School of Biology (Life Sciences)

Control of Sexual Reproduction in *Aspergillus* Species

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Abstract

The principal aim of the present study was to investigate biochemical, evolutionary and genetic factors that influence and control sexual reproduction in *Aspergillus* species. The aspergilli include species of major economic and medical importance, with some species reproducing by sexual means but many being only known to reproduce by asexual means. It was anticipated that an improved understanding of the factors controlling sexual reproduction would provide fundamental insights into biological processes controlling sex in ascomycete fungi as a whole, as well as providing insights to enable methods to be designed to promote sexual reproduction in the laboratory. The sexual cycle could then be used in classical genetic studies and strain improvement programmes. Studies were focussed mainly on three representative *Aspergillus* species, namely the homothallic (self fertile) *Aspergillus nidulans, Eurotium repens* and *Neosartorya fischeri.* Several isolates were obtained from different worldwide locations and their identity verified by phylogenetic means prior to main experimental work. Firstly, conditions were optimised for both sexual and asexual reproduction for each species to facilitate later experimental work. This involved growth under a variety of different environmental parameters including media type, temperature, light, dark, sealing or non-sealing of plates, and incubation in variable stack number, Interestingly it was found that highest levels of cleistothecia of the model species *A. nidulans* were produced at 32ºC, rather than the 37ºC routinely used in laboratories worldwide, and by incubating plates in a single layer.

Secondly, investigations were made to assess whether asexual conidia and sexual ascospores of *A. nidulans* exhibited differential resistance to environmental stress, which might be of importance for the evolutionary maintenance of different forms of reproduction in this species. Conidia and ascospores from a variety of isolates and strains were subjected to thermal stress, involving exposure to a range of temperatures between 50ºC and 75ºC for 30 min, and in addition to UV radiation stress, involving exposure to 254 nm for different time points between 10 and

60 min. Spore viability data for both heat and UV shock was converted to *D*-values, revealing that ascospores were slightly more resistant than conidia to thermal shock and had markedly greater resistance to UV shock. These results help to explain why homothallism might be retained in species that are able to produce asexual spores at much lower metabolic cost. Parallel studies with *E. repens* also showed that ascospores exhibited higher thermal resistance than conidia.

Thirdly, the possible evolution of asexuality was studied. Previous classic work by Mather and Jinks (1958) suggested that a gradual loss of sexuality occurs when fungi are subcultured solely by asexual transfer. This claim was tested by serial asexual transfer and subsequent assessment of sexual potential for a variety of *A. nidulans, E. repens* and *N. fischeri* isolates and strains. A 'slow decline' in sexual fertility was indeed observed in the majority when repeatedly subcultured by asexual conidia. Staining with Hoechst 33258 and Calcofluor was used to correlate the number of mitotic divisions occurred in the apical cells with the decrease in sexual fertility for all three species. Significantly, it was found that the sexual fertility of most long-term asexual strains could be restored either partly of fully back to initial levels by propagation by ascospore transfer. This suggested that a mixture of epigenetic and mutational factors might be partly responsible for the evolution of asexuality in fungi.

Finally, investigations were made to identify novel genes involved in the regulation of sexual fertility in the aspergilli. A series of gene disruption cassettes were used to delete putative high-mobility group transcription factors from the genome of *A. nidulans.* Deletion of one particular gene caused a loss of sexual fertility indicating that this gene acts as a sexual regulator. This gene was termed '*steD*'. Complementation work via the sexual cycle confirmed that sex could be restored by restoration of this gene. This discovery indicated that many so far unidentified genes are likely to have key roles regulating sexual fertility of *A. nidulans* and ascomycete fungi in general. Thus, the loss of function of a wide range of genes might lead to the evolution of asexuality in ascomycete fungi.

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Glossary

Anamorph: the asexual ('imperfect') form or morph characterised by the presence of conidia.

Ascoma: plural is ascomata, an ascus-containing structure.

Ascospore: A spore produced in an ascus by 'free cell formation'.

Ascus: a typical sac-like cell characteristic of the *Ascomycota* in which eight ascospores are produced after karyogamy and meiosis.

Conidiophore: a simple or branched hypha bearing or consisting of conidiogenous cells from which conidia are produced.

Holomorph: the whole fungus in all its morphs and phases

Hülle cells: terminal or intercalary thick-walled cells, which occur in large numbers in association with the ascomata of some aspergilli, for example *Aspergillus nidulans*

Karyogamy: the fusion of two nuclei after cell fusion, i.e. after plasmogamy.

Plasmogamy: the fusion of two cells without immediate karyogamy or a precursor to karyogamy

Teleomorph: the sexual ('perfect') form or morph, characterised by ascomata

❖The definitions are taken from Kirk and Ainsworth (2008).

Abbreviations

ACM	*Aspergillus* complete medium
AMM	*Aspergillus* minimal medium
ANOVA	Analysis of variance
APN2	Well conserved anaphase promoting gene bordering the *MAT* locus in ascomycete
AspGD	*Aspergillus* Genome Database
ATP	Adenosine triphosphate
aw	Water activity
BLAST	Basic local alignment search tool
Bt2a	Beta-tubulin 2a primer
Bt2b	Beta-tubulin 2b primer
CDA	Czapek dox agar
d	day(s)
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
***D*-value**	Decimal reduction time
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetraaceticacid
F	*F* factor
FGSC	Fungal Genetics Stock Centre

GMEA	Glucose malt extract agar
HCl	Hydrochloric acid
HMG	High-mobility group
KH_2PO_4	Monopotassium phosphate
K_2HPO_4	Dipotassium phosphate
MAT	Mating-type
MEA	Malt extract agar
MgSO4	Magnesium sulphate
M40Y	Malt extract medium
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
NaCl	Sodium chloride
Na_2HPO_4	Sodium phosphate
NaOH	Sodium hydroxide
OA	Oatmeal agar
ORF	Open reading frame
PABA	Para-Amino benzoic Acid
PCR	Polymerase chain reaction
PDOX	Pyridoxine HCL
PEG	Polyethylene glycol
psi	Precocious sexual inducer

QMC	Queen Medical Centre, University of Nottingham
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
RT	Room temperature
SDS	Sodium dodecyl sulphate
SLA2	Well conserved DNA lyase gene bordering the *MAT* locus in most filamentous ascomycetes
TBE	Tris borate EDTA buffer
TEM	Transmission electron microscopy
Tris	Tris-hydroxymethyl-methylamine or TRIZMA
Tris-HCl	Tris hydroxymethyl amino methane
TWA	Tap water agar
UV	Ultra violet
v/v	Volume fraction or volume concentration
VeA	Velvet gene
Wk	Week (s)
w/v	Weight per volume
YEG	Yeast extract glucose media
Δ	Deletion mutant

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Chapter 1 Introduction

1.1 Introduction

1.2 General background to the Ascomycota

The Ascomycota is the most important phylum of fungi, containing the majority of fungal species that have been described. By definition, the phylum encompasses species that reproduce by production of ascospores in asci. These are formed when two haploid nuclei fuse to yield a diploid nucleus, which is followed by the process of meiosis and mitosis to generate normally 8 haploid spores (ascospores or meiospores) (Burnett, 2003; Lee *et al.*, 2010).

