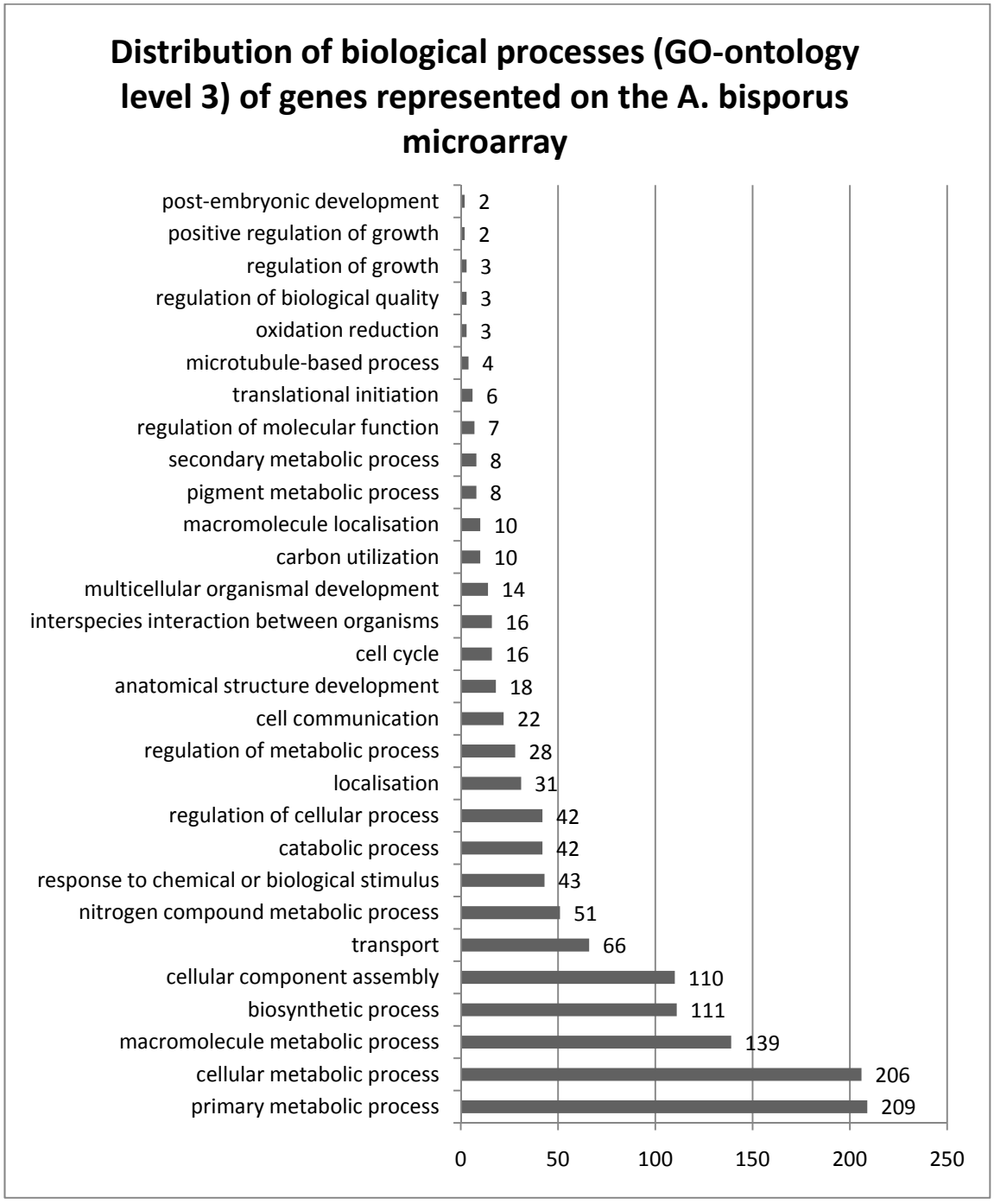
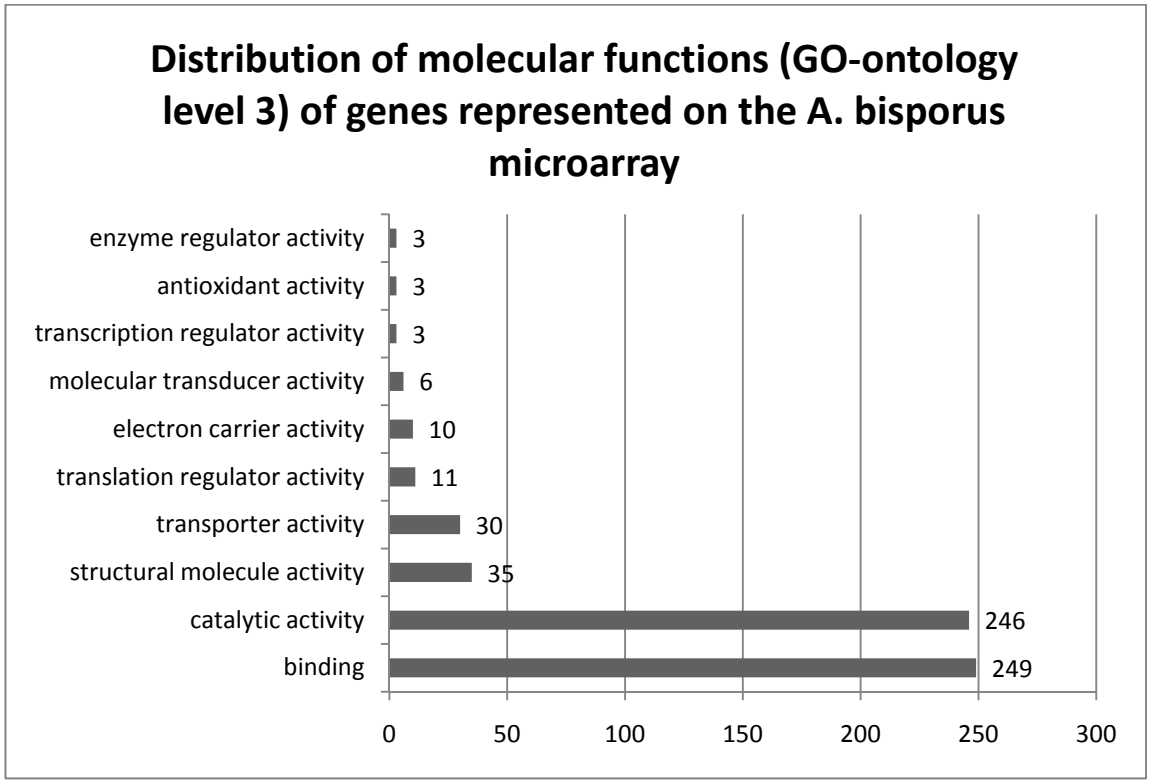


Table 4-4 Distribution of molecular functions (GO-ontology level 3) of genes represented on the *A. bisporus* microarray. Only processes represented by 2 or more sequences were considered.



4.9 *Discussion*

This chapter deals with the work to develop and customise tools and methodologies, , to investigate at the changes in gene expression of *A. bisporus* grown under standard (phase change stimulating conditions) and experimental conditions. The hyphal growth exhibited by *A. bisporus* means that massive and complex networks of hyphae colonise the soil and casing layer. The close interaction between the individual hyphae and soil or casing particles implies they are difficult to separate. It was considered to submerge a sample in a water and ice mixture, followed by manual disturbance of the sample but tests have shown that it was not possible to completely separate the fine mycelium from the substrate. Moreover, this process was time consuming and might cause changes in gene expression due to damage and exposure to low temperatures. For these reasons, it was decided to immediately halt activity and

development in the collected colonised casing samples by freezing in liquid nitrogen. The entire sample is then ground, with addition of dry ice, to achieve an initial disruption of the fungal cells and homogenization of the sample. A disadvantage of this approach is that the extraction of nucleic acids is notoriously difficult from high humic acids containing material such as peat colonised with *A. bisporus* mycelium. As described in section 4.1, humic acids have similar chemical properties to nucleic acids, which makes it difficult to separate these two products. In addition to this, they are powerful polymerase and reverse transcriptase inhibitors.

A number of methods have been developed to extract DNA and / or RNA from soil samples prior to this work (Hurt *et al.*, 2001; Jackson *et al.*, 1997; Miller *et al.*, 1999). Hurt *et al.* 2001 described a method which could be used to extract DNA and RNA simultaneously from forest soil (e.g. Great Smoky Mountains, US). For this method, soil samples are ground in a mortar under liquid nitrogen and a SDS-based lysis buffer. An extraction buffer (containing EDTA, CTAB, chloforom-isoamyl alcohol and SDS) is used to separate the nucleic acids from other cell components. A Sephadex G-75 resin slurry is used to separate the RNA from the DNA. The publication from Hurt *et al.* 2001 shows an electropherogram of samples extracted with this method. While the agarose gel is not as sensitive as the Bioanalyzer system (Agilent Technologies), it is possible to conclude that the 23 S rRNA is less intense than the 16S rRNA and that there is an elevated baseline below the 23S rRNA, indication a certain degree of degradation of the RNA fraction. Separation of RNA from the DNA was achieved through size separation on a Sephadex resin slurry. The use of Sephadex resin columns was also tested for the separation of humic acid from humic acid rich samples. It was however concluded that size separation methods are not feasible for mRNA purification as molecules of various sizes will not be purified with equal efficiency, potentially affecting transcript levels (Jackson *et al.*, 1997). Similarly, a phase separation method using an acid phenol:chloroform solution as described by Sreenivasaprasad (2000) and used for the extraction of total RNA from (soil-free) fungal samples was able to extract total RNA from colonised casing samples. The majority of the genomic DNA was retained in the acidic non-aqueous phase as suggested in the Applied Biosystems technical bulletin 158 and the RNA samples assessed on the Bioanalyzer (Agilent

Technologies). Total RNA extracted using this method was however contaminated with humic acids and partially degraded. Addition of CTAB (Doyle & Doyle, 1987), PVP or PVPP (Young *et al.*, 1993), additives commonly used in the extraction of total RNA (e.g. to remove melanin and polysaccharides) or purification through commercial silica matrix based columns such as the Qiagen RNeasy miniprep kit did not reduce the concentration of humic acid below inhibitory levels. Only few publications have become available recently discussing transcriptome analysis of soil samples. The extraction methods used in these publications are mainly commercial solutions. An example is presented by McGrath *et al*. (2008) who use the PowerSoil RNA extraction kit (MoBio, USA) to support the extraction and analysis of mRNA from environmental microbial communities using cDNA microarrays. They concluded that the PowerSoil RNA extraction kit (MoBio, USA) provided the best results. The agarose gel image presented did however indicate a certain degree of RNA degradation. This method was also not tested for humic acid rich samples. The FastRNA pro soil- direct kit (MP Biomedicals) claimed to remove inhibitory substances from soil samples, including humic acids. Modification of this kit to include an additional purification and concentrating step, presented in this work created a workflow that yields intact, polymerase- and reverse transcriptase- inhibitor free total RNA, verified using a sensitive, digital electrophoresis analysis (Bioanalyzer, Agilent Technologies) and oligonucleotide microarray analysis. The exact composition of the FastRNA pro soil – direct kit is proprietary but it is likely that the subsequent purification steps, also the principle of the PowerSoil RNA extraction kit mentioned previously (phase separation and selective binding of total RNA to a matrix) in combination with proprietary inhibitor removal solutions gradually enriches the total RNA fraction and reduces the inhibitor fraction while protecting the total RNA from the activity of degrading enzymes. The methodology based on a modified FastRNA Pro Soil-direct kit (MP Biomedicals) and the RNeasy MinElute cleanup kit (Qiagen) which achieves the extraction of high quality, un-degraded and inhibitor free total RNA that can be readily used for the enzymatic production of fluorescently labelled cRNA, required for microarray analysis, was developed.

At the beginning of this project (September 2005), no genome sequence information was available for *Agaricus bisporus*. The first confidential release of the

sequenced genome took place in May 2009. All available *A. bisporus* genomic and transcriptome data was collected in October 2005 and collated into a database. This database contained 1,319 sequences, of which 1,112 sequences were unique, representing approximately 13% of the predicted gene number for *A. bisporus* and was the largest available *A. bisporus* sequence database at the time.

An *A. bisporus* gene expression microarray based on long length oligonucleotides (probes) was not available prior to this project. Two bioinformatics programs, OLIGOPICKER and OLIGOSTAR were used to identify up to three unique 60-mer oligonucleotide sequences for each of the sequences in the *A. bisporus* sequence database. 2,708 candidate probes representing 1,105 were identified and organised into a custom 2 x 11,000 feature oligonucleotide validation microarray design.

The candidate probes, cRNA sample preparation and microarray hybridisation methodology was validated using a standard process. The quality of each microarray hybridisation was assessed using various metrics presented in the Feature Extraction quality control reports and the results from the control spike-ins. The cRNA sample preparation method and specificity of the candidate probes were assessed through a series of microarray hybridisations including self versus self hybridisations, dye-swap hybridisations and hybridisation with cRNA from various developmental stages. Ultimately, 1617 validated and highly specific probes representing 1079 *A. bisporus* sequences were selected and organised into the final custom, 8 X 15,000 60-mer oligonucleotide *A. bisporus* gene expression microarray design. Each validated probe was represented 5 times on this microarray design to further increase the strength of the gene expression results. An additional advantage of this multi-pack microarray format was that 8 samples (or multiples of 8 samples if hybridising multiple microarray slides at once) could be hybridised at the same time and under the same conditions, reducing experimental variability.

Differences between samples (e.g. concentration, integrity and presence of certain levels of inhibitory substances) and the slight differences in efficiency of the cRNA production and fluorescent labelling reaction do generally result in a (small) variation in yield and dye-incorporation of the fluorescently labelled cRNA or cDNA.

Before samples can be compared, the microarray data has to be "normalised". Whole genome normalisation methods such as a global normalisation or intensity-dependent normalisation expect that the majority of genes do not show differential expression between samples. The majority of genes present on a whole genome microarray, mostly representing the entire transcriptome of an organism, will generally change little over the course of an experiment. Technological advancements have made it possible to create custom microarrays whereby the user can specify the content of an array. As a consequence, it became possible to construct microarrays representing a subset of the transcriptome or include all available sequence information of a non-genome sequenced organism. This poses new challenges to microarray analysis, especially when the genes that are represented on the array are expected to be differentially expressed over the course of an experiment. It is therefore important to select a normalisation method that takes into account this higher frequency of differential expression. Currently, 6 different normalisation algorithms are widely used by researchers working with custom microarrays. These methods are 1) the **average** method whereby the intensities of each signal are adjusted so that the average signal of each array becomes the same (Thomson *et al.*, 2004); 2) the **rank invariant** method which has a similar approach to the average method but the algorithm identifies a subset of rank-invariant genes, which are used to correct the intensities of all signals (Jain *et al.*, 2005); 3) the **cubic spline** method which is a non-linear method present in BeadStudio software (Workman *et al.*, 2002); 4) the **quantile** method which tries to uniform the quantile distribution of each array signal population and is widely used as standard in single-channel arrays (Lopez-Romero *et al.*, 2010); 5) the **Lumi** method which was designed for Illumina BeadChips and is based on a variance stabilising normalisation algorithm (Du *et al.*, 2008) and 6) the **percentile** normalisation (Yauk *et al.*, 2004).

After analysis of the box-plots of the raw data for each of the microarrays that were performed during this project (data not shown), it was decided in discussion with biometrician Andrew Mead (Warwick HRI) to apply a custom normalisation whereby the microarray data was initially $\log_2$ transformed to balance out variability at various intensities and to achieve a more symmetrical data distribution. Pre-processing of the microarray data removed outlier probes and verified the absence of spatial hybridization

effects (section 2.6.1). Adjustments were made to the dataset as described in section 2.6.1 so that each array had the same median, similar to the **average** method and the same interquartile range, similar to the **quantile** method. It was assumed that the median and interquartile ranges should be relatively consistent across arrays with the "central" proportion of probes showing little variation in expression response. Box-plot analysis of the normalised data showed that the arrays had equal median intensities and interquartile ranges (data not shown). Quantitative reverse-transcriptase PCR on five genes, over three time points (three biological replicates, three technical replicates) and normalised to 18S rRNA expression levels as described in section 4.6 showed that the correlation between the PCR and microarray data increased from 0.931 (SD ± 0.037) to 0.976 (SD ± 0.025) after microarray normalisation. As mentioned in this discussion, the expression of various genes (e.g. hydrophobin genes 824, 854 and 1631) was studied in mycelial and primordia samples previously using other technologies and similar expression patterns were observed in the data created during this project.

The subsequent pre-processing of the microarray data and normalisation applied during this project was able to balance out average intensities and ranges of individual arrays, which allowed a inter-array comparison, despite the use of a microarray which was based on a small, biased sequence database.

The analysis of time course microarray data is relatively novel. The microarray data generated during this project is technically challenging to analyse due to the nature of the samples and the bias towards differential expression of the microarray probes. It was therefore decided to analyse the data using two distinct statistical methods. Only genes that were identified to be significantly differentially expressed by both statistical methods were considered for further analysis.

An *in silico* functional annotation experiment was performed using the Blast2GO software (Conesa *et al.*, 2005) to identify putative functions of sequences in this database, based on sequence similarity of the nucleic acid sequence and characterised sequences in other organisms. Of the 1079 represented on the final custom *A. bisporus* gene expression microarray, 619 sequences showed significant similarity to publicly

available sequences that are associated with a putative function, based on homology to characterised sequences.

The resources and methodologies presented in this chapter were used to analyse changes in gene expression in samples collected during the various growth experiments described in Chapter 3. The results of this gene expression study are presented and discussed in Chapter 5.