The asci, containing the ascospores, are produced either within ascomata (also termed 'ascocarps' or 'fruiting bodies') or can be borne naked, such as seen in yeasts. The cytoplasm within the asci becomes compartmentalized by double membranes leading to the eventual formation of the individual ascospore walls (Read and Beckett, 1996). In the case of filamentous ascomycetes, the asci usually arise from dikaryote ascogenous hyphae formed ascogonia (female) within ascoma (plural ascomata). Three different types of asci may be present in fungi, namely unitunicate, bitunicate and prototunicate In addition, four main forms of fruiting bodies can be recognised in the filamentous Ascomycota, namely cleistothecia, apothecia, perithecia and pseudothecia (**Figure 1-4**) (Burnett, 2003; Lee *et al.*, 2010).

Under classical fungal taxonomy rules, species in the Ascomycotina may have both an 'anamorphic' and a 'teleomorphic' state. Each has a different Latin name that refers to their respective different reproductive forms (Burnett, 2003). The anamorph refers to the asexual state and its name comes from the morphology of asexual spores, such as *Aspergillus*. Meanwhile, the teleomorph refers to the sexual state, and its name is

derived from the morphology of the sexual fruiting body, such as *Emericella* (Raper and Fennell, 1965; Malloch and Cain, 1972a; Malloch and Cain, 1972b). However, this 'dual nomenclature' is currently under debate with proposals for a 'one fungus, one name' convention (Hawksworth, 2011).

Furthermore, there are a significant number of species, which are placed phylogenetically within the Ascomycota but these are only known to generate asexual spores (mitospores). Thus, a sexual stage in these species has not been discovered yet and they have traditionally been termed 'imperfect' or 'anamorphic' fungi (Deuteromycotina), but are now included within the Ascomycota (Hennebert and Weresub, 1977; Guarro *et al.*, 1999).

Work in the current thesis will focus on reproduction in one particular grouping within the Ascomycotina, the genus *Aspergillus.* This group is also collectively termed 'the aspergilli'. Therefore there will be an especial focus on *Aspergillus* species in the following introduction concerning reproduction in ascomycete fungi.

1.3 Reproduction of the Ascomycotina

Sporulation is the main method of reproduction in ascomycete fungi and in filamentous species this involves the production of either conidia (asexual spores) via mitotic duplication or ascospores (sexual spores) by meiosis (**Figure 1-1**) (Nelson, 1996). Ascomycete species reproduce by sexual or asexual means according to the prevailing environmental conditions (e.g. pH level, temperature, light source, nutrition and moisture) (Nelson, 1996). The majority of fungal species can produce both sexual and asexual spores. However, perhaps 20% of all fungi are only known to reproduce by asexual means – although the number of sexual species has recently increased following studies of mating-type (*MAT*) genes and demonstration of functional sexual cycles *in vitro* (Kerenyi *et al.*, 2004; Ware *et al.*, 2007; Houbraken *et al.*, 2008; O'Gorman *et al.*, 2009; Horn *et al.*, 2009a; Horn *et al.*, 2009b)

1.3.1 Asexual reproduction

Almost all ascomycete fungi are able to reproduce asexually by mitotic duplication and as a result the offspring are normally genetically identical to their parents (Milgroom, 1996). Ascomycete yeasts (single-cell fungi) reproduce asexually by producing spores by budding, while filamentous fungi produce conidia borne on mycelia. Conidiophores (specialised branches of a hypha) produce specific so-called 'conidiogenous' cells, which are usually able to form the conidia as a single, a cluster or a sequence depending on the species involved and nutrients available (Moore-Landecker, 1996). In the case of the aspergilli, the conidiophore structure (stalk) involves the formation of a vesicle at the top, from which metulae and phialides (secondary structures) develop, these branches will finally form the different types of conidia (**Figure 1-1**) (Adams *et al.*, 1998).

There is considerable evidence that the sexual reproduction is the ancestral (original) state in fungi and many asexual species are therefore thought to be derived from sexual species (Guadet *et al.*, 1989; Chang *et al.*, 1991; Lobuglio *et al.*, 1993; Lobuglio and Taylor, 1993; Taylor, 1995; Geiser *et al.*, 1996; Turgeon, 1998; Geiser *et al.*, 1998a; Seifert *et al.*, 2007). The ascomycete grouping contains the highest proportion of species (estimated at around 40%) that are thought to reproduce only by asexual means and it is of much current interest to find out whether there might be extant sexual cycles in these fungi (Dyer and Paoletti, 2005). Interestingly, there is indeed evidence for previous or current sex in many of these supposed 'asexual' species from studies analysing their genomes. One clear example concerns the gene-silencing processes called 'repeat induced point' (Martin *et al.,* 2010) mutation, which acts to mutate duplicated genes in a genome during recombination. This was first described in *N. crassa* (Selker *et al.*, 1987), "RIPing" involves the mutation of duplicated DNA sequences, via C: G to T: A transitions in both the original sequence and the inserted sequence, which increases the chances of introducing amber and ochre stop codons. This leads to the alteration of the original and duplicate sequence and therefore

potentially results in non-functional gene products with premature stop codons or mutated or disabled regulator regions (Montiel *et al.*, 2006).