Chapter 5. Gene expression profiling of the phase change from vegetative to reproductive growth in A. bisporus

5.1 *Introduction*

Morphological development and differentiation of *A. bisporus* grown under standard and experimental cultivation conditions was studied during this project using time-lapse photography and microscope observations as described in Chapter 3. It was confirmed that the vegetative mycelium needs to experience a specific change in environmental conditions before the transition from vegetative to reproductive growth, the phase change, is initiated. Evidence was found that this process is controlled by 2 switches (temperature and level of mushroom volatiles) and a quantitative regulator (carbon dioxide level). A reduction of the level of mushroom volatiles is required to initiate the phase change. Subsequently, **fluffy mycelial cords**, **fluffy hyphal knots**, **fluffy undifferentiated primordia** and **smooth, undifferentiated primordia** develop. These structures do not develop further unless a reduction in temperature is perceived. When the temperature is reduced from 25°C to 18°C, **smooth undifferentiated primordia** continue their development and subsequently **elongated differentiating primordia** and **differentiating primordia with a distinct waist** develop. This is followed by further development and expansion into stage 2- 7 fruit bodies. Carbon dioxide levels influence the number of fruit bodies that develop with optimal concentrations below 1,000 p.p.m stimulating reproductive development. It was also found that primordia originating from mycelial cords on the surface of the casing layer will not develop into elongated differentiating primordia, in contrast to primordia developing below the surface of the casing layer.

While the information presented in Chapter 3 provided an insight into morphological development during the phase change and into the effects of individual environmental parameters on this process, very little is known on the molecular

mechanism that controls the phase change and how it is influenced by changes of individual environmental parameters.

Two series of gene expression profiling experiments were performed during this project, using the resources described in Chapter 4 to investigate the molecular mechanism active during the phase change.

The first series of experiments was designed to study the gene expression profile of *A. bisporus* under standard cultivation conditions whereby three environmental parameters (temperature, CO_2 level and level of mushroom volatiles) were modified simultaneously to optimally stimulate the phase change. It was expected that changes in gene expression recorded under these conditions, where the fungus switches from vegetative to reproductive growth, would provide an insight into the many interacting processes that regulate and support the phase change. The results of these experiments are presented in section 5.2. The second series of experiments was designed to separate out these 3 environmental parameters and investigate their individual effect on gene expression. The results of these experiments are presented in section 5.3. Analysis of differences in gene expression between *A. bisporus* developing under standard conditions (the first series of experiments) and experimental conditions (the second series of experiments) is expected to identify genes that might not be involved with the phase change but may be involved with the nutritional state of the fungus or other physiological processes.

The selection of time points used to construct gene expression profiles was based on observations made of *A. bisporus* grown during 4 separate growth experiments in mushroom trays and 2 growth experiments in the flask-based system, under phase change stimulating conditions, as described in Chapter 3.

From two of these crops, detailed time-lapse observations were made. The camera was positioned above a growth tray in the first experiment while it was positioned on the side of a growth pot in the second experiment. Images were taken at 1 h intervals. In addition to this, sections of casing were analysed at 24 h intervals from each of the four growth experiments to compare development inside the casing layer and

the presence of specific developmental structures was documented. These growth experiments showed very similar development rates whereby elongated differentiating primordia were seen, breaking through the surface of the casing layer at approximately 168 h after airing. The flask-based experiment where *A. bisporus* was grown under phase change stimulating conditions (section 2.2.3.1), standard growth conditions, was performed twice, separated in time and was therefore represented by two data-sets. The first replicate served as a comparison for the experiments described in sections 3.4.2, 3.4.3 and 3.4.4. The second replicate served as a comparison for the experiment described in section 3.4.5. It was decided not to examine samples taken from later stages as many of the primordia and fruit bodies that had developed on the surface of the casing layer did not develop into mature fruit bodies. Observations of flask-based experiments where *A. bisporus* was exposed to phase change stimulating conditions showed similar developmental processes, although at a slightly slower rate compared to *A. bisporus* grown in mushroom trays.

It was therefore decided to collect:

1) a reference sample at airing (0 h),

2) a sample at 24 h after airing to capture the initial response of the fungus to the change in environmental conditions and before any obvious macroscopic change in morphology occurred,

3) a sample at 72 h after airing to capture the change in gene expression present during the formation of hyphal knots and undifferentiated primordia, and

4) a sample at 168 h (mushroom house experiments) / 192 h (flask-based experiments) after airing to capture the changes in gene expression during the elongation and differentiation of primordia.

The second series of experiments, designed to separate out the effects of temperature, mushroom volatiles and carbon dioxide, were performed in a mushroom house as described in section 2.2.2 (temperature) and the flask-based system described in section 2.2.3 (mushroom volatiles and carbon dioxide). These experiments, with the

exception of the experiment where the volatile source was removed from the air-supply (section 3.4.4) did not stimulate the phase change or did not result in the development of mature fruit bodies. To allow comparison with the standard experiment where fruit bodies developed, it was decided to follow the same sampling regime for all experiments (at airing (0 h) and post-airing 24, 72, 168 (or 192 for flasks) hours.

Samples were collected, in triplicate (biological replicates), at the selected time-points for each of the growth experiments as described in sections 2.2.1.4 (mushroom house growth system) and 2.2.2.4 (flask-based growth system). No mushroom tray or Kilner jar was sampled more than once. Only mushroom trays at the same height in the mushroom house were sampled to avoid subtle differences in environmental conditions.

The various resources and methodologies developed in Chapter 4 were used to assist with this analysis. Total RNA from all colonised casing samples collected during these two series of experiments was extracted with the modified total RNA extraction method (section 2.3.2). The extracted total RNA was processed and hybridised on the *A. bisporus* gene expression microarray (sections 2.4.5 and 4.3) containing validated oligonucleotide probes representing 1079 *A. bisporus* genes (~ 13% of the predicted gene number for *A. bisporus*).

5.2 *Identification of genes that were significantly differentially expressed* in A. bisporus *during the phase change from vegetative to reproductive growth*

For this experiment, *A. bisporus* was grown under standard (commercial) cultivation conditions in 1) a growth tray in a mushroom house (performed once) and 2) flask-based system with controlled air-supply (performed in duplicate, separated in time).

When mycelium became visible on the surface of the casing layer in the growth trays or Kilner jars, the temperature was reduced from 25 °C to 18 C° and the system was ventilated in order to reduce CO_2 (< 1,000 p.p.m) and mushroom volatile levels as described in section 2.2.2.1 (mushroom house) and section 2.2.3.1 (flask-based system). These changes were applied simultaneously during airing. Time-lapse observations of

this experiment are described in section 3.2. Samples were collected as described in section 5.1 and section 2.8.1. Total RNA was extracted from these samples as described in section 2.3.2. Each of the total RNA samples was processed as described in section 2.3.4 to create fluorescently labelled cRNA which was hybridised onto *A. bisporus* 8 x 15,000 60-mer oligonucleotide microarrays as described in section 2.8.1 containing probes representing 1079 *A. bisporus* genes. Appendices 1 (mushroom house samples) and 2 (flask-based system samples) summarise the results of these steps (e.g. quality and concentration of the extracted total RNA; efficiency of the cRNA preparation step). All samples passed the labelling recommendation provided by Agilent Technologies. Gene expression microarray data was extracted from the microarray image files and processed as described in section 2.6.1.

Two analysis methods were used to identify genes that were differentially expressed in all three growth experiments where *A. bisporus* was grown under phase change stimulating conditions. Only genes that were identified to be differentially expressed by both analysis methods (BATS and MAANOVA) were retained for further analysis as discussed in section 2.6.2.3. The MAANOVA procedure identified 118 genes that were significantly differentially expressed over time, in all three phase change stimulating experiments while BATS identified 75 genes. Of these genes, 52 genes were identified by both analysis methods. The difference in the number of genes identified by both analysis methods can be explained partially by the difference in analysis approach and stringency. Figure 5-1 compares the number of genes identified to be differentially expressed in the three growth experiments, where *A. bisporus* was grown under phase change stimulating conditions, by the BATS-software and / or the MAANOVA ($p \leq 0.01$) procedures.

Figure 5-1 Comparison of the number of genes identified to be differentially expressed in *A. bisporus* grown under phase change stimulating conditions by BATS (section 2.6.2.2) and MAANOVA (section 2.6.2.1). This data only included genes that were identified to be differentially expressed by BATS or MAANOVA in 3 growth experiments (one mushroom house experiment and two flask-based growth experiments).

Another 20 other genes were significantly differentially expressed only in the mushroom house experiment (Appendix 3) and 9 genes were significantly differentially expressed only in one or both flask-based growth experiment(s) (Appendix 4). These 29 genes will however not be used for further analysis as they are not significantly differentially expressed in both growth systems.

Table 5-1 Genes that were differentially expressed in *A. bisporus* grown under phase change stimulating conditions in the mushroom house experiment and in the flask-based experiments (sequences with similar putative functions are grouped together).

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated genes				
22	argininosuccinate synthase **(nitrogen metabolism)**	+[##]	++	=
59	glutamine synthase **(nitrogen metabolism)**	=	+	=
318	Carboxypeptidase **(nitrogen metabolism)**	+	=	+
708	NADP-specific glutamate dehydrogenase **(nitrogen metabolism)**	+	++	+
1250	NADP-specific glutamate dehydrogenase **(nitrogen metabolism)**	+	+	=
1192	aminoadipate reductase **(nitrogen metabolism)**	=	=	++
1577	carbamoyl-phosphate synthase **(nitrogen metabolism)**	+	++	=
595	Metallothionein **(putative stress tolerance)**	+	-	++
824	hydrophobin *hypB* **(putative cell structure molecule)**	+++	+	+
854	hydrophobin *hypB* **(putative cell structure molecule)**	+++	+	+
1274	hydrophobin *hypA*, ABH1 **(putative cell structure molecule)**	=	+	+
994	mismatched base pair and cruciform DNA binding protein **(putative DNA replication regulator)**	+	++	=
1353	Major facilitator super family transporter **(transport protein)**	=	+	+
711	tyrosinase **(monooxygenase / oxidoreductase enzyme)**	=	++	+
748	cytochrome P450 **(monooxygenase/oxidoreductase enzyme)**	+	=	++
1555	Cytochrome P450 **(monooxygenase / oxidoreductase enzyme)**	=	++	+
998	riboflavin aldehyde-forming enzyme	+	=	++
94	gulonolactone oxidase **(glucose metabolism)**	+	++	=
358	gulonolactone oxidase **(glucose metabolism)**	+	++	=
1582	glutathione transferase	=	+	+
92	non-characterised sequence	+	++	+
208	non-characterised sequence	s>+	++	=
213	non-characterised sequence	+	++	=
240	non-characterised sequence	=	++	+
311	non-characterised sequence	+	+	+
474	putative protein	+	+	=

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h (growth tray) or 182 h (flask-based system) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing).
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point.. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

864	putative protein	+	+	++
896	putative protein	+	+	=
916	non-characterised sequence	++	-	++
956	non-characterised sequence	+	++	=
982	non-characterised sequence	+	+	+
990	non-characterised sequence	+	+	=
1198	putative protein	+	++	+
1231	non-characterised sequence	+	++	++
1251	non-characterised sequence	õ	+	=
1285	non-characterised sequence	+	++	-
1341	non-characterised sequence	++	+	-
1408	putative protein	+	+	++
1479	non-characterised sequence	+	+	=
1591	putative protein	=	=	++
1621	non-characterised sequence	+	+	=
down-regulated genes				
1131	isocitrate lyase (**carbohydrate / glyoxylate metabolism**)	=	--	-
1550	glycoside hydrolase family 5 protein	=	-	-
1631	hydrophobin ABH3 **(putative cell structure molecule)**	=	--	-
82	putative protein	-	-	=
289	non-characterised sequence	=	--	--
410	putative protein	-	-	-
907	non-characterised sequence	=	-	-
1134	non-characterised sequence	=	-	-
1181	non-characterised sequence	=	-	-
1534	putative protein	=	-	-
1542	non-characterised sequence	-	-	--

Where possible, *in silico* functional annotation information was used to link a putative function to a sequence and illustrated that the sequences represented in this table are thought to be involved in various functions. Examples of this are nitrogen metabolism genes (e.g. arginosuccinate synthase and glutamine synthase), genes that are potentially involved with (cellular) structure (e.g. hydrophobin), a transport protein (major facilitator superfamily transporter) and a potential transcription / DNA replication regulator. 20 sequences do not have a significant sequence homology to publicly available characterised sequences. These genes have been identified from a pool of 1079 genes based on their expression during this experiment and could be key to the regulation and / or support of the phase change. The gene expression profile of these

52 genes will be compared to the results from the experiments described in section 5.3, which were designed to investigate the effect of individual environmental parameters on gene expression. The result of this comparison will be discussed in depth in section 5.4.

5.3 *Investigating the effects of temperature, CO_2 and mushroom volatiles on gene expression during A. bisporus development*

The experiments described in section 5.2 were designed to study the gene expression profile of *A. bisporus* grown under phase change stimulating conditions. This involved a simultaneous change of three environmental parameters. A second series of experiments was performed to investigate the changes in gene expression in *A. bisporus* when only one or two these environmental parameters were not changed while the other parameter(s) were altered to phase change stimulating levels. In the first two experiments, the 1) effect of high temperature and 2) the combined effect of high levels of CO_2 and mushroom volatiles on *A. bisporus* in a mushroom house were tested.

It was then considered that the effect of different gasses in the head space should be analysed separately. Three experiments were performed whereby 1) the effect of high levels of volatiles, 2) the reversibility of inhibition caused by high levels of mushroom volatiles or 3) the effects of high CO_2 levels on gene expression in *A. bisporus* grown in the flask-based system while the other parameters were altered to phase change stimulating levels.

It was expected that a comparison of the gene expression profiles constructed during each of these experiments with the gene expression profile *A. bisporus* grown under standard cultivation conditions and observations of morphological development would identify genes that 1) respond to a change of specific environmental conditions and 2) might not be regulating the phase change (e.g. genes that were differentially expressed in conditions where no observable development or differentiation occurred).

Lists of significantly differentially expressed genes constructed for each of these experimental growth conditions and compared with the gene expression profile of the relevant control experiment (mushroom house or flask-based system) so that genes that

are differentially expressed only in the experimental treatment, only in the standard cultivation conditions experiment or in both cases could be identified.

5.3.1 Effect of high temperature but reduced levels of CO_2 and mushroom volatiles

This experiment was designed to investigate the gene expression profile of *A. bisporus* grown under high temperature (25 °C) in a mushroom house while the growth room was ventilated to reduce levels of CO_2 and mushroom volatiles. As a result of this treatment, the surface of the casing layer got covered by a matt of mycelium (stroma) and numerous **smooth, undifferentiated primordia** (2-4 mm diameter) as described in section 3.4.1 and illustrated in Figure 5-2.

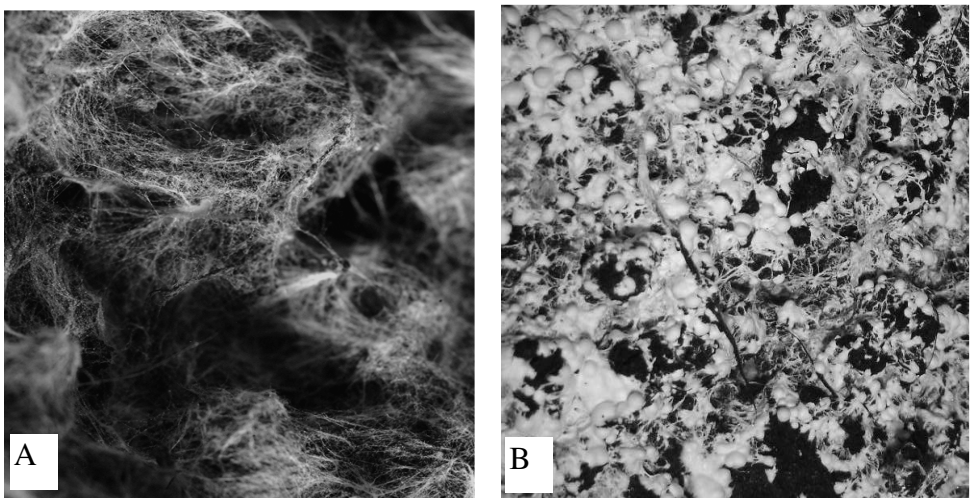


Figure 5-2 Images illustrating *A. bisporus* development under high temperature (reduced CO_2 and volatile levels at (A) airing and (B) 168 h after airing.

This treatment inhibited elongation and differentiation of the primordia and as a consequence, no mature fruit bodies developed. When the temperature was reduced to 18°C, at 312 h after airing, a significant number of fruit bodies started to develop (results not shown), illustrating the reversibility of this inhibition. Samples were collected at airing, 24 h, 72 h and 168 h after the change in environmental conditions and processed for gene expression analysis as described in section 2.8.2. Appendix 1 summarises the results of the RNA extraction and cRNA preparation step. Each sample had a yield and incorporation rate within the recommended values provided by Agilent Technologies.

The gene expression profile created for this experiment was then compared with the results of control experiment where *A. bisporus* was grown under standard conditions as described in section 5.2. The results of this experiment are:

- 24 genes were differentially expressed only in *A. bisporus* exposed to high temperature (reduced CO_2 and volatile levels) and not in the control treatment: Appendix 5. Of these genes, 13 were up-regulated and 11 were down-regulated. As these genes were only differentially expressed in this treatment it could be suggested that they are involved with a response to the extended exposure to high temperature rather than being involved with reproductive development;

- 39 other genes were differentially expressed in *A. bisporus* exposed to high temperature (reduced CO_2 and volatile levels) and under standard cultivation conditions: Appendix 6. These genes could be involved in the early phase change, where vegetative mycelium develops into smooth, undifferentiated primordia as they are differentially expressed in both treatments.

- 13 other genes were differentially expressed only *A. bisporus* grown under standard conditions and not when exposed to high temperature (reduced CO_2 and volatile levels): Appendix 7. These genes could be involved with the late phase change where smooth undifferentiated primordia develop into elongated differentiating primordia (and subsequent stages) as they were only differently expressed in *A. bisporus* exposed to phase change stimulating conditions.

5.3.2 Effect of high levels of CO_2 and mushroom volatiles but reduced temperature

This experiment was designed to investigate the effect of high levels of CO_2 (~ 3,000 p.p.m.) and mushroom volatiles on gene expression in *A. bisporus* grown in a mushroom house while the temperature was reduced to 18 °C as described in section 2.2.2.3. The high levels of CO_2 and mushroom volatiles were achieved by not ventilating the growth room and covering the growth trays with paper sheets. No sign of reproductive morphogenesis was observed in the centre of the growth tray as discussed previously in section 3.4.2 and illustrated in Figure 5-3.