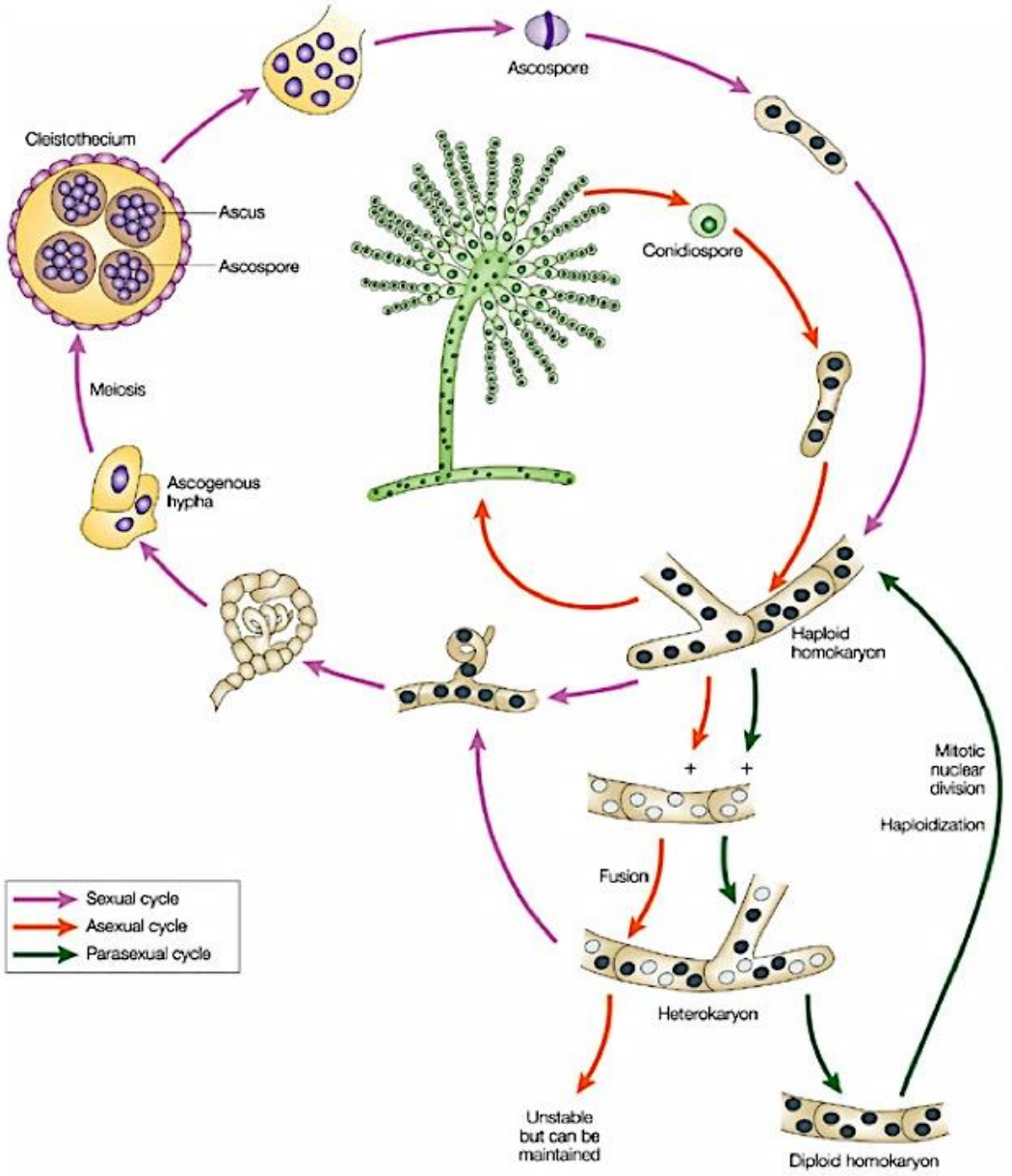

Figure 1-1 Two methods of sporulation of *A. nidulans*: Sexual, parasexual and asexual cycles are all shown. The sexual cycle can be further subdivided into the homothallic and heterothallic cycles (Casselton and Zolan, 2002).

Although the mechanistic process of RIPing is not fully understood, a gene encoding a putative cytosine DNA methyltransferase called RID (RIP defective) has been shown to be fundamental to the process (Freitag *et*

al., 2002). In addition to *N. crassa,* potential RID homologs have been found in various sexual species including *E. nidulans, N. fumigata, P. anserina* and also many asexual species, such as *A. oryzae*, *A. niger* and *P. chrysogenum* (Neuveglise *et al.*, 1996; Hamann *et al.*, 2000; Nielsen *et al.*, 2001; Clutterbuck, 2004; Galagan and Selker, 2004; Monroy and Sheppard, 2005; Montiel *et al.*, 2006; Braumann *et al.*, 2008). The latter observation is significant as it suggests that these 'asexual' species have a recent evolutionary history of sexual reproduction i.e. RIPing in these species being evidence of current possession or recent loss of a sexual cycle. There are differences between species in their mutation rates. For instance, *N. fumigata* has a very reduced mutation rate compared to *N. crassa*, which might reflect the relative rarity of sexual reproduction in the former species (Monroy and Sheppard, 2005; O'Gorman *et al.*, 2009).

Concerning asexual reproduction in the aspergilli, Adams *et al.* (1998) reported that a high number of different genes from 45 to 1,200 genes might be involved in asexual reproduction. Of particular importance are genes such as *brlA, abaA* and *wetA*, which have been shown to be fundamentally involved in conidia formation with the mRNA of *brlA* and *abaA* accumulating in the conidiophores. Notably, later observations illustrated that *brlA* is fundamental for conidiation (formation of conidiophores) in various aspergilli, such as the opportunistic human pathogen *A. fumigatus* (Mah and Yu, 2006), and also the industrial fungus *A. oryzae* (Yamada *et al.*, 1999). A full description of asexual reproduction is beyond the scope of the present study, which will focus mainly on sexual reproduction.

1.3.1.1 Advantages and disadvantages of asexual reproduction

There are many advantages to asexual development over sexual reproduction. One benefit is that the asexual development has lower metabolic demands compared with sexual propagation, which has high energy costs due to both the mating process and also the subsequent

costs of producing specific structures such as fruiting bodies for sexual reproduction (Lee *et al.*, 2010). Secondly, favourable combinations of genes can be broken up via gene recombination in sexual reproduction, whereas recombination very infrequent in asexual reproduction so beneficial combinations of genes will be maintained in asexual species (Milgroom, 1996). Thirdly, asexually propagating species are able to produce higher number of spores at a lower energy cost than sexual spores, and these asexual spores can normally germinate rapidly and efficiently (Nelson *et al.*, 1997; Wu and Miller, 1997). This may explain why many species favour their asexual rather than sexual cycle, with sex only occurring when it is necessary to increase genetic variation. Furthermore, many asexual species might retain the ability to generate variation and undergo recombination through 'the parasexual cycle', which provides a means for a low rate of recombination in a relatively short time compared to the longer and more metabolically costly sexual cycle (see following section **1.3.2**).

On the other hand, the progeny of asexual reproduction is often genetically identical to their parents unless rare mutational events occur (Milgroom, 1996). Such mutant clones have been identified in asexual species and can be defined as "repeatedly sampled, multilocus genotypes that are unlikely to have arisen by chance in sexual recombination" (Anderson and Kohn, 1998; Fillinger *et al.*, 2001). These mutations might be beneficial. However, many mutations are likely to be deleterious and can accumulate in the genome of asexual fungi, and be inherited from generation to generation, and are very hard to remove in the absence of recombination. Hence the concept of a 'ratchet' accumulation of mutational load in asexual species (Muller, 1964). This might explain why asexual lineages are apparently short-lived compared to long-term sexual lineages (Berbee and Taylor, 1993).

1.3.2 Parasexuality

Sexual stages have not been detected in many *Aspergillus*, *Penicillium*, *Fusarium* and *Trichoderma* species, whereas other species from the same

genera can reproduce sexual and asexual spores in specific conditions. However, alternative means of recombination might occur in asexual species because some fungi can recombine their genes in process termed 'parasexuality' (Deacon, 1997). Parasexuality can be distinguished as a different process from sexuality, which occurs in asexual species as result of vegetative hyphal fusion and can lead to recombination at a very low rate (Jinks, 1952; Tinline and Macneill, 1969; Anderson and Kohn, 1998). Parasexuality involves a sequence of three stages: heterokaryon formation, diploidization of nuclei, and final haploidization (**Figure 1-2**) and resembles sexuality but with a lower rate of recombination (Moore-Landecker, 1972). Indeed, it has been estimated that the frequency of mitotic crossing-over is 500-1000 times lower than that seen in meiotic crossing-over (Pontecorvo, 1956). Nevertheless, parasexuality has been used as a key tool to produce strains with desired combinations for industry. Also the parasexual cycle, although relying on rare events, can be induced during somatic growth unlike sexuality that often needs very specialised conditions to occur (Deacon, 1997).