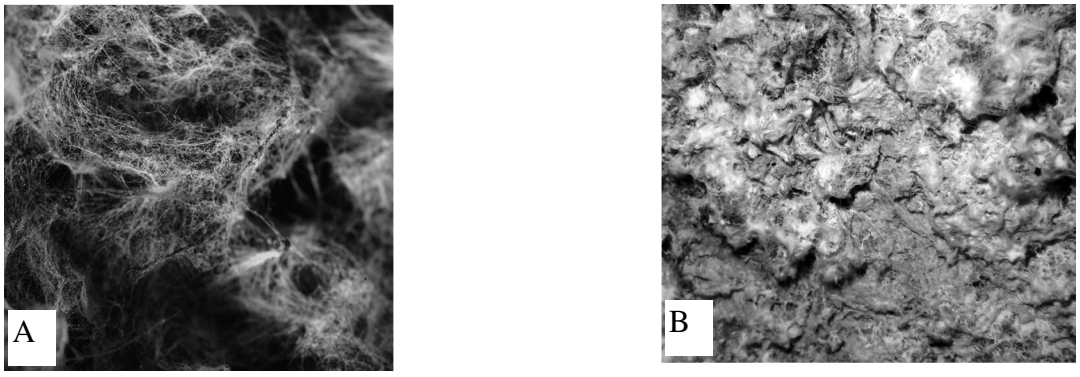


Figure 5-3 *A. bisporus* development under high CO_2 and volatile levels but reduced temperature (18 °C). Images were taken (A) at airing and (B) at 168 h after airing.

Samples were collected in triplicate at airing, 24 h, 72 h and 168 h after airing and used for gene expression analysis as described in section 2.8.3. Appendix 1 summarises the results of the RNA extraction and cRNA preparation step. Each sample had a yield and incorporation rate within the recommended values provided by Agilent Technologies. The gene expression profile created for this experiment was then compared with the results of control experiment where *A. bisporus* was grown under standard conditions as described in section 5.2. The results of this experiment are:

- 24 genes were differentially expressed only in *A. bisporus* exposed to high levels of CO_2 and mushroom volatiles (reduced temperature) and not when grown under standard conditions: Appendix 8. As these genes were not differentially expressed under standard conditions it could be suggested that they are more likely to be involved with general metabolism rather than reproductive development. Six of the 16 generally up-regulated genes were initially down-regulated but show a significant increase in gene expression between 72 h and 168 h after airing. The change in gene expression seen in the six genes that were generally down-regulated could be caused by increasing repression under these conditions;

- 25 other genes were differentially expressed in *A. bisporus* exposed high levels of CO_2 and mushroom volatiles (reduced temperature) and when grown under

standard conditions: Appendix 9. Of these genes 23 were up-regulated and 2 were down-regulated. As these genes are also differentially expressed under conditions where no reproductive development was observed it could be argued that these genes might not be involved with the regulating of the phase change. It could however be that they are involved with early development which was not detectable visually or they are involved in the general metabolism of the fungus. Examples of this are nitrogen metabolism genes (e.g. argininosuccinate synthase) and glucose metabolism genes (gluconolactone oxidase).

- 27 other genes were differentially expressed when grown under standard conditions and not when exposed to high levels of CO_2 and mushroom volatiles (reduced temperature): Appendix 10. As these genes are only differentially expressed under standard cultivation conditions in this comparison, they could potentially be "key" to the regulation of the phase change.

This last experiment studied the combined effect of high levels of CO_2 and mushroom volatiles on gene expression. Visual observations confirmed that no indication of reproductive development could be observed in the centre of the growth trays. A flask-based system was used to carefully control the gaseous headspace in the next experiments whereby the individual effects of mushroom volatiles (sections 5.3.3 and 5.3.4) and CO_2 (section 5.3.5) on gene expression were studied.

5.3.3 Effect of high level of 1-octen-3-ol but reduced CO_2 level and reduced temperature

A flask-based system with controlled air supply was used to study the effects of high level of 1-octen-3-ol on gene expression while CO_2 level and temperature were reduced to phase change stimulating levels as described in section 2.2.3.2. These elevated 1-octen-3-ol levels completely inhibited reproductive morphogenesis as described in section 3.4.3 and illustrated in Figure 5-4.

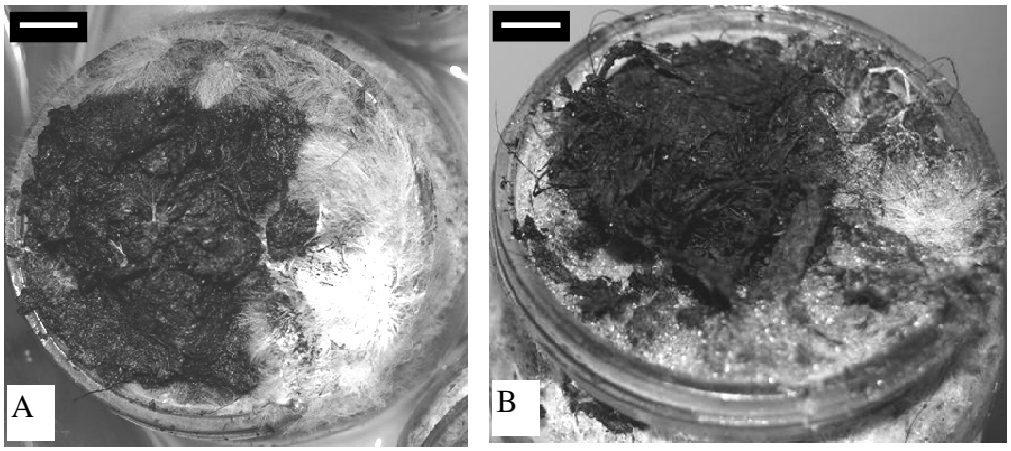


Figure 5-4 ***A. bisporus*** **development under high level of 1-octen-3-ol but under reduced temperature and CO_2 level. Images were taken (A) at airing and (B) at 192 h after airing. Scale bars = 10 mm**

Samples were collected in triplicate at airing, 24 h, 72 h and 192 h after airing and used for gene expression analysis as described in section 2.8.4. Appendix 2 summarises the results of the RNA extraction and cRNA preparation step. Each sample had a yield and incorporation rate within the recommended values provided by Agilent Technologies. The gene expression profile created for this experiment was then compared with the results of *A. bisporus* grown under standard conditions as described in section 5.2. The results of this experiment are:

- 47 genes were differentially expressed in *A. bisporus* exposed to high level of 1-octen-3-ol level (reduced temperature, reduced CO_2 levels) and not when grown under standard conditions: Appendix 11. Remarkably, the majority of genes (29) were down-regulated under these conditions, including two putative transporter proteins that were initially up-regulated but show significant down-regulation for the remainder of the experiment.

- 38 other genes were differentially expressed in *A. bisporus* exposed to high levels of 1-octen-3-ol (reduced temperature, reduced CO_2 levels) and when grown under standard conditions: Appendix 12. Of these genes, 30 were generally up-regulated and 8 were generally down-regulated. Various nitrogen metabolism genes and hydrophobins were up-regulated. A putative transcription

regulation factor / DNA replication regulation protein is up-regulated over three-fold between airing and 24 h after airing and down-regulated over three-fold between 24 h and 72 h after airing, followed by up-regulation (<2.8 fold) between 72 h and 168 h after airing. This pattern is different to the expression pattern for this gene in *A. bisporus* grown under standard cultivation conditions where this gene is up-regulated between airing and 24 h after airing (<2.8 fold) and between 24 h and 72 h after airing (>8 fold).

- 14 other genes were differentially expressed when grown under standard conditions and not while exposed to high levels of 1-octen-3-ol levels (reduced temperature, reduced CO_2 levels): Appendix 13. As these genes are only differentially expressed under standard cultivation conditions in this comparison, they could potentially be "key" to the regulation of the phase change.

5.3.4 Effect of a reduction of the level of 1-octen-3-ol following removal of the 1-octen-3-ol source

This experiment is the continuation from the previous experiment (section 5.3.3). At 192 h after airing, the volatile source was removed from the air-supply. This resulted in the development of normal fruit bodies, which confirmed that the inhibition of reproductive growth observed under high volatile levels was reversible as discussed in section 3.4.4 and as illustrated in Figure 5-5.

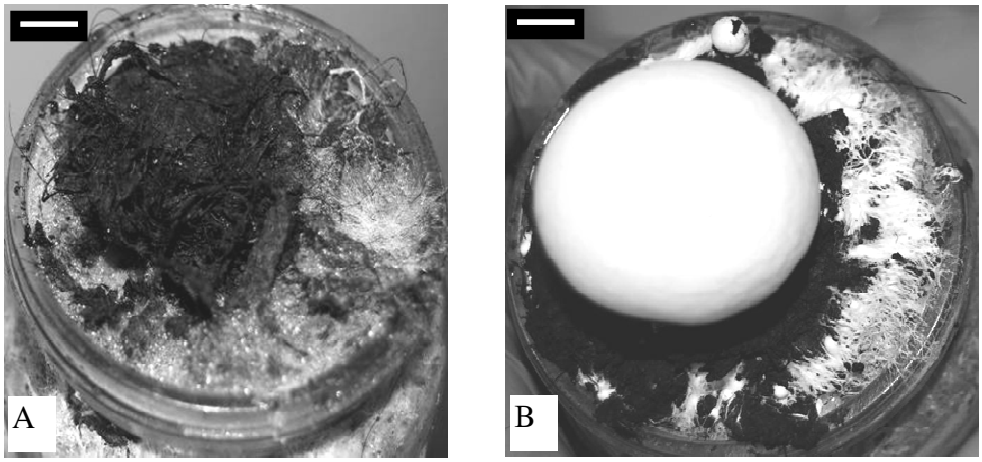


Figure 5-5 Illustrating the reversibility of the volatile related phase change inhibition: (A) *A. bisporus* grown in a flask-based system with controlled air supply after exposure to elevated 1-octen-3-ol concentrations for 192 h. At this point, the volatile source was removed from the airflow; (B) Approximately 240 h later, fruit bodies had developed. Scale bar = 10 mm.

Samples were collected at airing, 24 h, 72 h and 168 h after removal of the volatile source and used as for gene expression analysis as described in section 2.8.5. Appendix 2 summarises the results of the RNA extraction and cRNA preparation step. Each sample had a yield and incorporation rate within the recommended values provided by Agilent Technologies. The gene expression profile created for this experiment was then compared with the results of *A. bisporus* grown under standard conditions as described in section 5.2. The results of this experiment are:

- 9 genes were only differentially expressed in *A. bisporus* exposed to reduced 1-octen-3-ol levels after extended exposure to high 1-octen-3-ol levels (reduced temperature, reduced CO_2 levels) and not when grown under standard conditions: Appendix 14;

- 15 other genes were differentially expressed in *A. bisporus* exposed to reduced 1-octen-3-ol levels after extended exposure to high 1-octen-3-ol levels (reduced temperature, reduced CO_2 levels) and when grown under standard conditions: Appendix 15. Of these genes, 7 were generally up-regulated and 8 were generally down-regulated;

- 37 other genes were differentially expressed only when grown under standard conditions and not while exposed to reduced 1-octen-3-ol levels after extended exposure to high 1-octen-3-ol levels (reduced temperature, reduced CO_2 levels): Appendix 16. As these genes were only differentially expressed under standard cultivation conditions in this comparison, they could potentially be involved with the regulation of the phase change. Of these genes, 33 were generally up-regulated and 4 were generally down-regulated.

Only 9 genes were differentially expressed only in *A. bisporus* exposed to significantly reduced levels of 1-octen-3-ol after extended exposure to high levels of 1-octen-3-ol levels (reduced temperature, reduced CO_2 levels) and not when grown standard conditions. This low number of genes that were only differentially expressed under these conditions (compared to standard cultivation conditions) was not unexpected as after removal of the volatile source, the fungus developed fruit bodies as under standard cultivation conditions.

5.3.5 Effect of high level of CO_2 but reduced temperature and reduced level of mushroom volatiles

This experiment was designed to study the effects of elevated CO_2 level (reduced temperature and reduced level of mushroom volatiles) on gene expression levels in *A. bisporus*. CO_2 concentration in the flask-based growth system were therefore artificially increased to ~ 5,000 p.p.m. while volatile levels and temperature were reduced to phase change stimulating levels as described in section 2.2.3.4. Under these conditions, fruit body development occurred at lower rates as discussed in section 3.4.5. It was therefore concluded that carbon dioxide is not an on/off switch of reproductive development as was seen with temperature and mushroom volatiles but acts as a quantitative regulator.

Samples were collected at airing, 24 h, 72 h and 192 h after airing as described in 2.2.3.5 and used for gene expression analysis as described in section 2.8.6. Appendix 2 summarises the results of the RNA extraction and cRNA preparation step. Each sample had a yield and incorporation rate within the recommended values provided by Agilent Technologies. The gene expression profile created for this experiment was then

compared with the results of *A. bisporus* grown under standard conditions as described in section 5.2. The results of this experiment are:

- 34 genes were differentially expressed only in *A. bisporus* exposed to high CO_2 levels (reduced temperature, reduced volatile levels) and not when grown under standard conditions: Appendix 17. Of these genes, 23 were generally up-regulated and 11 were generally down-regulated. Even though fruit bodies developed under these conditions, there was no indication that these genes were differentially expressed under control conditions so they are most likely involved with non phase change related physiological processes, in response to the elevated CO_2 levels. Interestingly, only sequences 115, 262, 1297 and 1425 were also found to be uniquely differentially expressed in the conditions where high levels of CO_2 and mushroom volatiles were maintained.

- 37 other genes were differentially expressed in *A. bisporus* exposed to elevated CO_2 levels (reduced temperature, reduced volatile levels) and when grown under standard conditions Appendix 18. As these genes are identified to be differentially expressed in both treatments, they could be involved with reproductive growth, as fruit bodies developed in both treatments. They could however be involved with the response to elevated carbon dioxide levels, which might not relate directly to the phase change.

- 15 genes other were differentially expressed when grown under standard conditions and not when exposed to artificially elevated CO_2 levels (reduced temperature, reduced volatile levels): Appendix 19. As these genes were only differentially expressed under standard cultivation conditions in this comparison, they could potentially be "key" to the regulation of the phase change.

5.4 *Discussion*

Comparison of the gene expression profiles of A. bisporus grown under standard conditions and experimental conditions where specific environmental factors were varied

A first series of experiments was designed to study the gene expression profile of *A. bisporus* grown under standard, phase change stimulating conditions in growth trays in a mushroom house and in a flask-based growth system with controlled air-supply. At "airing", the temperature was reduced from 25 °C to 18 °C and the growth system was ventilated to reduce levels of CO_2 (< 1,000 p.p.m) and mushroom volatiles. As this project aimed to investigate the gene expression profile during the phase change from vegetative to reproductive growth and subsequent development of primordia, it was decided to collect colonised casing samples at airing (0 h), 24h, 72 h and 168 h after airing. Samples collected at airing represented vegetative mycelium (hyphae and mycelial cords). Samples collected at 24 h after airing were expected to represent the initial response of the fungus after the change in environmental conditions whereby intense localised branching, resulting in **fluffy mycelial cords,** was initiated (early phase change). Samples collected at 72 h after airing were expected to capture changes in gene expression during development from **fluffy hyphal knots** to **fluffy** and **smooth undifferentiated primordia** (early phase change). The time point, 168 h after airing is thought to capture the gene expression between the undifferentiated primordia stages and the development of **elongated differentiating primordia** (late phase change).

Each sample was a mixture of vegetative (supporting) mycelium and reproductive mycelium but it was expected that by performing a time-course analysis, subtle but significant changes in gene expression related to reproductive growth would be identified. For this purpose, a normalisation procedure was developed as described in section 4.6 and two statistical approaches were followed to identify genes that were differentially expressed over the course of the experiment as described in section 4.7. The analysis of samples from the mushroom house experiment and the flask-based growth system experiment, exposed to standard, phase change stimulating conditions identified 52 genes (selected by both statistical analysis methods) that were differentially

expressed during reproductive development. A representation of the expression levels of these genes during the four investigated time-points is given in Figure 5-6. This gene expression profile shows two distinct expression patterns, one series of genes were up-regulated at airing and showed a significant drop in relative expression over the course of this experiment and a second series of genes were down-regulated at airing and showed a significant increase in gene expression over the course of the experiment. Interestingly, the largest change in gene expression is observed between 24 h and 72 h after airing, at which point fluffy mycelial cords and fluffy hyphal knots were observed and when fluffy and (subsequently) smooth undifferentiated primordia start to develop.