In order for a heterokaryon to form, isolates must be of the same 'vegetative incompatibility (vic)' or 'heterokaryon (het)' grouping. Genome sequencing studies of various ascomycete species [including *P. chrysogenum, N. fumigata, E. nidulans, N. crassa* and *P. anserina* (Pontecorvo *et al.*, 1953; Debeaupuis *et al.*, 1997; Xiang and Glass, 2002; Fedorova *et al.*, 2005)] have shown a variety of genes to be involved with determination of heterokaryon grouping, with around 50 genes found to encode proteins containing HET domains. Interestingly, in some *Neurospora* species the *MAT* genes (*MATA-1* and *MATa-1*) can act as allelic *het* incompatibility factors. Homologs of these genes and other HET domain encoding genes have been described from various asexual and sexual *Aspergillus* species (Pal *et al.*, 2007).

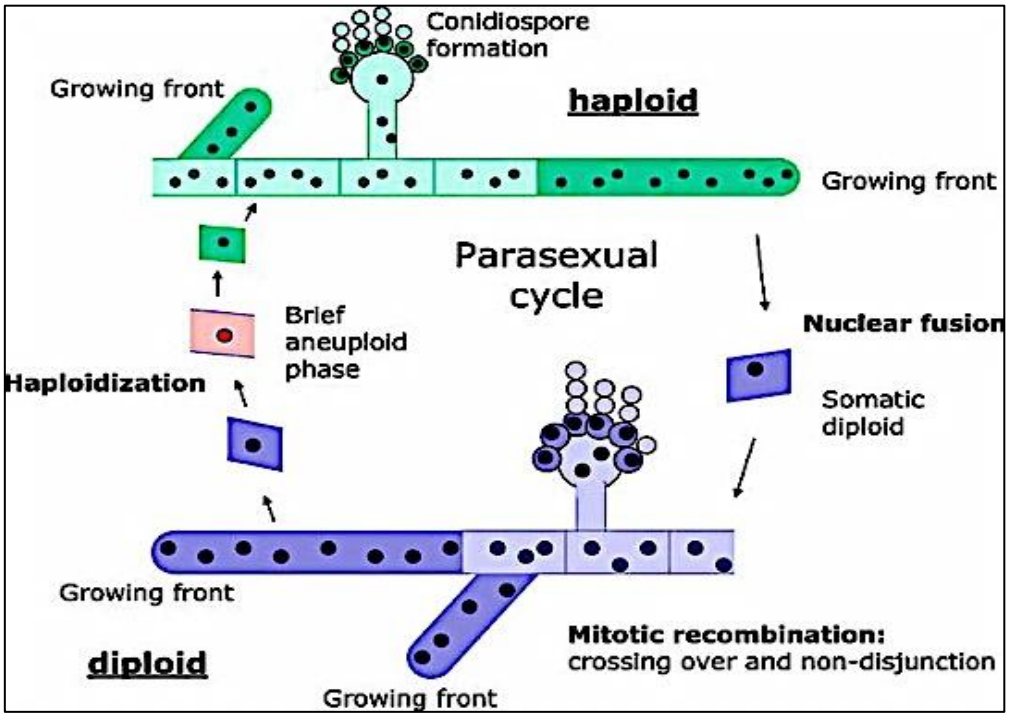

Figure 1-2 Schematic overview of the parasexual cycle in the filamentous fungus *A. nidulans* showing an alternation between vegetative growth with haploid or diploid nuclei [Adapted from (Schoustra *et al.*, 2007)]. The parasexual cycle involves the formation of heterokaryotic mycelium containing genetically different nuclei within the same cytoplasm, which are able to fuse and form a diploid nucleus, which then undergoes sequential loss of chromosomes to restore the haploid state.

BLAST searches have focused on homologs of one incompatibility gene (*het-6*), one suppressor gene (*tol*) and an incompatibility related gene (*pin-c*) which is linked to *het-c*. Significantly, sexual *Aspergillus* species were found to have fewer homologs of the *het-6*, *tol* and *pin-c* genes than asexual species, with possible implications for gene flow by the parasexual cycle.

The parasexual cycle might also provide a means to prevent the spread of myco-viruses, with an increased number of different het groupings likely to inhibit the spread of viruses. For example, *A. niger* has a large number of homologs of the three genes aforementioned (the *het-6*, *tol* and *pin-c* genes), which might offer a means to counter infection by

mycoviruses compared to the sexual species *E. nidulans* which can remove such viruses via the production of sexual progeny (Van Diepeningen *et al.*, 1997; Coenen *et al.*, 1997; Glass *et al.*, 2000; Galagan *et al.*, 2003; Galagan *et al.*, 2005; Fedorova *et al.*, 2005; Glass and Dementhon, 2006; Paoletti and Clave, 2007).

Furthermore, the parasexual cycle may allow a fungus to survive conditions that are too harsh for existence of the parental homokaryons by formation of a more resistant heterokaryon (Jinks, 1952; Burnett, 2003). However, such adverse conditions might still cause damage to the vegetative mycelium, whereas ascospores are often more resistant to harsh stresses another advantage of sexual over parasexual reproduction (Jinks, 1952).

The exact reasons for cell death following formation of a vegetatively incompatible heterokaryon are still not fully understood. However, Ishikawa *et al.* (2012) recently investigated this issue using different vegetatively compatible and incompatible strains of the bean pathogen *Colletotrichum lindemuthianum*. Nuclei from strains were labelled with either a green or red fluorescent protein and observed microscopically during cell fusion and heterokaryon formation. It was found that the fusion of hyphae between incompatible mature colonies resulted in cell death within 3 hr. However, fusion involving conidial anastomosis tubes (CATs) between (Galagan *et al.*, 2003) incompatible strains during colony initiation did not trigger a strong vegetative incompatibility response. Colonies from these fusions were formed with phenotypes and other characteristics different to those of either parental strain. It was therefore hypothesised that the CAT system during the initiation of colony repressed the vegetative incompatibility response in this fungus and, if this occurs more widely in the fungal kingdom, might provide a mechanism to increase the genetic diversity of many asexual fungal species. Indeed, the CAT system has been identified during the early stages of colonisation in various filamentous fungi including plant pathogenic species (Roca *et al.*, 2005a; Roca *et al.*, 2005b; Read *et al.*, 2009; 2010).

1.3.3 Sexual reproduction

The fungal kingdom has been divided into four phyla (**Figure 1-3**) depending on how sexual spores are formed: Chytridiomycetes, Zygomycetes, Ascomycetes and Basidiomycetes (Lee *et al.*, 2010).