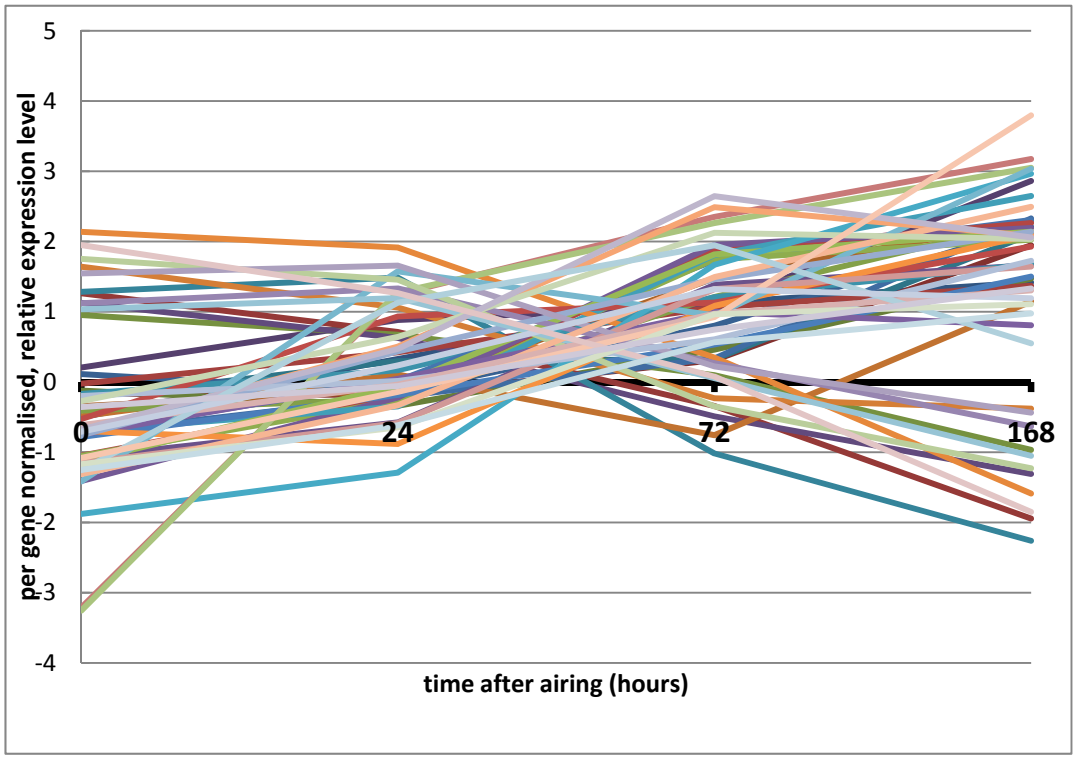


Figure 5-6 Microarray expression profile of the 52 genes that were identified to be differentially expressed in *A. bisporus* grown under phase change stimulating conditions in both a mushroom house and a flask-based system. Samples were collected at airing, 24 h, 72 h and 168 h after airing. Each line shows the expression levels of specific gene, at a specific time-point.

A second series of experiments was performed to investigate effect of individual environmental parameters such as temperature, CO_2 and mushroom volatiles on morphogenesis and gene expression as described in section 5.3. Genes that were identified to be differentially expressed in one or more of these treatments were then compared with the 52 genes identified to be differentially expressed in the control treatment. Based on observations of morphological development for each treatment, as discussed in Chapter 3, it was possible to organise these 52 genes into 3 clusters:

- **cluster one**: genes that were differentially expressed in conditions where fruit bodies developed but not in conditions where no reproductive growth was observed or where only smooth undifferentiated primordia developed. This cluster contained **11 genes;**

- **cluster two**: genes that were differentially expressed in conditions where smooth, undifferentiated primordia developed without further differentiation but not in conditions where no reproductive growth was observed. This cluster contained **4 genes**;

- **cluster three**: genes that were differentially expressed in conditions where no reproductive growth was observed: **37 genes.**

Figure 5.7 outlines the biological association of these clusters in relation to the "black box" of the phase change. These genes are presented in Table 5-2 which provides the gene expression profile of each of the 52 genes constructed from the standard (mushroom house) growth experiment where temperature, CO_2 levels and mushroom volatile levels were modified simultaneously to optimally stimulate the phase change. This table also indicates whether these genes were generally up- or down-regulated in one or more of the experimental growth conditions and what their putative function could be, based on sequence similarity to previously characterised, publicly available sequences.

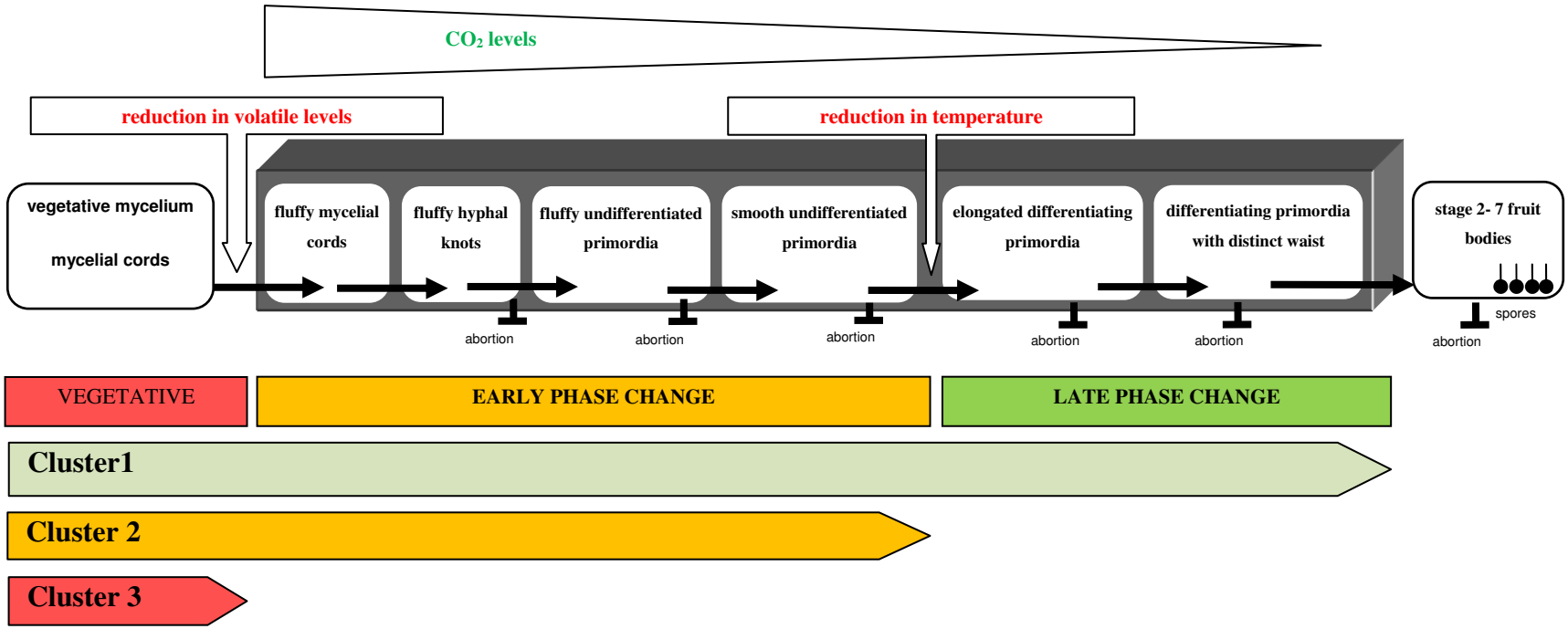


Figure 5-7 Updated "black box" representing the key morphological stages observed in *A. bisporus* developing and differentiating from vegetative mycelium to fruit bodies. This process is controlled by 2 on/off switches (red text) and one quantitative regulator (green text). Genes identified to be significantly differentially expressed in *A. bisporus* grown under phase change stimulating conditions in both a mushroom house and the flask-based growth system could be organised into 3 clusters, based on gene expression profiles and morphological development of experiments designed to study the effect of individual environmental parameters.

Table 5-2 Gene expression profiles of the 52 genes that are significantly differentially expressed in *A. bisporus* grown under (A[#]) phase change stimulating conditions and their response when grown under experimental conditions: (B) reduced level of 1-octen-3-ol after removal of the 1-octen-3-ol source; (C) high CO_2 level (reduced temperature and reduced level of mushroom volatiles); (D) high temperature (reduced levels of CO_2 and mushroom volatiles); (E) high levels of CO_2 and mushroom volatiles (reduced temperature); (F) high level of 1-octen-3-ol (reduced temperature and CO_2 level).

ID	A1[#]	A2[#]	A3[#]	B	C	D	E	F	putative functional annotation
Cluster 1									
1192	**=**[##]	**=**	**++**						aminoadipate reductase **(nitrogen metabolism)**
1353	**=**	**+**	**+**	**U**	**U**				major facilitator superfamily transporter **(transport)**
998	**+**	**=**	**++**	**U**					riboflavin-aldehyde forming enzyme
748	**+**	**=**	**++**						cytochrome P450 **(monooxygenase/oxidoreductase enzyme)**
1555	**=**	**++**	**+**	**U**					cytochrome P450 **(monooxygenase/oxidoreductase enzyme)**
896	**+**	**+**	**=**	**U**					putative protein
1591	**=**	**=**	**++**	**U**					putative protein
1550	**=**	**-**	**-**						glycoside hydrolase family 5 protein **(glucose metabolism)**
1631	**=**	**--**	**-**						hydrophobin ABH3
410	**-**	**-**	**-**	**D**					putative protein
1534	**=**	**-**	**-**	**D**					putative protein
Cluster 2									
595	**+**	**-**	**++**		**U**	**U**			Metallothionein **(stress metabolism)**
1274	**=**	**Ă**	**+**	**U**		**U**			hydrophobin ABH1 *hypA*
864	**+**	**+**	**++**		**U**	**U**			putative protein
990	**+**	**+**	**=**			**U**			non-characterised sequence
Cluster 3									
22	**+**	**++**	**=**		**U**	**U**	**U**	**U**	argininosuccinate synthase **(nitrogen metabolism)**
59	**=**	**+**	**=**		**U**			**U**	glutamine synthetase **(nitrogen metabolism)**
318	**+**	**=**	**+**	**U**	**U**			**U**	vacuolar carboxypeptidase **(nitrogen metabolism)**

[#] Indication of changes in gene expression from (A1) airing (0h) to 24 h after airing, (A2) 24 h -72 h after airing and (A3) 72-168 h after airing in *A. bisporus* grown under standard, phase change stimulating conditions in a mushroom house.
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression in the second time point, compared to the first time point. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points."U" and "D" indicate respectively general up- and down-regulation over the course of the experiment.

708	**+**	**++**	**+**		**U**	**U**	**U**	**U**	NADP glutamate dehydrogenase **(nitrogen metabolism)**
1250	**+**	**+**	**=**			**U**	**U**		NADP glutamate dehydrogenase **(nitrogen metabolism)**
1577	**+**	**++**	**=**	**D**	**U**	**U**	**U**	**U**	carbamoyl-phosphate synthase **(nitrogen metabolism)**
94	**+**	**++**	**=**		**U**	**U**	**U**	**U**	gulonolactone oxidase **(glucose metabolism)**
358	**+**	**++**	**=**		**U**	**U**	**U**	**U**	gulonolactone oxidase **(glucose metabolism)**
711	**=**	**++**	**+**		**U**	**U**	**U**	**U**	tyrosinase
824	**+++**	**+**	**+**		**U**	**U**	**U**	**U**	hydrophobin *hypB*
854	**+++**	**+**	**+**		**U**	**U**	**U**	**U**	hydrophobin *hypB*
994	**+**	**++**	**=**		**U**	**U**		**U**	mismatched base pair and cruciform DNA binding protein
1582	**=**	**+**	**+**			**U**	**U**	**U**	putative glutathione transferase
92	**+**	**++**	**+**		**U**	**U**		**U**	non-characterised sequence
208	**+**	**++**	**=**		**U**	**U**	**U**	**U**	non-characterised sequence
213	**+**	**++**	**=**		**U**	**U**	**U**	**U**	non-characterised sequence
240	**=**	**封**	**+**		**U**	**U**		**U**	non-characterised sequence
311	**+**	**+**	**+**		**U**	**U**	**U**	**U**	non-characterised sequence
474	**+**	**+**	**=**		**U**	**U**	**U**	**U**	putative protein
916	**++**	**-**	**++**		**U**	**U**		**U**	non-characterised sequence
956	**+**	**++**	**=**		**U**	**U**	**U**	**U**	non-characterised sequence
982	**+**	**+**	**+**		**U**	**U**	**U**	**U**	non-characterised sequence
1198	**+**	**++**	**+**		**U**	**U**	**U**	**U**	putative protein
1231	**+**	**++**	**++**		**U**	**U**	**U**	**U**	non-characterised sequence
1251	**+**	**+**	**=**		**U**	**U**	**U**	**U**	non-characterised sequence
1285	**+**	**++**	**-**		**U**	**U**	**U**	**U**	non-characterised sequence
1341	**++**	**+**	**-**	**D**	**U**	**U**	**U**	**U**	non-characterised sequence
1408	**+**	**+**	**++**		**U**	**U**		**U**	putative protein
1479	**+**	**+**	**=**		**U**	**U**	**U**	**U**	non-characterised sequence
1621	**+**	**+**	**=**		**U**	**U**		**U**	non-characterised sequence
1131	**=**	**--**	**-**	**D**		**D**		**D**	isocitrate lyase **(carbohydrate / glyoxylate metabolism)**
82	**-**	**-**	**=**		**D**	**D**		**D**	putative protein
289	**=**	**--**	**--**	**D**	**D**	**D**		**D**	non-characterised sequence
907	**=**	**-**	**-**		**D**	**D**	**D**	**D**	non-characterised sequence
1134	**=**	**-**	**-**					**D**	non-characterised sequence
1181	**=**	**-**	**-**		**D**	**D**		**D**	non-characterised sequence
1542	**-**	**-**	**--**	**D**	**D**	**D**	**D**	**D**	non-characterised sequence

Cluster 1: Genes that were differentially expressed under conditions where fruit bodies developed (A) but not under conditions where only smooth undifferentiated primordia developed (D) or where no reproductive growth was observed (E and F)

This cluster contains genes that are potentially regulating and / or supporting the late phase change as they were differentially expressed in conditions where fruit bodies developed but not in conditions where only smooth differentiated primordia developed. It would therefore be expected that these genes were differentially expressed between 72 h – 168 h after airing, when elongated differentiating primordia were first observed. Down-regulated genes could be involved with earlier development (e.g. vegetative growth or the early phase change).

Of the 11 genes organized in cluster 1, 7 were generally up-regulated. Genes **748**, **1192, 998** and **1591** showed a significant increase in expression between 72 h and 168 h after airing. Genes **1353** and **1555** showed a gradual up- regulation from 24 h and 72 h after airing.

Gene **1192** shows significant similarity (blastn E-value of 2.3 E-42) to an aminoadipate reductase. Aminoadipate reductase converts 2-aminoadipate to 2-aminoadipate 6-semialdehyde in fungi and is thought to be involved in lysine biosynthesis (An *et al.*, 2002). Lysine can represent 10 % of the amino acid content in a fruit body (Flegg *et al.*, 1985) and this amino acid could be required to support development of the primordium.

Gene **1353** shows similarity (blastx E-value of 3.4 E-09) to a major facilitator superfamily transporter. The corresponding protein could have a role in the transport of amino acids, nutrients and / or water to support development and differentiation. A similar transporter protein was found to be up-regulated in developing fruit bodies of *Lentinula edodes* (Sakamoto *et al.*, 2009).

Genes **748** and **1555** show similarity (blastx E-value of 7.24 E-133 and 1.04 E-21) to a cytochrome P450 (*cypA*). De Groot *et al*. (1997) previously found that gene 748 (*cypA*) was up-regulated between elongated differentiating primordia and differentiating primordia with a distinct waist. Eastwood *et al.* (2001) found a similar increase of

related cytochrome P450 proteins in stage 2 fruit bodies, comparing gene expression at harvest and two days after harvest. These enzymes are monooxygenase, oxidoreductase enzymes and are thought to be involved in the metabolism of a wider range of substrates such as fatty acids. Muraguchi and Kamada (2000) found that *C. cinereus* mutants with a defective *eln2*, a cytochrome P450 gene showed a significant reduction in stipe elongation. This *eln2* gene has significant sequence similarity (tblastx E-value of 4.0 E-047) to gene 748 and (tblastx E-value of 3.0 E-0.13) to gene 1555. The first indication of a hyphal organisation representing a stipe is seen during the elongated differentiating primordia stage. It could therefore be suggested that this gene might also have a function in the stipe development and / or elongation in *A. bisporus*.

Gene **998** showed an increase in expression level between airing - 24 h after airing and 72 -168 h after airing. This gene shows similarity (blastx E-value of 1.0 E-14) to a riboflavin aldehyde forming enzyme. Sreenivasaprasad *et al.*(2006) previously identified this gene in *A. bisporus* and found that *raf* transcript levels could not be detected in vegetative mycelium by northern blot but showed increased expression from stage 1 fruit bodies (elongated differentiating primordia) onwards and highest activity was found in stipe tissue. The technology used during this project allowed to investigate expression in the stages between vegetative mycelium and elongated differentiating primordia and confirmed that gene 998 is up-regulated during the (early and late) phase change. Similar expression of a riboflavin aldehyde forming enzyme was found in primordia of *L. edodes,* compared to dikaryotic mycelium (Chum *et al.*, 2008; Hirano *et al.*, 2004). Even though there is strong evidence that this gene is involved with reproductive development based on previous work and results from this project, its function is not yet identified.

Four genes in this cluster, **1550, 1631, 410** and **1534**, were down-regulated over the course of the control treatment.

Gene **1631** showed a significant reduction in gene expression from 24 h after airing up to 168 h after airing. Gene 1631 is the consensus sequence of a gene cluster, which shows a match to the *A. bisporus* hydrophobin ABH3 sequence (blastx E-value of 7.0 E-19). Lugones *et al.* (1998) reported that this protein is specifically secreted by

vegetative hyphae of *A. bisporus* while it is not expressed in fruit bodies. This is similar to the microarray expression pattern which shows a clear down-regulation from 72 h after airing and onwards. The ABH3 was found to form a rodlet layer on hyphae and mycelial cords. This amphipathic molecule is thought to have two functions: 1) assist with the aggregation of mycelial cords to switch from exploitative to explorative growth when crossing non-nutritive areas such as gas spaces and 2) make hydrophobic substrate such as lignin, a main nutrient source for *A. bisporus*, hydrophilic in order to promote a better attachment between the vegetative, assimilative hyphae and lignin (Lugones *et al.*, 1998).

Genes **1534** and **1550** were gradually down- regulated from 24 h after airing up to 168 h after airing. This down-regulation could potentially indicate that these genes were involved with vegetative development, nutrient acquisition or repression of phase change driving genes. Gene **1550** matches to a glycoside hydrolase family 5 protein (blast x E-value: 8.17E-19). This protein family contains proteins that have the ability to hydrolase glycosidic bonds between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate complex. The matching protein is suggested to be involved with lignin polymer breakdown (Valaskova & Baldrian, 2006).

The up-regulated genes in this cluster generally show a distinct increase in expression between 72 h and 168 h after airing (e.g. sequences 998, 748, 1192 and 1591). As these sequences were only differentially expressed in conditions where fruit bodies developed and not in conditions where only fluffy undifferentiated primordia developed or where no reproductive growth was observed, it could be suggested that they regulate and / or support of the late phase change where smooth undifferentiated primordia start to differentiate into elongated differentiating primordia (and subsequent stages). Of these genes, 748, 896, 998 showed a small but significant increase in expression between airing and 24 h after airing. It is yet unclear what the function of this early (small) increase in gene expression could be as it is interestingly followed by a second significant increase in gene expression between 72 h and 168 h after airing.

Cluster 2: Genes that were differentially expressed under conditions where fruit bodies developed (A) and under conditions where only smooth undifferentiated primordia developed (D) but not under conditions where no reproductive growth was observed (E and F)

Genes organised in this cluster could potentially be involved with the regulation or support of the early phase change as they were differentially expressed in conditions where the development of smooth undifferentiated primordia is stimulated. It could be expected that these genes are differentially expressed shortly after airing.

Four genes, **595**, **864, 990** and **1274** were generally up-regulated under conditions where fluffy undifferentiated primordia developed but not under conditions where further development was observed. And remarkably, there were no down-regulated genes in this cluster.

Gene **595** showed an initial increase transcription from airing to 24 h past airing, drops in transcription between 24 h after airing and 72 h after airing and shows a significant increase in transcription between 72 h after airing and 168 h after airing. This initial drop was not seen in samples from the high temperature treatment (reduced CO_2 and reduced volatile levels) where a continuous increase in gene expression was observed from airing up to 168 h after airing. The sequence of this gene shows a significant sequence similarity to a putative *A. bisporus* metallothionein (tblastx E-value 4E-41). Eastwood *et al.* (2001) investigated differences in gene expression between stage 4 mushrooms at harvest and after two days of storage at room temperature of these stage 4 mushrooms using differential screening and found that a metallothionein was up-regulated during storage. Putative metallothionein genes have also been identified in other basidiomycetes and are up-regulated during fruit body development of the truffle *Tuber borchii* (Pierleoni *et al.*, 2004) and *L. edodes* (Chum *et al.*, 2008). This family of proteins is responsible for metal ion chelation through the formation of tetrahedrically coordinated metal-thiolate clusters (Palmiter, 1998). These proteins might therefore have a role in protecting the cell against toxic metals that accumulate during active growth or development.

Gene **1274** shows a gradual increase an increase in transcription levels from 24 h after airing up to 168 h after airing. This increase is more intense in the control experiment, compared to the maintained high temperature treatment. Sequence analysis shows a significant similarity to the *A. bisporus* hydrophobin ABH1 *hypA* (blastx E-value 2.33E-40). De Groot *et al.* (1996) have studied the transcription levels of this gene in *A. bisporus* previously and found that it is highly up-regulated in developing primordia and fruit-bodies, compared to vegetative mycelium. The gene is expressed mostly in the cap peel tissue and almost absent in stipe tissue. The highest expression levels were observed in stage 3 fruit bodies. However, the expression pattern constructed during this project shows that this gene might also have a role in the early stages of the phase change, when the fluffy hyphal knots and fluffy undifferentiated primordia start to form.

Gene **990** shows an initial increase in gene expression from airing up to 72 h after airing, after which no further up-regulation was observed. This sequence does not show a significant similarity to a publicly available sequence.

Genes **595**, **864** and **990** showed an early response to the change in environmental conditions in both *A. bisporus* grown under standard growth conditions and conditions where the temperature was maintained at 25 °C. This could suggest that these genes are involved with the initial response of the fungus (early phase change) to the reduction of mushroom volatiles concentration and possibly also in the maintenance of subsequent structures such as fruit bodies (e.g. the putative metallothionein and hydrophobin sequence).

Cluster 3: Genes that were differentially expressed under conditions where fruit bodies developed (A) and under conditions where no reproductive growth was observed (E and F)

Genes collated in this cluster were differentially expressed in *A. bisporus* grown under phase change stimulating conditions, where fruit bodies developed normally. However, these genes were differentially expressed under conditions where no evidence of reproductive growth could be observed (e.g. when exposed to high level of 1-octen-3-ol

or high levels of CO_2 and mushroom volatiles). These genes might therefore not be "key" genes, controlling the phase change but they could be involved with supporting this process. 30 genes in this cluster were generally up-regulated while 7 genes were generally down-regulated in *A. bisporus* grown under standard cultivation conditions.

Genes **824** and **854** show sequence similarity to the *A. bisporus hypB* hydrophobin (blastx E-value 0.0, respectively 96% identities and 100% identities) and only differ from each other by 15 nucleotides. Each gene is represented by 2 probes on the array who give highly comparable gene expression levels. De Groot *et al.* (1999) found that *hypB* was very lowly expressed in vegetative mycelium while it was significantly up-regulated in smooth undifferentiated primordia after which expression levels gradually drop. Expression in fruit bodies was localised to stipe tissue. Based on this information, De Groot *et al.* (1999) concluded that *hypB* is instrumental in the differentiation process and could have a role in coating air channels in the stipe of the fruit body. Microarray expression patterns for this gene constructed during this project confirm observations made by these researchers and shows that these genes are significantly up-regulated 24 h after airing, in the standard cultivation conditions experiment. Microarray data, however, also showed that *hypB* is up-regulated in conditions where no fruit bodies developed but where temperature was reduced. This could suggest that does not regulate primordia development but supports it.

Other genes in this cluster appear to be related to the nitrogen metabolism. These genes are **22** (argininosuccinate synthase, EC 6.3.4.5, blastx E-value 0.0), **59** (glutamine synthetase, EC 6.3.1.2, 1E-9), **318** (carboxypeptidase, blastx E-value 9.0 E-25), **708** (NADP-glutamate dehydrogenase, blastx E-value 0.0, EC 1.4.1.4), **1250** (NADP-glutamate dehydrogenase, blastx E-value 1.2 E-67, EC 1.4.1.4), **1577** (carbamoyl-phosphate synthase, blastx E-value 4.8E-82). Expression levels of these (general) nitrogen metabolism genes were gradually up-regulated during standard growth conditions but also in treatments where no fruit bodies developed. Activity of glutamine synthase and argininosuccinate synthase has been studied previously and both showed increased activity during fruit body development and are thought to be involved in the accumulation of urea in the fruitbody, which was thought to increase the osmotic

gradient seen in developing fruit bodies (Ewaze *et al.*, 1978; Wagemaker *et al.*, 2007a; Wagemaker *et al.*, 2007b).

Genes **94** and **358** (gulonolactone oxidase, blastx E-value 4.0 E-34 and 2.7 E-09, EC:1.1.3.8) show a significant up-regulation between airing – 24 h after airing and between 24 h - 72 h after airing. This enzyme catalyses the reaction from D-glucuronolactone with oxygen, leading to the production of ascorbic acid. The antioxidant properties of ascorbic acid could have a function in scavenging of reactive oxygen species (Kranner *et al.*, 2005). Ascorbic acid prevents the production of survival structures in *Sclerotinia minor* by reducing oxidative stress which can trigger the differentiation of these structures (Georgiou & Petropoulou, 2002). Increasing concentrations of this molecule are found in rapidly expanding cells (Smirnoff, 1996). These genes might therefore have a function in protecting the developing and differentiating cells against reactive oxygen species.

Gene **711** (tyrosinase, blastx E-vale 0.0, EC:1.14.18.1; EC:1.10.3.2) is significantly up-regulated between 24 h -72 h after airing in conditions where fruit bodies develop and in lesser degree in treatments where fruit bodies did not develop. This gene is represented by two probes covering different locations of the gene with a high degree of correlation (0.9958) in expression profile, which underlines the robustness of the technology used to investigate the gene expression levels. This enzyme showed increased activity in fruit bodies after damage and during storage as it is thought to be involved with browning of the fruit bodies. It is also thought to have a role in melanin production in the spores and has been identified in various filamentous fungi such as *L. edodes* and *Neurospora crassa* (Flegg *et al.*, 1985; Halaouli *et al.*, 2006). This does however not explain why this enzyme has shown to be expressed in the cap and stipe tissue (Flegg *et al.*, 1985) and is identified to be differentially expressed in *A. bisporus* grown under various environmental conditions during this project.

Gene **994** (mismatched base pair and cruciform DNA binding protein, blastx E-value 3.0 E-37) show a gradual increase in expression from airing – 24 h and from 24 h - 72 h after airing. Endo *et al* (1994) have identified a gene (*priB*c) encoding for a DNA-binding protein that is highly expressed in developing primordia while it is barely

detectable in vegetative mycelium and mature fruit bodies of *L. edodes.* Similarly, Chum *et al.* (2008) identified a second gene (*priA*) in *L. edodes* that encodes for a DNA-binding transcription factor and has a similar expression profile to the *priB*c gene. Chum *et al.* (2008) therefore suggested that this gene might have a function in regulation of fruit body development. Eastwood *et al.* (2001) reported the identification of gene encoding for a cruciform DNA binding protein that showed increased expression during storage of stage 2 fruit bodies. The gene expression profiles for this gene reported here are in line with expression of the *priA* and *priB*c gene found in *L. edodes*. Microarray data generated during this project did however reveal that this gene is also up-regulated in treatments where no fruit bodies developed.

Gene **1582** (putative glutathione transferase, blastx E-value 2.5 E-28, EC 2.5.1.18) shows a gradual increase in gene expression from 24 h-72 h after airing and 72 h -168 h after airing. This enzyme family is proposed to be involved with protection against oxidative stress (Adnane *et al.*, 2006).

A further 17 genes in this third cluster were up-regulated at various time points in *A. bisporus* grown under phase change stimulating conditions and under conditions where no reproductive growth was observed or where only smooth differentiated primordia developed. These sequences did not show sequence similarity to characterised sequences and did not have a distinct protein motif.

Seven genes in this cluster were found to be down-regulated in *A. bisporus* grown under phase change stimulating conditions. Of these genes, only gene **1131** showed a significant similarity to a characterised sequence (isocitrate lyase, blastx E-value 1.1 E-45, EC 4.1.3.1). This enzyme lyses isocitrate into succinate and glyoxylate. Flegg *et al.* (1985) reported that succinate concentrations increased under elevated CO_2 levels (2,000- 4,000 p.p.m) due to an increased activity of the Krebs (glyoxylate) cyclus. This pathway is potentially involved with the conversion of lipids or fatty acids into glucose. Interestingly, a significant reduction in gene expression levels were observed at 24h -72h after airing and 72h 168 h after airing in *A. bisporus* grown under phase change stimulating conditions. Gene expression was also down-regulated in the high temperature treatment (reduced levels of CO_2 and mushroom volatiles), the high level of

1-octen-3-ol treatment (reduced temperature and reduced CO_2 level) and the reduced level of 1-octen-3-ol after removal of the 1-octen-3-ol source treatment (reduced temperature and reduced CO_2 level) but was continuously expressed in the high levels of CO_2 and mushroom volatiles treatment and the high level of CO_2 treatment (reduced level of mushroom volatiles and reduced temperature). This is in line with observation made by Flegg *et al.* (1985). Even though this gene was differentially expressed in conditions where no fruit bodies developed, it could still play an important role in causing a change in metabolism in response to changing CO_2 levels.

Conclusion

This project found that 52 genes out of a collection of 1079 genes were significantly differentially expressed in *A. bisporus* grown under phase change stimulating conditions where the temperature was reduced and the growth system was ventilated to reduce levels of CO_2 and mushroom volatiles, using the first *A. bisporus* oligonucleotide gene expression microarray.

Analysis of reproductive morphogenesis in *A. bisporus* and characterisation of the responses to specific environmental conditions revealed that temperature, CO_2 and mushroom volatiles influence the phase change in different ways. A second series of experiments was performed to identify the effects of these individual parameters on the gene expression levels of 1079 sequences present on the microarray. The gene expression profiles constructed for each of these experimental growth conditions was compared with the gene expression profile of *A. bisporus* grown under phase change stimulating conditions. After correlation of these molecular analyses to the results from the morphological study presented in Chapter 3, it was possible to organise the 52 genes into 3 clusters:

- Cluster 1: genes that are potentially involved with the regulation and / or support of the “late” phase change where smooth undifferentiated primordia developed into elongated differentiating primordia;

- Cluster 2: genes that are potentially involved with the regulation and / or support of the "early" phase change where the vegetative mycelium developed into smooth undifferentiated primordia, but not further;
- Cluster 3: genes that are differentially expressed both under phase change stimulating conditions and in conditions where no reproductive growth could be observed.

While the genes in Cluster 3 are also differentially expressed under conditions where no reproductive growth is observed, they could have a function in supporting the phase change rather than in the regulation of this process and might be involved with changes in metabolism that are not translated directly in morphological development.

These 52 genes are therefore ideal candidates for further study whereby functional characterisation could verify the hypotheses on their function stated in this work.

Analysis of the GO ontology terminology assigned during the *in silico* annotation of the *A. bisporus* sequences (section 2.4.3 and 4.8) represented on the gene expression microarray revealed that over 200 sequences were putatively involved with binding and catalytic activity. A similar analysis of the 52 genes identified to be differentially expressed in *A. bisporus* grown under phase change stimulating conditions gives a similar distribution as shown in Figure 5-8 (biological processes) and Figure 5-9 (molecular functions) whereby the majority of genes that could be annotated *in silico* are putatively involved with catalytic activity (10) and binding (8). It was not unexpected to see this similarity in frequencies of functions as the *A. bisporus* microarray, mainly including genes related to various developmental processes. The binding activity of these 8 genes is more metabolic then regulatory as none of the corresponding transcripts have a DNA / RNA binding function, with a possible exception of the cruciform DNA binding protein. It is, however, important to stress that a considerable proportion of sequences (63%) could not be annotated as discussed in section 4.8. If the putative function of these genes was known, the distribution of functions represented on the microarray could cover a much wider range.

Figure 5-8 Comparison of the distribution of biological processes (GO-ontology level 3) of genes represented on the *A. bisporus* microarray and genes that are differentially expressed during the phase change

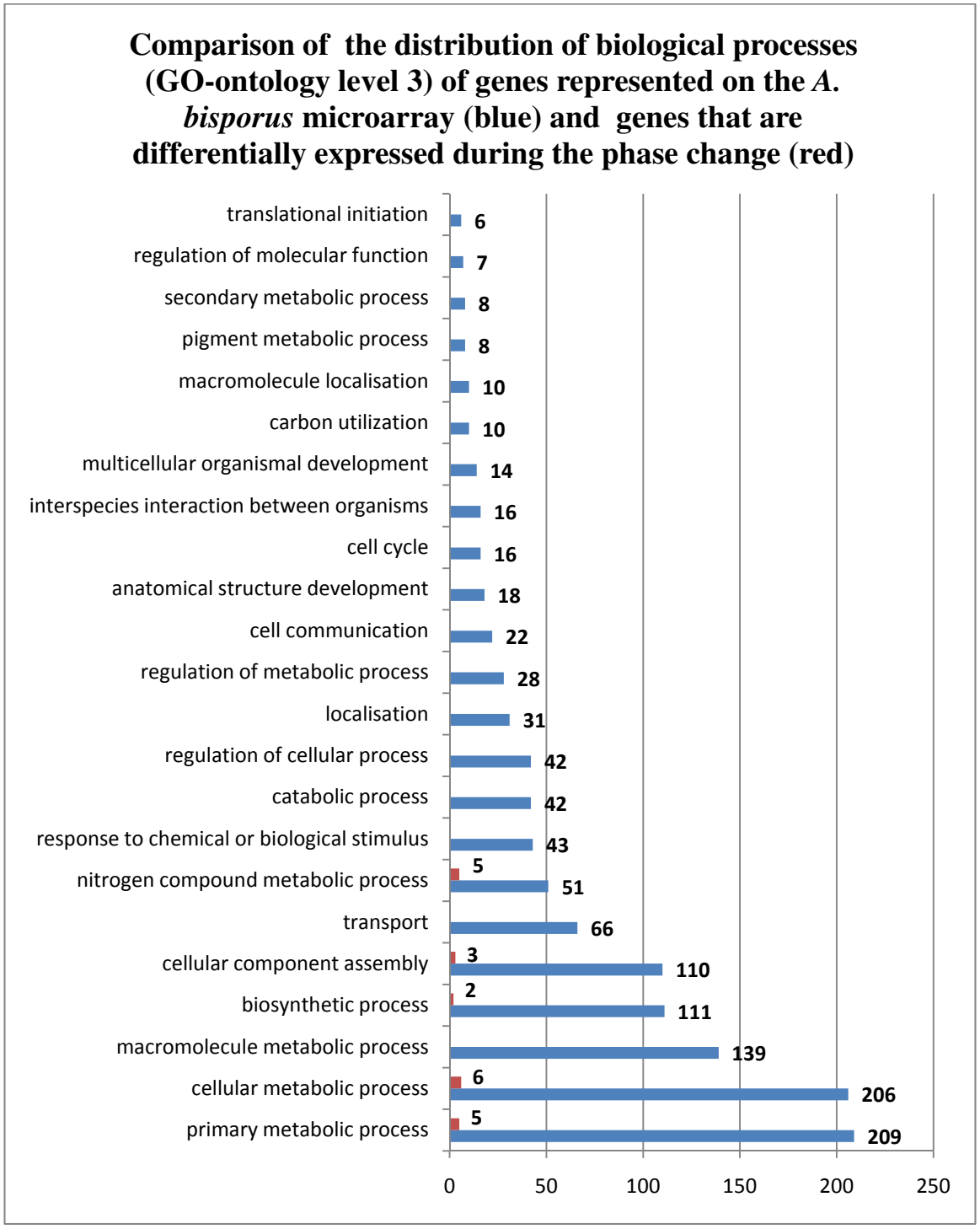
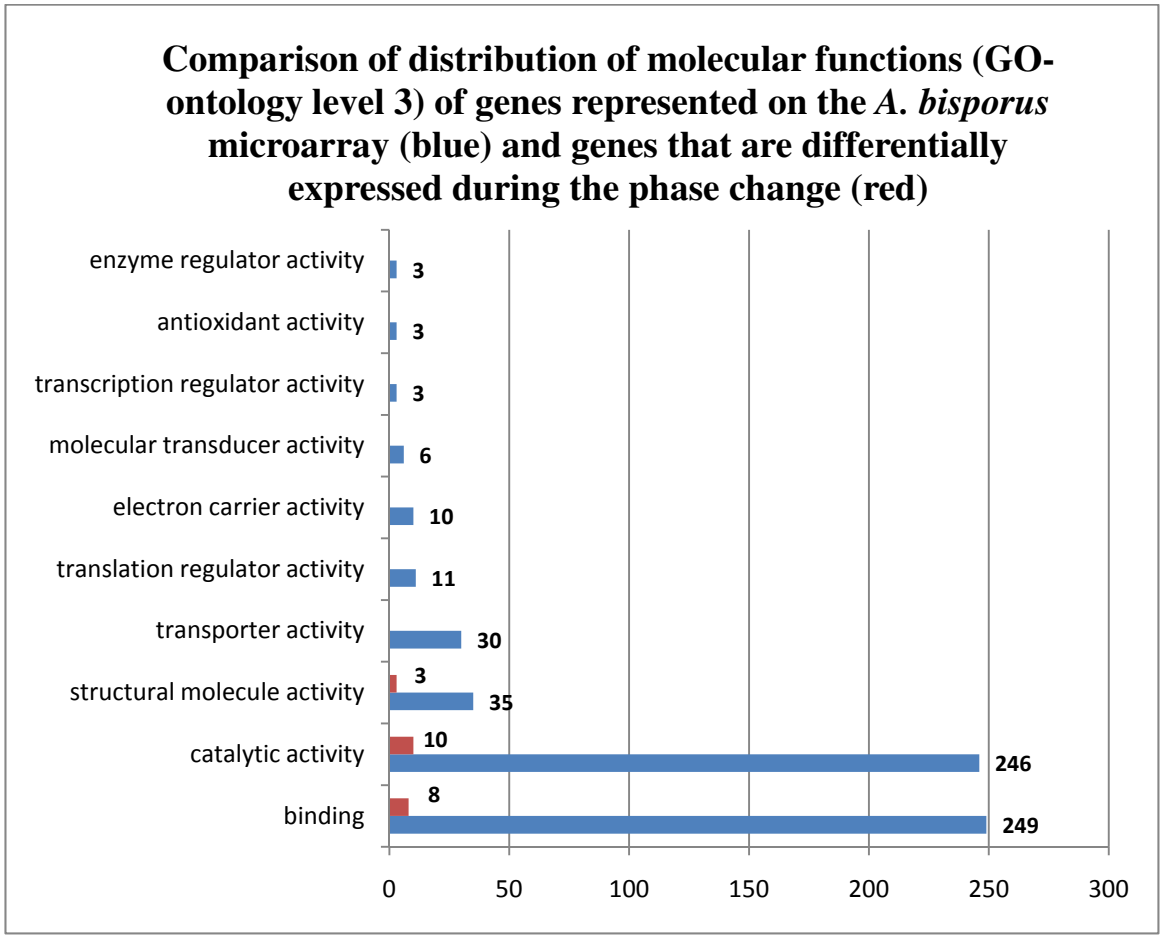