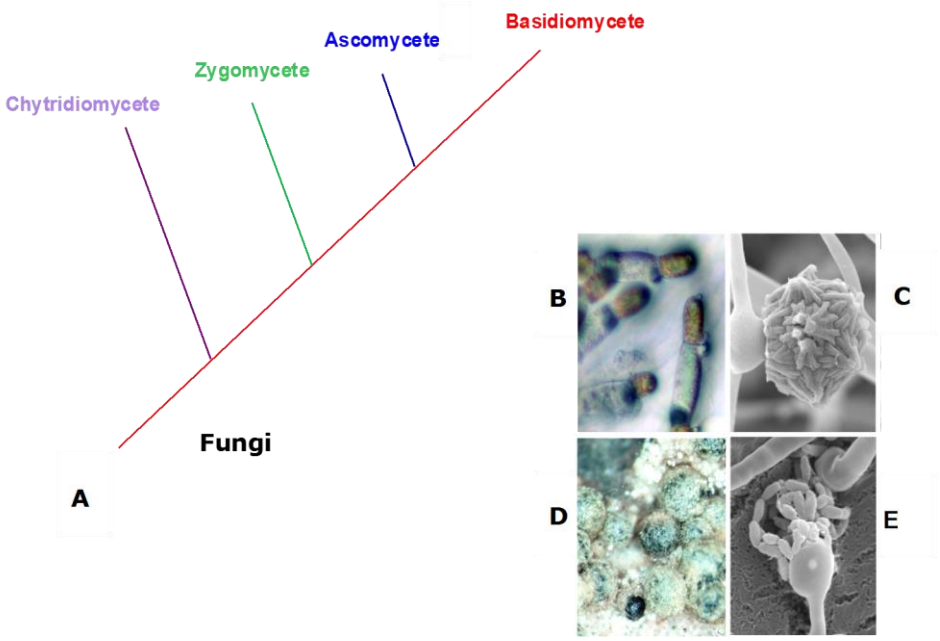

Figure 1-3 Shows A. four phyla of the Fungi Kingdom. B. Chytridiomycete sexual hyphae. The male is orange and female hyaline: *Allomyces macrogynus.* C. Zygomycete zygospore: *Mucor circinelloides.* D. Ascomycete cleistothecia: *Aspergillus nidulans*. E. Basidiomycete basidia and sexual spore chains: *Cryptococcus neoformans.* [Adapted from (Lee *et al.*, 2010)].

With particular regards to the Ascomycotina, filamentous ascomycetes normally grow vegetatively in the haploid state and it is only during sexual reproduction that two haploid nuclei fuse to form a diploid nucleus, which rapidly undergoes meiosis to produce ascospores under appropriate condition. Conversely, many yeast species can exist as diploids in the vegetative state, with diploid nuclei undergoing meiosis to generate ascospores, which are released after the ascus wall breaks down. In filamentous ascomycetes fusion of the two haploid nuclei occurs

in so called 'ascogenous' hyphae and fusion of compatible nuclei is delayed as dikaryotic nuclei divide several times, whereas in yeast species the two compatible nuclei normally fuse immediately after plasmogamy (Lee *et al.*, 2010).

1.3.3.1 Classification of the Ascomycotina and morphology of teleomorph states

As mentioned in section **1.2**, ascomycete sexual species produce sexual spores (ascospores) within asci, each ascus commonly containing up to 8 spores depending on the species (Lee *et al.*, 2010). These asci may be surrounded by an ascocarp (fruiting body) such as the cleistothecium in *Aspergillus nidulans*, or can be naked as seen in yeasts including *Saccharomyces cerevisiae*, with different forms of sexual development occurring depending on each phylum or even subgroup (Lee *et al.*, 2010). Species of ascomycetes have traditionally been classified into six classes based on structure of fruiting bodies as follows (**Figure 1-4**):

Hemiascomycetes: has no fruiting body, including the yeasts *S. cerevisiae* and *S. pombe.*

Pyrenomycetes: produce fruiting bodies termed perithecia (flask-shaped fruiting bodies) containing unitunicate (single-walled) asci, for example *Neurospora crassa*.

Loculoascomycetes: form fruiting bodies similar to perithecia but which contain bitunicate (double-walled) asci, for example *Cochliobolus heterostrophus*.

Plectomycetes: produce closed fruiting bodies called cleistothecium with many unitunicate asci, for example *Eurotium repens*.

Discomycetes: form cup-shaped fruiting bodies termed apothecia containing exposed asci. For example, *Peziza vesiculosa*

Laboulbeniomycetes: this class has been poorly studied and includes some insect parasites.

This classification has proved useful for general typing. However, recent phylogenetic studies have shown that many of these groupings are not supported by molecular evidence and that some of these groups are paraphyletic (James *et al.*, 2006; Schoch *et al.*, 2009).

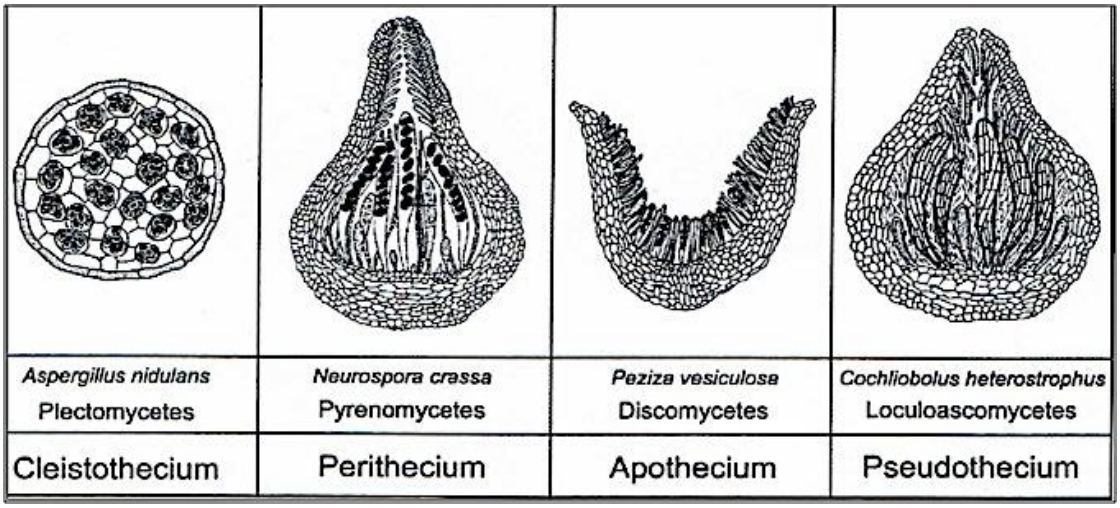

Figure 1-4 Four types of fruiting bodies of filamentous Ascomycotina [Adapted from (Nelson, 1996)].

1.3.4 Homothallism, heterothallism and pseudohomothallism in the Ascomycotina

A variety of different sexual breeding systems are evident in the Ascomycotina. Some ascomycetes species have two (or more) mating types and compatible isolates are required to complete sexual reproduction; these species are termed '**heterothallic**' fungi and are by definition self–sterile. By contrast, other fungal species can perform their sexual cycle individually without the need for a partner, these are termed '**homothallic**' fungi and are by definition self-fertile (Dyer *et al.*, 2003; Burnett, 2003; Lee *et al.*, 2010). Meanwhile, other fungal species are termed '**pseudohomothallic**' and are very similar functionally to homothallic species because they can also complete the sexual process individually and hence are self-fertile – though unlike true homothallics, it is achieved by the presence of two compatible nuclei in a single

ascospore (Metzenberg and Glass, 1990; Corcoran *et al.*, 2012). In some yeast species cells can switch mating type to allow mating with a closely 'un-switched' sister cell (Herskowitz, 1992; Klar, 1992), whereas in filamentous ascomycetes switching of mating type does not occur normally (Glass *et al.*, 1990b).