Figure 5-9 Comparison of distribution of molecular functions (GO-ontology level 3) of genes represented on the *A. bisporus* microarray and genes that are differentially expressed during the phase change



This project has documented the changes on a molecular (gene expression) and macroscopic level (morphological development) during the phase change from vegetative mycelium to reproductive fruit bodies and the effects of regulatory environmental factors on that development. After careful consideration, 52 genes were identified to be differentially expressed during the phase change and "clustered" based on whether this differential expression also occurred under specific, environmental conditions. Following this approach, it was possible to separate these 52 genes in 3 "clusters", representing genes that are potentially key regulators or drivers of the (early or late) phase change or are more likely to have a supporting role. These three clusters were then subdivided to indicate which genes were generally up- or down regulated.

The more traditional approach of analyzing gene expression data is to cluster genes based on expression levels or patterns. Various algorithms have been developed for this purpose. Examples are hierarchical clustering (Eisen *et al.*, 1999), k-means clustering (Tavazoie *et al.*, 1999), Bayesian networks (Friedman *et al.*, 2000) and self organising maps (Tamayo *et al.*, 1999). These methods are capable of identifying biologically relevant patterns in whole genome experiments, as demonstrated for hierarchical clustering by Savage *et al.* (2009). They do however not consider the sequential nature of time, a fact discussed previously in section 4.7.1, for the identification of differentially expressed genes in time course experiments. More recently, clustering methods for time-course experiments are being investigated. From a statistical point, there are two classes of time-course experiments, short time-course experiments (3-8 time points) and long time-course experiments (> 10 time points). Most of these time-course clustering methods work well with long time-course experiments. Examples of this are hidden Markov models (Schliep *et al.*, 2003) and the model developed by Peddada *et al*. (2003). These methods can identify biologically significant patterns but do not work optimally with small time-course experiments, such as the data created in this project where the various treatments, growth nature of *A. bisporus* as well as budgetary and resource limitations reduced the number of available time-points for each treatment. The method described by Tchagang *et al*. (2009) is designed to extract biologically significant patterns from short time-series gene expression data. Future work on the data produced to this work might include analysis using this novel method. A comparison between the clusters created, based on expression data over time using the method described in Tchagang *et al*. (2009), and the clusters presented in this work, would be of scientific interest.

The analysis presented in this work and future analyses based on the expression data collected during this project is and will however be limited by the sequences present on the 1st generation *A. bisporus* gene expression array. This limitation means that major biological pathways are only represented by single or only a small number of genes.

Differential expression of individual genes can give an indication that a particular pathway might be activated or deactivated but does not give an indication of the activity of genes that feed into or depend on that pathway. An advantage of the 2nd generation *A. bisporus* gene expression array, containing probes representing the entire transcriptome available for the *A. bisporus* genome sequencing programme (January 2010), is that differential expression of an entire pathway or genes with a specific function can be considered, rather than to focus on the expression of a single gene of that pathway or gene with a specific function. This approach, referred to as gene set enrichment analysis (Subramanian *et al.*, 2005), will increase the statistical significance of the expression data (e.g. smaller fold changes in expression of the various genes of a pathway is more significant and biologically relevant than a large change in expression of a single gene of that pathway). Similarity of these pathways with other organisms could also identify potential regulatory mechanisms.

Chapter 6. General discussion

The homobasidiomycete, *Agaricus bisporus* is a secondary decomposer of plant litter in temperate forest soils. Initial decomposition of plant litter by primary colonisers exposes the complex lignin polymers which are exploited by *A. bisporus* as a substrate. *A. bisporus* uses these complex molecules as a growth substrate by producing a whole series of enzymes that break down these polymers into small, easily absorbable molecules. The search for these nutrients and subsequent enzyme production is an energy intensive process. The hyphal mode of growth from *A. bisporus* means that the fungus can colonise the soil with a web of filamentous hyphae. Once the resources are found, localised branching ensures that the resource is colonised and utilised efficiently. When crossing an area depleted of resources, less branching occurs and hyphae can aggregate into mycelial cords. Once a new resource is found, increased branching occurs and the mycelial cords assist with transport of various molecules. This specialised mode of growth is currently being investigated in detail in related basidiomycete fungi (Tlalka *et al.*, 2008; Watkinson *et al.*, 2005) and the fungal colony acts as one complex organism that is focussed on nutrient uptake, storage and translocation to support development. *A. bisporus* switches from vegetative to reproductive growth depending on the environmental conditions, whereby in less than 300 h, spore releasing fruit bodies develop which can be over 6 cm high and 15 cm wide.

The phase change is a resource intensive process and *A. bisporus* therefore has to be able to sense the conditions which will allow optimal dispersion and survival of its spores before this process is initiated. The commercial strain used in this project is a dikaryotic strain containing both mating-types, and is capable of initiating reproductive growth in response to environmental cues. This strongly implies the existence of a molecular mechanism that controls and drives this process. Similar mechanisms are thought to be present in various other basidiomycetes which alter their growth mode in response to changes in environmental parameters such as light, temperature and gasses (Bahn *et al.*, 2007).

Research into *A. bisporus* has been mainly driven by commercial application whereby various cultivation conditions were investigated to find optimal, fruit body development stimulating growth conditions (Flegg, 1979; Flegg *et al.*, 1985). This research has established that *A. bisporus* grown commercially on compost needs to be covered with a moist casing layer, generally peat, which promotes mycelial cord formation. A reduction in temperature from 25° C to 18 °C and ventilation of the growth rooms to reduce the levels of carbon dioxide and mushroom volatiles has to be applied to stimulate reproductive growth. It was also suggested that *Pseudomonas putida*¸ a bacterium that can metabolise eight carbon volatiles such as the mushroom volatile 1-octen-3-ol is required to remove the inhibitory effects of these volatiles on mushroom phase change (Noble *et al.*, 2003). The function of *P. putida* in the stimulation of the phase change by reducing levels of volatiles is currently under discussion based on work performed as part of a larger project including this PhD programme and reported by Noble *et al.* (2009).

In contrast to these well defined growth conditions, little research has been done on the molecular mechanism controlling the phase change in *A. bisporus*. The non-availability of the genome sequence of *A. bisporus* and related resources as well as the molecular methodologies to analyse the casing-based fungal structures are the key issues that were impeding this research. The study presented here is the first major effort integrating molecular analyses with morphological investigations into the phase change process in *A. bisporus*.

Time-lapse photography and microscopic observations led to the definition of key stages of morphological differentiation and development during the reproductive phase change in *A. bisporus* grown under standard (commercial) cultivation conditions. Additionally, a series of growth experiments whereby one or two factors were not maintained at the standard cultivation levels led to the identification of temperature and mushroom volatiles as on/off switches and CO_2 as a quantitative regulator that affect the phase change process. Moreover, these switches affect different stages of development, indicating two major steps - “early” and “late”, in phase change.

A reduction in the level of mushroom volatiles (e.g. 1-octen-3-ol) is required to initiate the “early” phase change whereby vegetative mycelium (mycelial cords)

starts to exhibit heavy localised branching and form fluffy mycelial cords. At apparently random locations on these fluffy mycelial cords, the heavy localised branching continues and fluffy two-dimensional hyphal knots develop. These fluffy hyphal knots continue their development and form smooth undifferentiated primordia. If the temperature is not reduced at this point, no further differentiation and development occurs. If the temperature is reduced, differentiation occurs and elongated differentiating primordia develop. These structures gradually develop further and form differentiating primordia with a distinct waist, which ultimately form mature fruit bodies. Carbon dioxide concentrations over 1,000 p.p.m do not inhibit reproductive development but increasing levels of this gas reduce the number of developing primordia and fruit bodies, hence it is referred to as a quantitative regulator. The general understanding previous to this study has been that high CO_2 levels (> 2,000 p.p.m) in the growth environment stimulate vegetative growth but inhibit fruit body development (De Groot *et al.*, 1998; Flegg *et al.*, 1985). The airing process is thought to reduce the levels of this gas, which together with a reduction of temperature initiates the phase change. This project has shown that primordia and fruit bodies can develop under CO_2 levels as high as 5,000 p.p.m., as long as the levels of mushroom volatiles are reduced by ventilation and the temperature is reduced.

Testing of a range of RNA extraction methods and appropriate modifications led to the development of a methodology which can be used to extract un-degraded, inhibitor free total RNA from peat samples which is microarray-based analysis. This enabled microarray-based investigations into changes in gene expression of *A. bisporus* grown under phase change stimulating conditions and under experimental conditions, designed to investigate the individual effects of environmental factors such as temperature, CO_2 and mushroom volatiles. Microarray-based analyses are being increasingly applied to understand functional and environmental adaptation in fungi (Breakspear & Momany, 2007).

The custom gene expression microarray developed in this study contains 1079 genes of *A. bisporus* representing approximately 13 % of the predicted total gene set. The microarray includes a wide range of genes such as various classes of hydrophobins that show different expression patterns in vegetative mycelium and fruit bodies (Lugones *et al.*, 1996; Lugones *et al.*, 1998), 10 genes or gene families

associated with mushroom development (Sreenivasaprasad *et al.*, 2006), 19 genes that were up-regulated during storage of stage 2 fruit bodies including a Cytochrome P450, a putative metallothionein and various nitrogen metabolism genes (Eastwood *et al.*, 2001), 477 expressed sequence tags of genes expressed in primordia and fruit bodies or both stages (Ospina-Giraldo *et al.*, 2000) and over 400 genes identified from certain stages of the mushroom phase change (Eastwood, D.E., Warwick HRI).

Comparative gene expression profiling of *A. bisporus* grown under standard phase change conditions and the experimental conditions which impact on the phase change stages, discussed above, led to the identification of a set of 52 genes which could be categorised into three clusters relatable to the "early" and "late" phase change stages identified based on morphological investigations. Some of the genes represented within Clusters 1, 2 and 3 have been described previously in *A. bisporus* and other fungi in relation to reproductive development. Examples of this are the hydrophobin *hybB* (De Groot *et al.*, 1999), a major facilitator superfamily transporter (Sakamoto *et al.*, 2009) and a putative metallothionein (Chum *et al.*, 2008; Pierleoni *et al.*, 2004). However, the present results suggest that the genes categorised in Cluster 3 might not be involved with the regulation of reproductive growth but rather support this process. Also, 29 of these 52 genes do not show any sequence similarity to characterised genes and could be specific to the regulation or support of the phase change in *A. bisporus*. These 52 genes and particularly the genes in Clusters 1 and 2 would form ideal candidates for *in vivo* functional analysis. Eastwood *et al.* (2008) have developed hairpin expression vectors that, in combination with *Agrobacterium tumefaciens*–mediated transformation have been used to down-regulate the expression of argininosuccinate lyase in *A. bisporus*. This technology could be used to down-regulate (a selection of) these key genes to investigate (and confirm) their function in the regulation or support of the reproductive phase change.

The results presented here, though based on a commercial strain grown under commercial cultivation conditions, provide us with an insight into the molecular mechanisms regulating and supporting the growth processes in *A. bisporus* and related fungi such as *Agaricus bitorquis.* The temperature switch was found to be absolute as elongated differentiating primordia developed when the temperature was not reduced. This implies that the predicted temperature increases due to global

warming could impact on the ability of various fungi such as *A. bisporus* and *Flammulina velutipes* to develop fruit bodies.

The *A. bisporus* genome (and derived transcriptome) sequence information is expected to be in the public domain in early 2010. Once the transcriptome has been annotated *in silico*, a microarray representing the full gene set and / or the whole genome could be created building on the tools and resources developed in the present study. This would also allow gene set enrichment analysis, as discussed in section 5.4.

Interestingly, no instances occurred whereby the direction of a change in gene expression was inverted (e.g. up-regulated in one experiment and down-regulated in another experiment) in *A. bisporus* grown under different conditions. This could indicate that the genes present on the array are either up- or down-regulated when stimulated by a specific treatment. An exception to this was seen in a small portion of "antisense" probes which were retained on the final microarray design (data not discussed here). For approximately 10% of the genes represented on the microarray, antisense probes were retained. The majority of these antisense probes did not show any measurable expression or changes of expression while a fraction of antisense probes showed differential regulation. The changes in gene expression of these antisense probes were either similar or inverse to the changes in expression of the corresponding sense transcripts. Eastwood *et al.* (2008) have previously shown that transcriptional regulation (silencing) using hairpin-mediated dsRNA can occur. Smith *et al*. (2008) found evidence of naturally occurring antisense in *Aspergillus flavus*. Moreover, the level of antisense transcripts was linked to changes in temperature. A more in-depth study and validation of the antisense levels in *A. bisporus* in the standard experiment and the experiment where the temperature was not reduced might suggest a similar link in *A. bisporus*, which could further improve our understanding of the effect of the various environmental parameters on the morphological development seen in *A. bisporus* and other fungi. The genomic sequence should also reveal small non-conding RNA (ncRNA) . This class of RNA includes transfer RNA, ribosomal RNA, small nuclear and small nucleolar RNA, microRNA (miRNA) and small interfering RNA (siRNA). While these miRNA and siRNA's are not translated into proteins, they are thought to be involved in

posttranscriptional regulation of gene expression (Xie *et al.*, 2004). *In silico* identified miRNA and siRNA's (Patzel, 2007; Van Der Burgt *et al.*, 2009) could be organised onto a miRNA gene expression array such as the customer miRNA gene expression platform provided by Agilent Technologies. A correlation of the transcription of specific miRNA or siRNA's with the gene expression data collected during this project could identify additional regulation, potentially in response by a change in an environmental parameter.

Whole genome analysis will also reveal putative promoter regions, transcription factors and transcription factor DNA binding domains. Chromatin immunoprecipitation with microarray technology (ChIP-on-chip) can be used to investigate *in vivo* interactions between transcription factors and their corresponding DNA domains (Aparicio *et al.*, 2005). When a protein or gene of interest is identified, e.g. the 52 genes identified to be differentially expressed in *A. bisporus* during the phase change, and a potential transcription factor is found, an antibody to that transcription factor can be designed. Genomic DNA is then stabilised, sheared and the antibody binds to the transcription factor. This antibody-transcription factor-(genomic DNA) complex is then isolated. The antibody is removed and the DNA is denatured and fluorescently labelled. The Cy-5 labelled ChIP enriched DNA is mixed with Cy-3 labelled non-enriched genomic DNA and hybridized onto a CGH microarray. Fluorescence analysis will identify whether a specific transcription factor was bound to a specific genomics sequence. A comparison of results of a ChIP analysis in samples from *A. bisporus* grown under various experimental conditions could reveal regulation on transcriptional level and potentially help to understand the differential expression observed during this project. An alternative approach, avoiding the use of a microarray is ChIP-sequencing (Mardis, 2007) whereby a DNA-protein complex is isolated using a protein specific antibody after which the DNA is sequenced e.g. using a next-generation sequencing platform.

The *A. bisporus* mutant strain B430 curiously forms undifferentiated primordia in axenic cultivation conditions whereby no ventilation is applied and CO_2 and more importantly volatiles are not reduced (Flegg *et al.*, 1985; Noble *et al.*, 2003). In addition to this, no elongated differentiating primordia are formed, even after reduction of the temperature indicating that the control mechanism in this

mutant is severely disrupted. Array-based comparative genome hybridisations (aCGH) of the mutant and commercial strains might therefore add to our understanding of the control of reproductive phase change in *A. bisporus*.

Improved knowledge and the resources developed from the present study lay a platform for further in-depth molecular investigations leading to mushroom strain improvement, improved growth systems and efficient utilisation of resources such as energy, water and peat.

Appendix 1 - Total RNA extraction and labelling results from experiments with* A. bisporus *grown under various environmental conditions in growth trays in a mushroom house. (A) time point, hours after airing; (B) biological replicate; (C) RNA concentration, ng/µl (12 µl final volume) ; (D) 260 nm /280 nm absorbance ratio; (E) 260 nm / 230 nm absorbance ratio; (F) Cy-3 labelled cRNA concentration, ng/µl; (G) Cy-3 incorporation rate, ; (H) cRNA yield, µg; (I) cRNA used / hybridisation, ng; (J) average signal intensity from hybridised sample.

A	B	C	D	E	F	G	H	I	J
Phase change stimulating conditions (section 5.2)									
0*	1	253	2.07	1.45	115	3.45	9.6	1000	9792
0*	2	117.2	2.06	1.40	144	4.32	11.8	1000	9841
0*	3	144	2.06	1.44	102	3.06	11.8	1000	10820
0*	1	72	2	0.85	238	10.5	11.9	1000	9946
0*	2	47	2	0.57	230	8.3	6.9	1000	7899
0*	3	86	1.98	1.22	190	10.5	5.7	1000	6597
24	1	72.6	2.11	1.52	172	5.16	9.9	1000	8637
24	2	243.2	2.11	1.70	116	3.48	11.2	1000	11452
24	3	101.3	2.02	0.91	124	3.72	11.3	1000	9827
72	1	194	1.66	1.07	137	4.11	9.5	800	10422
72	2	178.6	2.06	0.65	108	3.24	12.0	800	12539
72	3	190	2.03	1.14	55	1.65	10.9	800	10803
168	1	113	2.38	1.53	113	3.39	8.8	600	7921
168	2	345.9	2.08	1.76	165	4.95	10.3	600	7516
168	3	385.2	2.02	0.91	187	5.61	10.7	600	6733
high temperature treatment (section 5.3.1)									
24	1	141.3	2	0.66	**226**	7.5	6.8	1000	3212
24	2	170	2.06	0.57	**210**	11.0	6.3	1000	7190
24	3	53	2.04	0.79	**107**	13.1	3.2	1000	10600
72	1	118	2.1	1.57	**177**	11.3	5.3	800	7984
72	2	119	2.04	0.46	**177**	11.3	5.3	800	7987
72	3	126	2.13	0.96	**228**	11.8	6.8	800	8942
168	1	166	2.17	0.28	**184**	10.3	5.5	600	7273
168	2	284	2	1	**65**	6.2	3.9	600	3568
168	3	237	2	1.52	**191**	13.6	5.7	600	7288
high levels of CO_2 and mushroom volatiles treatment (section 5.3.2)									
24	**1**	64	1.82	0.68	101	**8.9**	3.03	1000	6815
24	**2**	105	1.93	0.66	188	**8.5**	5.64	1000	1709
24	**3**	47	2	1.9	261	**13.0**	7.83	1000	13158
72	**1**	113	1.93	1.19	235	**7.2**	7.05	800	4720
72	**2**	138	1.98	0.86	168	**13.1**	5.04	800	8364
72	**3**	88	1.97	1.18	73	**10.5**	2.19	800	10459
168	**1**	175	1.97	0.92	112	**8.3**	3.36	600	5603
168	**2**	80	1.95	1	140	**10.5**	4.2	600	4594
168	**3**	65	2	0.73	123	**8.9**	3.69	600	6111

Appendix 2 - Total RNA extraction and labelling results from experiments with* A. bisporus *grown under various environmental conditions in flask-based growth system with controlled air supply. (A) time point, hours after airing; (B) biological replicate; (C) RNA concentration, ng/µl (12 µl final volume); (D) 260 nm /280 nm absorbance ratio; (E) 260 nm / 230 nm absorbance ratio; (F) Cy-3 labelled cRNA concentration, ng/µl; (G) Cy-3 incorporation rate, ; (H) cRNA yield, µg; (I) cRNA used / hybridisation, ng; (J) average signal intensity from hybridised sample.

A	B	C	D	E	F	G	H	I	J
Phase change stimulating conditions (section 5.2)									
0	1	129	1.92	0.38	125	9.6	3.8	1000	7493
0	2	67	1.94	0.3	251	7.2	7.5	1000	4902
24	1	129	1.75	0.78	129	12.4	3.9	1000	6517
24	2	148	1.92	0.23	123	10.3	3.8	1000	8242
24	3	88	1.74	0.83	102	11.8	3.1	1000	8508
72	1	154	1.83	0.9	218	10.6	6.5	800	7959
72	2	86	2	0.23	153	12.4	4.6	800	7283
72	3	140	1.8	0.99	106	10.4	3.2	800	9119
168	1	77	2.13	1.66	164	11.6	4.9	600	4933
168	2	153	2	0.9	136	13.2	4.1	600	5929
168	3	93	2	0.5	105	13.3	3.2	600	7450
Phase change stimulating conditions (section 5.2), duplicate experiment									
0	1	45.1	1.75	0.39	103	8.7	3.09	1100	3261
0	2	66.3	1.92	0.48	55	10.91	1.65	1100	4289
0	3	66.9	1.9	0.59	71	8.45	2.13	1100	5644
24	1	39.4	2.02	0.93	83	8.43	2.49	800	5608
24	2	156	1.92	0.24	70	8.57	2.1	800	4254
24	3	39.4	2.01	0.55	93	9.68	2.79	800	4889
72	1	76.1	2.07	0.41	40	17.50	1.2	800	2399
72	2	126.5	2	0.91	94	10.64	2.82	800	6193
72	3	112	1.91	0.28	103	11.65	3.09	800	6304
191	1	132.6	2.12	1.57	173	10.40	5.19	600	4290
191	2	220	2.1	0.99	140	10.00	4.2	600	5225
191	3	282.6	2.01	1.56	144	11.11	4.32	600	5416
High level of 1-octen-3-ol (section 5.3.3)									
0	1	110.00	1.75	0.87	98	2.94	11.22	1000	4973
0	2	58.00	1.96	0.66	139	4.17	12.23	1000	9178
24	1	*54.00*	1.73	0.79	100	3.00	10.00	1000	7239
24	2	100.00	1.90	1.32	147	4.41	9.52	1000	4467
24	3	140.00	1.75	0.75	115	3.45	11.30	1000	9070
72	1	133.00	1.73	0.69	163	4.89	10.43	800	4813
72	2	78.00	1.90	0.30	102	3.06	11.76	800	7058
72	3	98.00	1.99	0.92	73	2.19	9.59	800	4382
168	1	83.00	1.98	0.71	133	3.99	8.27	600	2665

168	2	174.00	1.93	1.50	144	4.32	14.58	600	3773
168	3	218.00	1.99	1.72	186	5.58	12.90	600	6448
Reduction of the level of 1-octen-3-ol by removal of the 1-octen-3-ol source (section 5.3.4)									
24	1	101.00	2.00	0.78	295	8.85	8.81	600	1493
24	2	156.00	2.00	1.57	127	3.81	9.45	600	4292
24	3	157.00	2.00	0.77	104	3.12	12.50	600	4552
72	1	168.00	2.00	1.80	249	7.47	9.24	600	4849
72	2	186.00	1.98	1.00	147	4.41	10.88	600	6095
72	3	88.00	2.00	1.21	114	3.42	12.28	600	5541
120	1	177.00	2.00	1.60	172	5.16	11.63	600	4397
120	2	71.00	2.00	1.10	131	3.93	13.74	600	5349
120	3	73.00	2.00	1.33	139	8.34	11.51	600	7045
High level of CO_2 treatment (section 5.3.5)									
24	1	42.8	1.94	0.4	140	4.2	9	1000	2848
24	2	79	1.88	0.21	190	5.7	6.84	1000	6004
24	3	91	1.84	0.66	140	4.2	8.57	1000	6184
72	1	147	1.75	0.47	210	6.3	7.62	800	7519
72	2	130	2.02	1.03	170	5.1	9.41	800	6002
72	3	138.5	2.05	0.28	180	5.4	10.00	800	6051
191	1	232.8	2.12	1.48	115	3.45	7.83	800	3864
191	2	272.5	2.12	0.51	110	3.3	8.18	800	5163
191	3	246.1	2.02	1.47	100	3	7.00	800	3043

Appendix 3 Genes that are differentially expressed in* A. bisporus *grown under phase change stimulating conditions in a mushroom house <u>but not</u> in* A. bisporus *grown in a flask-based system with controlled air supply.

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated gens				
818	Cdc25-like phosphatase **(general metabolism)**	+[##]	=	+
1540	para-aminobenzoate synthase	=	=	+
205	putative protein	+	+	=
237	non-characterised sequence	Ā	+	=
1259	non-characterised sequence	=	+	=
1388	non-characterised sequence	=	+	+
down-regulated genes				
111	hsp70 chaperone **(putative stress response)**	=	-	=
695	laccase-2 **(nutrient acquisition)**	--	--	-
729	chitin synthase **(general metabolism)**	-	=	-
922	utp-glucose-1-phosphate uridylyltransferase	-	-	=
1099	hydrophobin ABH3	=	--	-
1119	non-characterised sequence	=	---	-
108	putative protein	-	-	-
142	non-characterised sequence	=	-	=
372	putative protein	-	--	=
489	non-characterised sequence	=	--	-
602	putative protein	-	-	-
606	putative protein	-	-	=
821	non-characterised sequence	-	-	-
1139	non-characterised sequence	-	-	=
1521	putative protein	-	-	=

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h (house) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

Appendix 4 Genes that are differentially expressed in* A. bisporus *grown under phase change stimulating conditions in a flask-based system with controlled air supply <u>but not</u> in a mushroom house.

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated genes				
381	Cellulose binding protein (cel1) precursor	=[##]	=	++
694	salicylate hydroxylase/ 4-aminobenzoate hydroxylase	---	+++	+
713	phosphoglycerate kinase	-	+	++
1006	glycoprotein I	=	+	=
900	non-characterised sequence	=	+	+
939	non-characterised sequence	-	++	+++
1537	putative protein	=	+	+
down-regulated genes				
450	polysaccharide lyase family 14 protein	+	--	-
1116	non-characterised sequence	=	-	-

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h (house) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

Appendix 5 Genes identified to be differentially expressed in* A. bisporus *exposed to high temperature levels (reduced CO_2 and volatile levels) <u>and not</u> under phase change stimulating conditions.

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated genes				
262	carbohydrate-binding module family 21 protein	=[##]	+	+
350	4-aminobenzoate hydroxylase	=	=	+
377	glutamine synthetase	=	=	++
694	salicylate hydroxylase/ 4-aminobenzoate hydroxylase	=	+	+++
700	glyceraldehyde-3-phosphate dehydrogenase	=	+	+++
777	synthetic construct gpdii promoter region	=	=	+++
993	non-characterised sequence	=	+	+
1159	putative protein	-	=	+++
1230	non-characterised sequence	=	=	++
1259	non-characterised sequence	=	+	++
1297	pantothenate transporter liz1	-	+	++
1388	non-characterised sequence	=	+	+
1499	putative protein	=	+	++
down-regulated genes				
111	hsp70 chaperone	+	-	-
133	histone h3	+	-	-
156	protein transport protein sec61 alpha subunit	=	-	--
372	putative protein	-	-	--
450	polysaccharide lyase family 14 protein	-	=	--
660	potential stress response protein [	-	-	-
865	putative protein	=	-	--
922	utp-glucose-1-phosphate uridylyltransferase	-	-	-
1116	non-characterised sequence	+	-	-
1383	non-characterised sequence	+	--	=
1521	putative protein	-	-	--

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h (house) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

Appendix 6 Genes identified to be differentially expressed in* A. bisporus *exposed to high temperature levels (reduced CO_2 and volatile levels) and under phase change stimulating conditions.

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated genes				
22	argininosuccinate synthase	+[##]	++	+
92	non-characterised sequence	=	+	+
94	gulonolactone oxidase	=	+	=
208	non-characterised sequence	+	+	=
213	non-characterised sequence	+	+	+
240	non-characterised sequence	=	+	=
311	non-characterised sequence	+	=	+
358	gulonolactone oxidase lgo1	+	+	=
474	putative protein	+	+	+
595	metallothionein	=	+	++
708	nadp-specific glutamate dehydrogenase	=	++	++
711	tyrosinase	+	+	+
717	hydrophobin-c	++	+++	++
824	hydrophobin 1	+++	+	++
854	hydrophobin-b	++	+	++
864	putative protein	=	+	+
916	non-characterised sequence	+	++	+
956	non-characterised sequence	=	++	++
982	non-characterised sequence	=	=	++
990	non-characterised sequence	=	+	+
994	mismatched base pair and cruciform DNA recognition	=	+	+++
1198	putative protein	-	++	++
1231	non-characterised sequence	=	+	++
1250	nadp-specific glutamate dehydrogenase	=	+	+
1251	non-characterised sequence	=	+	+
1274	hydrophobin-2	=	=	+
1285	non-characterised sequence	=	++	++
1341	non-characterised sequence	+	++	++
1408	putative protein	=	+	++
1479	non-characterised sequence	+	+	-
1577	carbamoyl-phosphate large subunit	++	+	=
1582	putative glutathione transferase	=	+	+
1621	non-characterised sequence	-	++	+++
down-regulated genes				
82	putative protein	=	-	-
289	non-characterised sequence	+	-	--
907	non-characterised sequence	+	--	-
1131	isocitrate lyase	-	=	-
1181	non-characterised sequence	=	-	-
1542	non-characterised sequence	+	--	--

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point. One, 2 or 3 symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

Appendix 7 Genes identified to be differentially expressed only in* A. bisporus *grown under phase change stimulating conditions and not while grown under maintained high temperature (reduced CO_2 and volatile levels).

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated genes				
59	glutamine synthase	=[##]	+	=
318	Carboxypeptidase	+	=	+
998	riboflavin aldehyde-forming enzyme	+	=	++
896	agglutinin B (1.0 E-2)	+	+	=
1192	aminoadipate reductase	=	=	++
1353	MFS exporter	=	+	+
1555	Cytochrome P450 (Eastwood 2001)	=	++	+
1591	putative protein	=	=	++
down-regulated genes				
410	putative protein	-	-	-
1134	non-characterised sequence	=	-	-
1534	putative protein	=	-	-
1550	glycoside hydrolase family 5 protein	=	-	-
1631	hydrophobin probe ABH3	=	--	-

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h (house) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

Appendix 8 Genes identified to be differentially expressed in* A. bisporus *exposed to maintained (high) CO_2 and volatile levels while the temperature was reduced <u>and not</u> in* A. bisporus *exposed to phase change stimulating conditions.

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated gens				
205	putative protein	+[##]	+	-
234	class i glutamine	+	+	=
262	carbohydrate-binding module family 21 protein	=	+	+
414	non-characterised sequence	-	+	++
620	endo-1,4-beta-xylanase	--	=	++
623	anthranilate synthase component ii	-	=	++
658	hexose transporter	-	-	++
742	glycosyl hydrolase family 7 protein	=	=	+++
929	Tyrosinase	=	+	+
1022	non-characterised sequence	-	=	++
1130	non-characterised sequence	+	=	+
1259	non-characterised sequence	=	=	+
1297	pantothenate transporter liz1	-	=	++
1416	putative protein	+	+	-
1425	non-characterised sequence	=	+	+
1540	para-aminobenzoate synthase	+	=	-
down-regulated genes				
77	hydroxymethylglutaryl-CoA synthase 1	=	=	--
115	putative protein	=	=	--
332	non-characterised sequence	-	-	=
368	putative terpene synthase	-	--	=
589	chitin synthase	=	=	-
602	putative protein	-	=	+
729	chitin synthase	=	-	=
1306	histone h3	-	+	--

 # Samples were collected at aring (0h), 24 h, 72 h and 168 h (house) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

Appendix 9 Genes identified to be differentially expressed in* A. bisporus *exposed to maintained (high) CO_2 and volatile levels while the temperature was reduced and in* A. bisporus *exposed to phase change stimulating conditions.

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated gens				
22	argininosuccinate synthase	+[##]	++	+
94	gulonolactone oxidase	+	+	=
208	non-characterised sequence	+	+	=
213	non-characterised sequence	+	++	-
311	non-characterised sequence	+	+	=
358	gulonolactone oxidase lgo1	+	+	=
474	putative protein	=	+	+
708	nadp-specific glutamate dehydrogenase	+	+	+
711	tyrosinase	+	=	=
717	hydrophobin-c	++	++	=
824	hydrophobin 1	++	++	-
854	hydrophobin-b	++	++	-
956	non-characterised sequence	=	++	+
982	non-characterised sequence	++	=	++
1198	putative protein	+	=	++
1231	non-characterised sequence	+	=	++
1250	nadp-specific glutamate dehydrogenase	+	=	+
1251	non-characterised sequence	=	++	=
1285	non-characterised sequence	=	++	+
1341	non-characterised sequence	+	++	--
1479	non-characterised sequence	+	+	=
1577	carbamoyl-phosphate large subunit	+	++	=
1582	putative glutathione transferase	-	++	+
down-regulated genes				
907	non-characterised sequence	=	-	-
1542	▪ ▪ n-characterised sequence	+	-	--

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h (house) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

Appendix 10 Genes identified to be differentially expressed only in* A. bisporus *exposed to phase change stimulating conditions and not in* A. bisporus *exposed to high CO_2 and volatile levels while the temperature was reduced.