There is a close evolutionary relationship between homothallic and heterothallic species. It has long been suggested that homothallic species have evolved from heterothallic species but not vice versa (Nelson, 1996). Indeed recent molecular investigations have supported this hypothesis, with the demonstration of heterothallic "sexual machinery" apparently being retained in homothallic species (Paoletti *et al.*, 2007).

Major insights into the molecular-genetic basis of homothallism and heterothallism have come from studies of so-called 'mating-type' (*MAT*) genes (also see section **6.1**). These *MAT* genes have a key role in determining sexual identity in fungi, and the presence of different organisations of *MAT* genes in ascomycete fungi results in a species being either homothallic (self-fertile) or heterothallic (self-incompatible) (**Figure 1-5**) (Lee *et al.*, 2010; Dyer and O'Gorman, 2012). The mating system of the yeast *S. cerevisiae* was first to be studied in detail at the molecular-genetic level and provided the initial model for understanding fungal mating systems. *S. cerevisiae* is a heterothallic species with two alternative mating type cells termed **a** and α. In **a** mating-type cells the **a**1 gene encodes an HD2 class homeodomain (HD) transcription factor, whilst in α mating-type cells the α1 gene encodes an alpha box transcription factor and the α2 gene encodes a HD1 class homeodomain transcription factor. In the α and **a** haploid cell types, expression of these genes is mating-type cell-specific and these genes regulate other genes involved with sexual development (Herskowitz *et al.*, 1992). Haploid **a** cells produce an **a** factor pheromone to signal to α cells. Likewise, haploid α cells produce an α factor pheromone to signal **a** cells. Three possible types of cells can be found in *S. cerevisiae* in terms of mating identity, these are **a**, α (both haploid) and **a**/α diploid. The **a**1 and α2 gene products in the diploid cell form a heterodimer that helps to

transcriptionally repress haploid-specific genes. The **a**/α diploid cells produce haploid meiotic progeny (two α and two **a** cells in an ascus) after undergoing meiosis (Herskowitz *et al.*, 1992; Johnson, 1995; Galitski *et al.*, 1999; Galgoczy *et al.*, 2004).

By contrast, a slightly different organisation of *MAT* genes is evident in the filamentous Ascomycotina. Mating-type (*MAT*) genes again act as master regulators of sexual identity and development (Coppin *et al.*, 1997a; Turgeon and Yoder, 2000). In heterothallic species, a single *MAT* locus is present, with alternative mating-type sequence information or 'idiomorphs' being found here. *MAT-1* isolates characteristically contain a *MAT* locus with a *MAT1-1* gene that encodes an α-domain protein, whereas *MAT-2* isolates characteristically contain a *MAT-1-2* gene which encodes a high-mobility group (HMG) protein (**Figure 1-5**). Moreover, other genes such as *MAT1-1-2, MAT 1-1-3*, can be detected in the *MAT* idiomorph locus of certain ascomycete species (Coppin *et al.*, 1997b; Pöggeler, 2001). As with the *S. cerevisiae* yeast model, one key role of the *MAT* genes is to regulate the production of pheromones, which are diffusible mating-type-specific substances produced in one individual that elicit a response in individuals of opposite mating type due to the presence of complementary pheromone receptor proteins, which are linked to a MAP kinase signalling cascade (Nelson, 1996; Wei *et al.*, 2003; Seo *et al.*, 2004). Mating-type genes have also been found in homothallic species. For example, a *MAT-1* α-domain homologue was identified by BLAST searching in the homothallic species *A. nidulans* (Dyer *et al.*, 2003) and was later shown to be functional (Paoletti *et al.*, 2007). *MAT1* and *MAT2*-specific genes have also been shown to be located in sequences in other homothallic ascomycete species (**Figure 1-5**). (Pöggeler *et al.*, 1997; Lin and Heitman, 2007) and it has been suggested that it is the presence of both types of mating-type gene within the same genome that allows a species to become self fertile (Paoletti *et al.*, 2007). As far as expression of genes related to sexuality is concerned, interestingly *MAT* gene expression has been found in both asexual *Alternaria* and *Fusarium* species (Arie *et al.*, 2000; Yun *et al.*,

2000). Analysis of a range of filamentous ascomycete species has shown that the position within the genome of the *MAT* locus is fairly well conserved, normally bordering a SLA2 and/or APN2 gene within the genome i.e. there is conserved synteny (**Figure 1-6**).

Nelson (1996) has pointed out some relative benefits of homothallism and heterothallism. These include the fact that heterothallism promotes the generation of new gene combinations as it involves obligate outcrossing. By contrast, homothallism can maintain linkage between favourable sets of genes that might be broken up during outcrossing and has significant biological benefits in allowing the production of sexual spores, that are often more resistant than vegetative cells to environmental stresses, without the need for a compatible mate.