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated genes				
59	glutamine synthase	=[##]	+	=
92	non-characterised sequence	+	++	+
240	non-characterised sequence	=	++	+
318	Carboxypeptidase	+	=	+
595	Metallothionein (Eastwood 2001)	+	-	++
998	riboflavin aldehyde-forming enzyme	+	=	++
864	putative protein	+	+	++
896	agglutinin B (1.0 E-2)	+	+	=
916	non-characterised sequence	++	-	++
990	non-characterised sequence	+	+	=
994	mismatched base pair and cruciform DNA recognition	+	++	=
1192	aminoadipate reductase	=	=	++
1274	hydrophobin (HYPA ABH1)	=	+	+
1353	MFS exporter	=	+	+
1408	putative protein	+	+	++
1555	Cytochrome P450 (Eastwood 2001)	=	++	+
1591	putative protein	=	=	++
1621	non-characterised sequence	+	+	=
down-regulated genes				
82	putative protein	-	-	=
289	non-characterised sequence	=	--	--
410	putative protein	-	-	-
1131	isocitrate lyase	=	--	-
1134	non-characterised sequence	=	-	-
1181	non-characterised sequence	=	-	-
1534	putative protein	=	-	-
1550	glycoside hydrolase family 5 protein	=	-	-
1631	hydrophobin probe ABH3	=	--	-

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h (house) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

Appendix 11 Genes that are differentially expressed in* A. bisporus *exposed to high levels of 1-octen-3-ol (reduced CO_2 levels and reduced temperature) and not in* A. bisporus *grown under phase change stimulating conditions.

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated genes				
234	class i glutamine	=[##]	+	+
513	putative protein	+	+	+
514	probable oxidoreductase	+	=	+
694	salicylate hydroxylase/ 4-aminobenzoate hydroxylase	---	+++	+
829	putative protein	++	=	+
890	putative protein	=	+	+
929	tyrosinase	++	=	++
939	non-characterised sequence	=	=	++
1006	glycoprotein I*	=	+	+
1130	non-characterised sequence	++	=	+
1259	non-characterised sequence	=	=	++
1262	non-characterised sequence	=	+	+
1298	tyrosinase	++	+	++
1522	60s ribosomal protein l10	++	-	-
1537	putative protein	=	+	+
1562	tyrosinase	+	+	++
1590	putative protein	-	+	++
1592	60s ribosomal protein l10	++	-	=
down-regulated genes				
40	mucin 17	-	=	-
112	pantothenate transporter liz1	+	=	--
113	putative glyoxylate pathway regulator	---	++	--
123	putative protein	-	=	=
296	scp160 protein	-	=	=
305	sphingosine n-acyltransferase lac1	-	=	=
313	calnexin homolog precursor	=	=	-
377	glutamine synthetase	-	=	-
446	phospholipid-translocating atpase	+	-	-
483	aconitate hydratase	-	=	=
602	putative protein	+	-	--
674	arginase	--	+	=
675	manganese peroxidase enzyme	+	-	-
700	glyceraldehyde-3-phosphate dehydrogenase	+	--	=
744	5-aminolevulinic acid synthase	-	+	--
880	non-characterised sequence	-	=	=
905	non-characterised sequence	-	=	--
964	dag8 gene [agaricus bisporus]	-	=	-

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h (house) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

1009	copper transporter	++	--	-
1035	putative protein	-	=	-
1119	Acyl transferase subunit	=	=	--
1126	non-characterised sequence	-	=	=
1139	non-characterised sequence	-	-	=
1159	putative protein	--	++	-
1305	thioredoxin domain containing 8	--	+	-
1383	non-characterised sequence	=	--	-
1399	heme steroid binding domain	-	=	-
1426	non-characterised sequence	-	=	-
1475	non-characterised sequence	-	=	=

Appendix 12 Genes that are differentially expressed in* A. bisporus *grown under phase change stimulating conditions and in* A. bisporus *exposed to high levels of 1-octen-3-ol (reduced CO_2 levels and reduced temperature).

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated genes				
22	argininosuccinate synthase	=[##]	+	++
59	putative glutamate-ammonia ligase (e-9)/ glutamine	=	+	+
92	non-characterised sequence	=	=	++
94	gulonolactone oxidase	+	=	++
208	non-characterised sequence	=	+	+
213	non-characterised sequence	=	++	++
240	non-characterised sequence	=	+	++
311	non-characterised sequence	=	+	++
318	vacuolar carboxypeptidase	=	+	=
358	gulonolactone oxidase lgo1	++	=	++
474	putative protein	+++	-	-
708	nadp-specific glutamate dehydrogenase	+	+	++
711	tyrosinase	+	=	+++
717	hydrophobin-c	+	+	+++
824	hydrophobin 1	+	+++	+++
854	hydrophobin-b	=	++	++
916	non-characterised sequence	=	+	+
956	non-characterised sequence	+	=	++
982	non-characterised sequence	++	+	+++
994	mismatched base pair and cruciform DNA recognition	+++	---	+
1198	putative protein	+	=	+++
1231	non-characterised sequence	=	=	+
1251	non-characterised sequence	+	+	++
1285	non-characterised sequence	=	=	++
1341	non-characterised sequence	=	++	++
1408	putative protein	+	=	+++
1479	non-characterised sequence	=	+	+
1577	carbamoyl-phosphate large subunit	=	++	++
1582	putative glutathione transferase	+	=	+
1621	non-characterised sequence	+++	---	+
down-regulated genes				
82	putative protein	-	-	-
289	non-characterised sequence	-	-	--
907	non-characterised sequence	-	=	=
1131	isocitrate lyase	=	-	-
1134	non-characterised sequence	+	-	-
1181	non-characterised sequence	=	-	-
1542	non-characterised sequence	-	-	-
1631	hydrophobin probe** sim to 1080 (HYP3/1) Lugones 1998	=	--	--

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h (house) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

Appendix 13 Genes that are differentially expressed only in* A. bisporus *grown under phase change stimulating conditions <u>and not</u> while exposed to high levels of 1-octen-3-ol (reduced CO_2 levels and reduced temperature

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated gens				
595	metallothionein	=[##]	-	++
864	putative protein	=	++	+
896	agglutinin B*	=	++	++
990	non-characterised sequence	=	=	++
998	riboflavin aldehyde-forming enzyme	+	=	+
1192	aminoadipate reductase	=	=	++
1250	nadp-specific glutamate dehydrogenase	=	=	++
1274	hydrophobin (HYPA) sim to 721 201 de groot 1996 cap	=	=	+++
1353	MFS dha1 amino acid exporter	=	=	++
1555	Cytochrome P450	=	+	++
1591	putative protein	=	=	++
down-regulated genes				
410	putative protein	+	-	--
1534	putative protein	+	-	-
1550	glycoside hydrolase family 5 protein	=	--	-

Appendix 14 Genes identified to be differentially expressed in* A. bisporus *after removal of 1-octen-3-ol and reduced temperature and CO_2 levels but not in the control treatment.

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated genes				
205	putative protein	=[##]	=	+
300	cytochrome P450	=	+	+
381	cel1 protein precursor	=	=	+
748	riboflavin aldehyde-forming enzyme	=	++	+
939	non-characterised sequence	-	++	+
998	riboflavin aldehyde-forming enzyme	+	=	+
1259	non-characterised sequence	=	+	+
down-regulated genes				
1540	putative protein	=	--	-
1627	non-characterised sequence	=	-	=

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h (house) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h (house) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

Appendix 15 Genes identified to be differentially expressed in* A. bisporus *grown after removal of 1-octen-3-ol and reduced temperature and CO_2 levels <u>and in</u>* A. bisporus *during the phase change

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated genes				
318	vacuolar carboxypeptidase	=[##]	+	+
896	agglutinin B*	-	+	+
998	riboflavin aldehyde-forming enzyme	+	=	+
1274	hydrophobin-2	+	+	=
1353	mfs dha1 amino acid exporter	+	=	=
1555	Cytochrome P450	=	+	=
1591	putative protein	=	+	++
down-regulated genes				
289	non-characterised sequence	--	=	-
410	putative protein	=	--	-
1131	isocitrate lyase	-	=	-
1341	non-characterised sequence	=	-	-
1534	putative protein	=	--	=
1542	non-characterised sequence	--	=	-
1577	carbamoyl-phosphate large subunit	+	--	=
1631	hydrophobin	=	-	--

Appendix 16 Genes identified to be differentially expressed only in* A. bisporus *during the phase change and not after removal of 1-octen-3-ol and reduced temperature and CO_2 levels

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated genes				
22	argininosuccinate synthase	=[##]	++	++
59	glutamate-ammonia ligase (e-9)/ glutamine synthase (e-10)	=	++	=
92	non-characterised sequence	=	++	+
94	gulonolactone oxidase	=	++	=
208	non-characterised sequence	=	++	=
213	non-characterised sequence	=	+++	+
240	non-characterised sequence	=	++	++
311	non-characterised sequence	=	++	+
358	gulonolactone oxidase lgo1	+	++	+
474	putative protein	=	++	=
595	metallothionein	=	-	++
708	nadp-specific glutamate dehydrogenase	+	++	+++
711	tyrosinase	=	+	++
717	hydrophobin-c (HYPC) sim to -	=	+++	++
824	hydrophobin (HYPB) sim to 854	-	+++	+
854	Hydrophobin (HYPB) sim to 824	-	+++	+
864	putative protein	=	++	+
916	non-characterised sequence	-	+++	++
956	non-characterised sequence	=	++	++
982	non-characterised sequence	++	+	+++
990	non-characterised sequence	=	=	++
994	mismatched base pair and cruciform DNA recognition	+	=	+++
1192	aminoadipate reductase	=	=	++
1198	putative protein	+	=	+++
1231	non-characterised sequence	+	=	+++
1250	nadp-specific glutamate dehydrogenase	=	=	++
1251	non-characterised sequence	+	++	+
1285	non-characterised sequence	=	++	++
1408	putative protein	=	++	+++
1479	non-characterised sequence	=	++	=
1582	putative glutathione transferase	=	+	++
1621	non-characterised sequence	+	-	+++
down-regulated genes				
82	putative protein	-	-	-
907	non-characterised sequence	-	=	--
1134	non-characterised sequence	+	--	--
1181	non-characterised sequence	=	-	-
1550	glycoside hydrolase family 5 protein	=	--	-

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h (house) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

Appendix 17 Genes identified to be differentially expressed in* A. bisporus *exposed to elevated CO_2 levels (reduced temperature, reduced volatile levels) <u>but not</u> in the control treatment.

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated genes				
248	non-characterised sequence	-	--	++
262	carbohydrate-binding module family 21 protein	-	=	++
270	3-oxoacyl-(acyl-carrier-protein) reductase*	=	=	++
503	putative protein	=	=	++
513	putative protein	=	+	+
628	metallothionein [Agaricus bisporus]	=	=	+++
629	metallothionein 2	--	=	++
694	salicylate hydroxylase/ 4-aminobenzoate hydroxylase	=	++	+
713	phosphoglycerate kinase	=	=	++
829	putative protein	+	=	++
857	non-characterised sequence	-	=	++
890	putative protein	+	+	--
900	non-characterised sequence	+	=	=
986	peptidyl-prolyl cis-trans isomerase	-	=	++
993	non-characterised sequence	=	=	+++
1032	von willebrand factor	++	-	++
1230	non-characterised sequence	=	=	++
1262	non-characterised sequence	=	++	=
1297	pantothenate transporter liz1	+	=	++
1380	sugar transporter	-	-	+++
1537	putative protein	+	=	+
1562	Tyrosinase	+	+	++
1590	putative protein	=	--	++
down-regulated genes				
69	beta tubulin	=	=	--
113	putative glyoxylate pathway regulator	---	++	=
115	putative protein	-	+	--
133	histone h3	=	=	--
302	heat shock protein hsp90	=	=	-
888	putative protein	-	-	+
931	putative protein	=	=	--
1378	putative protein	-	-	++
1425	non-characterised sequence	--	-	++
1504	gtp binding protein	+	=	--
1538	aromatic peroxygenase precursor	+	=	-

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h (house) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)

Appendix 18 Genes identified to be differentially expressed in* A. bisporus *exposed to high CO_2 levels (reduced temperature, reduced volatile levels) and when grown under phase change stimulating conditions

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated genes				
22	argininosuccinate synthase	=[##]	=	+++
59	glutamate-ammonia ligase (e-9)/ glutamine synthase (e-	+	++	--
92	non-characterised sequence	=	+	=
94	gulonolactone oxidase	=	++	-
208	non-characterised sequence	=	++	=
213	non-characterised sequence	=	++	=
240	non-characterised sequence	=	+	++
311	non-characterised sequence	=	++	+
318	vacuolar carboxypeptidase	=	++	-
358	gulonolactone oxidase lgo1	=	++	=
474	putative protein	=	++	+
595	metallothionein [Agaricus bisporus]	--	=	+++
708	nadp-specific glutamate dehydrogenase	+	+	++
711	Tyrosinase	+	=	+++
717	hydrophobin-c	=	+++	+++
824	hydrophobin 1	=	+++	=
854	hydrophobin-b	=	+++	-
864	putative protein	=	++	=
916	non-characterised sequence	=	+++	=
956	non-characterised sequence	=	++	++
982	non-characterised sequence	++	=	+++
994	mismatched base pair and cruciform DNA recognition	=	=	+++
1198	putative protein	=	=	+++
1231	non-characterised sequence	+	=	+++
1251	non-characterised sequence	+	++	++
1285	non-characterised sequence	=	++	++
1341	non-characterised sequence	++	+	-
1353	mfs dha1 amino acid exporter	=	=	++
1408	putative protein	=	+	++
1479	non-characterised sequence	=	++	=
1577	carbamoyl-phosphate large subunit	=	+	+
1621	non-characterised sequence	=	=	++
down-regulated genes				
82	putative protein	=	--	=
289	non-characterised sequence	=	--	-
907	non-characterised sequence	=	-	--
1181	non-characterised sequence	=	-	-
1542	non-characterised sequence	=	--	-

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h (house) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

Appendix 19 Genes identified to be differentially expressed only in* A. bisporus *when grown under phase change stimulating conditions and not when exposed to high CO_2 levels (reduced temperature, reduced volatile levels)

ID	putative functional annotation	0-24[#]	24-72	72-168
up-regulated genes				
998	riboflavin aldehyde-forming enzyme	+[##]	=	+
896	agglutinin B*	=	++	++
990	non-characterised sequence	=	=	++
1192	aminoadipate reductase	=	=	++
1250	nadp-specific glutamate dehydrogenase	=	=	++
1274	hydrophobin (HYPA) sim to 721 201 de groot 1996 cap	=	=	+++
1555	Cytochrome P450	=	+	++
1582	putative glutathione transferase	=	+	++
1591	putative protein	=	=	++
down-regulated genes				
410	putative protein	+	-	--
1131	isocitrate lyase	=	-	--
1134	non-characterised sequence	+	--	--
1534	putative protein	+	-	-
1550	glycoside hydrolase family 5 protein	=	--	-
1631	hydrophobin probe** sim to 1080 (HYP3/1) Lugones	=	--	--

 # Samples were collected at airing (0h), 24 h, 72 h and 168 h (house) after airing and expression patterns between two successive samples were compared (e.g. airing versus 24 h after airing)
The symbols "-", "=" and "+" indicated down-regulation, equal level and up-regulation of gene expression from the first to second time point. One, two or three symbols indicate respectively between 1.4 - 2.8, 2.8 - 8 or > 8 fold difference in expression between two time points.

Appendix 20 Correlation of qRT-PCR data with non-normalised and normalised microarray data. Transcript levels of 5 genes in colonised casing samples of* A. bisporus *grown under phase change stimulating conditions were investigated using both technologies. (A) Time point at which the colonised casing samples were collected (h after airing). (B) Non-normalised microarray data. (C) Correlation coefficient of qRT-PCR data with non-normalised data. (D) Normalised microarray data. (E) Correlation coefficient of qRT-PCR data with normalised data.

GENE	A	Average ΔCt	SD (ΔCt) [#]	B	C	D	E
1119	0	0.003264	0.000448	1.017794	0.904027	1.630474	0.988977
	72	0.000646	3.26E-05	-0.70411		-1.35767	
	168	0.000281	2.13E-05	-2.61841		-2.44757	
982	0	8.81E-05	7.8E-05	-1.93274	0.992026	-0.75987	0.94055
	72	0.000592	0.000152	2.182109		1.463974	
	168	0.000525	1.7E-05	2.201264		2.138315	
1542	0	0.00052	3.15E-05	0.871491	0.919896	1.949195	0.99996
	72	0.000314	7.71E-05	0.438426		0.077632	
	168	0.000109	2.54E-05	-2.4288		-1.8499	
1550	0	0.000605	0.000123	0.533227	0.938422	1.125966	0.990831
	72	0.000388	4.97E-05	0.09713		-0.48923	
	168	0.000207	1.59E-05	-1.3592		-1.3086	
318	0	6.64E-06	6.99E-06	-1.56883	0.901736	-0.52698	0.961306
	72	2.77E-05	6.4E-06	1.211181		1.132234	
	168	5.55E-05	6.84E-06	1.76536		1.92928	

[#] The standard deviation on the average ΔCt is based on the difference between the ΔCt of the biological and technical replicates for that time point.

Additional material

A DVD containing additional documentation is attached to the back cover of this thesis. The DVD contains the following files:

- The file "**sequence and probe information *Agaricus bisporus* gene expression microarray v1.xls**" contains the full length sequence and probe sequences of each of the *A. bisporus* sequences present on the custom 8 X 15,000 60-mer oligonucleotide *Agaricus bisporus* gene expression microarray design;
- The file "***in silico* annotation results of *A. bisporus* genes.xls**" gives the *in silico* annotation information for the sequences present on the custom 8 X 15,000 60-mer oligonucleotide *Agaricus bisporus* gene expression microarray design. The annotation information was collected using Blast2GO (Conesa *et al.*, 2005);
- The file "R**-script for the processing and normalisation of Agaricus bisporus microarray data**";
- The media file "**Top-view time-lapse recording of *Agaricus bisporus* development**";
- The media file "**Side-view time-lapse recording of *Agaricus bisporus* development**";
- The media file "**abortion of a differentiating primordium (top-view) 1**";
- The media file "**abortion of a differentiating primordium (top-view) 2**";
- The one- and two-colour microarray-based gene expression analysis protocols (Agilent Technologies) used during this project;
- The Feature Extraction Software v 9.5 reference guide.

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