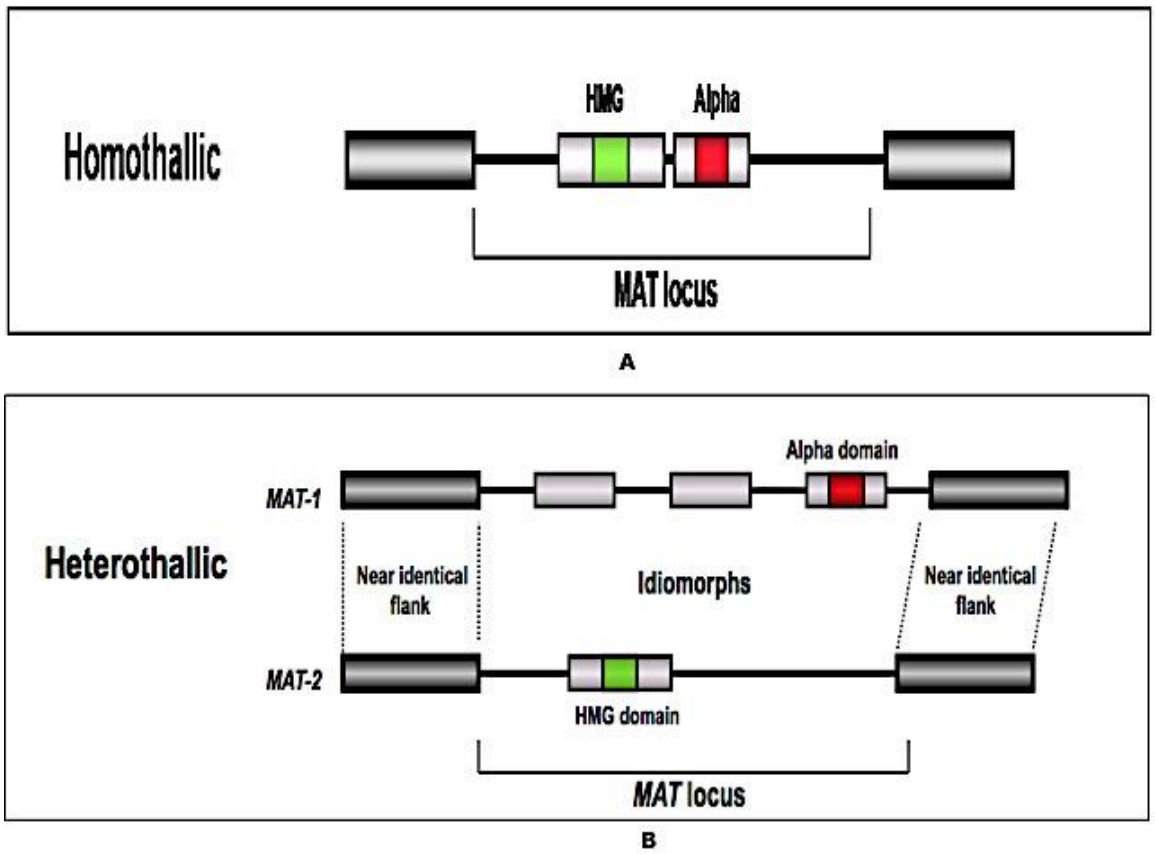

Figure 1-5 Different arrangements of *MAT* gene seen in filamentous ascomycetes resulting in (A) homothallic (self-fertile), or (B) heterothallic (self–sterile) breeding systems (Dyer, 2007).

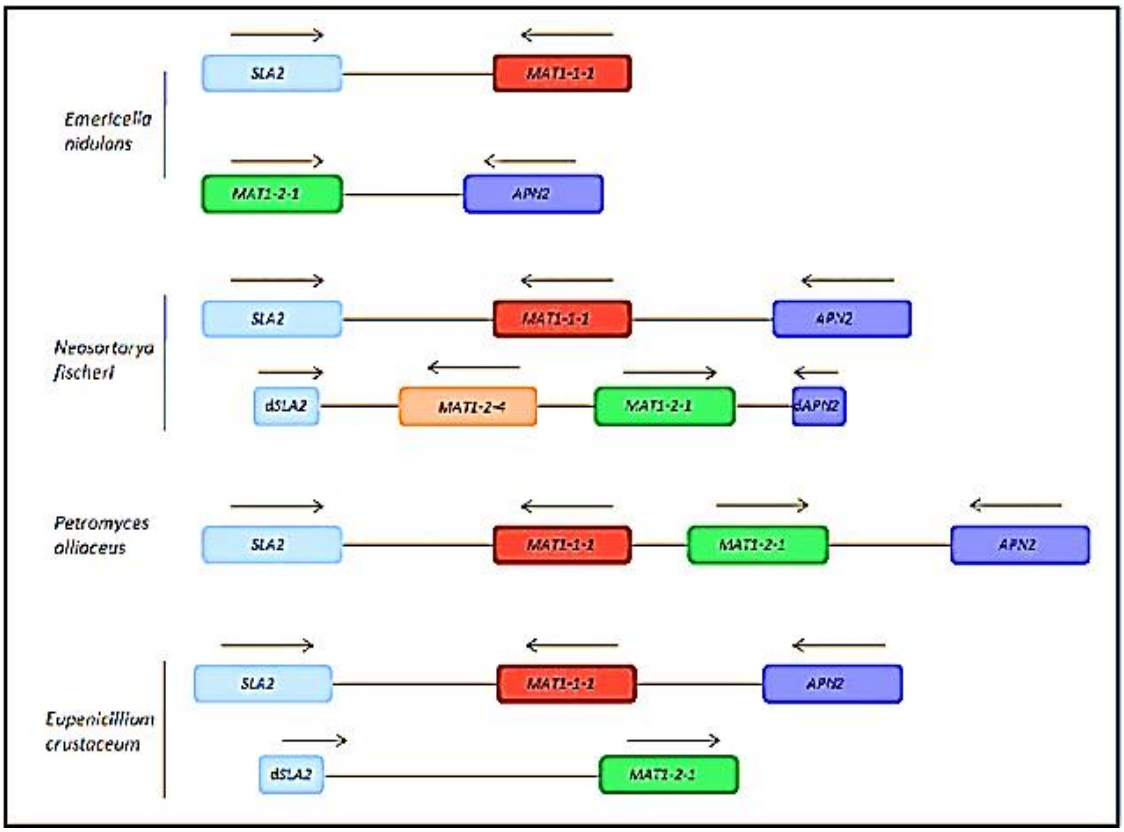

Figure 1-6 Organisation of *MAT* genes in the *MAT* loci of homothallic pezizomycete species: *E. nidulans*, *N. fischeri* and *E. crustaceum* have unlinked *MAT* loci, with the *MAT* genes appearing on different chromosomes, whereas *P. alliaceus* has a single *MAT* locus. Arrows indicate gene orientation. Prefix 'd' refers to a mutated partial ORF. [Adapted from (Rydholm *et al.*, 2007; Paoletti *et al.*, 2007; Ramirez-Prado *et al.*, 2008)].

1.3.4.1 Evolution of sex and the advantages and disadvantages of sexual reproduction:

"Sexuality in fungi has long been recognised as one of the more perplexing yet intriguing facets of the biology of this large and varied group of micro-organisms" [(Raper, 1966; Dyer and O'Gorman, 2012)]. The evolution of sex in fungi has been scientifically studied over many years to gain a better understanding of the evolutionary mystery of sexual development. Whitton *et al.* (2008), Otto (2009) and Schoustra *et*

al. (2010) have argued that most eukaryotes should reproduce sexually at least occasionally as part of their life cycle. Sexuality is considered to be evolutionary the ancestral state in fungi and has been maintained in the majority of species, despite the fact that there are many fitness costs related to sexual reproduction and that it requires many generations to overcome such expenses (West *et al.*, 1999; Agrawal, 2001; Schoustra *et al.*, 2010; Scharnweber *et al.*, 2011).

Sexuality in fungi has many benefits to the species involved. One of these advantages is that, in the case of outbreeding, it allows production of a range of recombinant offspring meaning that useful mutations can be maintained whilst harmful ones can be deleted (Milgroom, 1996; Hurst and Peck, 1996; Kück and Pöggeler, 2009). This can result in production of some progeny with higher fitness (Stearn, 1987; Otto and Lenormand, 2002). Thus, sexual recombination contributes to increased genetic diversity and likely survival in adverse or changing environmental conditions (Barton and Charlesworth, 1998; Goddard *et al.*, 2005). The fact that sex increases genetic variation has been described in the 'lottery hypothesis' of Williams (1975), who hypothesised that asexuality might be analogous to purchasing a huge number of tickets to win a lottery only to find that all tickets were of the same number, whereas sexuality allowed purchase of a much smaller number of tickets but at least these were all of a different number so increasing the opportunity of winning accordingly (Harrub and Thompson, 2004). Related to this generation of novel genetic diversity, sexual recombination can enable species to evolve in order to overcome the resistance of host organisms and other external factors (Maor and Shirasu, 2005). This ability to generate increased variation through recombination might be particularly significant in pathogenic fungi (Dyer and Paoletti, 2005) and the 'Red Queen' hypothesis illuminates the importance of sex in the evolutionary cycle between hosts and parasites (Salathe *et al.*, 2009). Furthermore, sexual reproduction can prevent the accumulation of deleterious mutations that may occur in purely asexual species as described by the phenomenon of 'Muller's Ratchet' (Soderberg and Berg, 2011), and can

overcome the accumulation of potentially deleterious hitchhiking genes (Chun and Fay, 2011). In addition, sex rapidly produces novel gene combinations, whereas asexual species require numerous successive mutations (Crow, 1994).

It is noted that the presence of sexual reproduction also provides benefits for mankind. For example, the sexual cycle is a useful laboratory tool for classical genetical analysis allowing researchers to determine the genetic basis (whether monogenic or polygenic) of traits of interest. There is the great example of Dyer *et al.* (2000) who determined the genetic basis of resistance to triazole fungicides in plant pathogenic *Tapesia* species by analysis of sexual progeny. In addition, genetic crosses can be used for strain improvement purposes for species that have a fundamental role in industry and biotechnology such as shown in recent work with *Penicillium chrysogenum* (Böhm *et al.*, 2013). The sexual cycle can also be used to distinguish between very closely related species such as *A. flavus* and *A. oryzae*, and *A. fumigatus* and *A. lentulus,* which are otherwise very similar in morphology but mating is not possible between species (Ramirez-Prado *et al.*, 2008; Swilaiman *et al.*, 2013). Similarly, *A. fischeri* and *A*. *fumigatus* are phylogenetically almost identical, but they have different sexual breeding systems with *A. fischeri* being homothallic, while its sister *A*. *fumigatus* is heterothallic (Peterson, 2000; Tamura *et al.*, 2000; Udagawa and Uchiyama, 2002). Additionally, certain aspects of molecular investigations such as gene complementation following gene disruption are easier and faster in sexual species than asexual species (Sharon *et al.*, 1996; Dyer *et al.*, 2000; Grosse and Krappmann, 2008; Pyrzak *et al.*, 2008). On the other hand, there are many costs of sex. Outbreeding sexual species must find an opposite partner and attract it, which is both energetically costly and involves passing up opportunities to gain resources, thus a life history trade-off (Reznick, 1983; Otto, 2009). Also whereas asexual species transmit 100% of their genes to their offspring, in comparison outcrossing sexual parents only transmit 50% (part of the '2-fold' cost of sex) (Bell, 1982). Moreover, sexual reproduction breaks down the beneficial combinations of genes that built

within past selection (Agrawal, 2006; Otto, 2009). Thus there is thought to be an evolutionary trend away from sex in some pathogenic fungi such as *Fusarium sp*. (Yang *et al.*, 2011) in order to prevent the loss of pathogenic genes. Furthermore, sexual development can take a far longer period than asexual development. A clear example is the case of *Sacchromyces cerevisiae* that undergoes mitotic replication in 90 min, but needs several days for meiosis to occur (Otto, 2009). Thus, overall there seems to be an evolutionary balance between factors favouring asexual and sexual reproduction. One recent study intriguingly found that the fitness of a species in certain environmental conditions is a key factor in the evolution and maintenance of a sexual cycle, with disproportionately more resources allocated to sex when fitness is low. This is termed fitness-associated-sex (abbreviated FAS) (Schoustra *et al.*, 2010).

1.3.5 The genus *Aspergillus*

Species of the genus *Aspergillus* are to be the main models for experimental work in the present study. This genus was chosen both due to its economic and medical importance and also its versatility as an amenable laboratory organism. *Aspergillus* is one of largest genera of the Ascomycotina, It comprises at least 250 species (Geiser *et al.*, 2007). The genus *Aspergillus* is characterised by the production of aseptate conidiophores with a vesicle that produces two layers of cells termed metulae and phialides (sterigmata). These cells finally produce conidiospores (**Figure 1-7**).

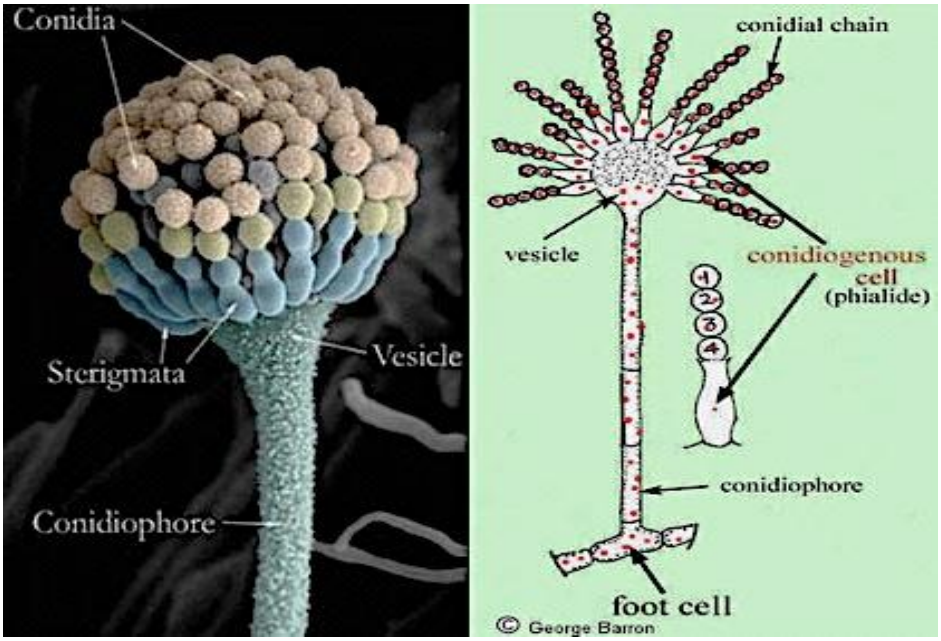

Figure 1-7 Morphology of the asexual structure of the genus *Aspergillus*.(http://www.uoguelph.ca/~gbarron/2008/parasex.htm).

According to Hibbett *et al.* (2007) the genus *Aspergillus* is classified as follows.

Kingdom: Fungi

Subkingdom:Dikarya

Phylum: Ascomycota

Subphylum: pezizomycotina

Class: Eurotiomycetes

Subclass: Eurotiomycetidae

Order: Eurotiales

Family: mitosporic Trichocomaceae

Genus: *Aspergillus*

Although the majority of species are only known to reproduce by asexual means, the genus nevertheless includes more than 70 teleomorphic species divided into homothallic and heterothallic taxa. Samson *et al.* (2007) placed the teleomorphic species into several different taxanomic

genera, these being namely *Neosartorya, Petromyces, Hemicarpenteles, Dichlaena, Eurotium, Warcupiella, Chaetosartorya, Emericella, Fennellia* and *Neopetromyces.*

The most common teleomorphic genera in terms of number of species are *Eurotium*, *Emericella*, *Neosartorya*, and *Petromyces*. By convention the anamorphic and teleomorphic states have different names. For example, *Aspergillus glaucus* is the asexual (anamorphic) state of the sexual (teleomorphic state) species *Eurotium herbariorum*. The vast majority of *Aspergillus* sexual species are homothallic, with only nine heterothallic species described so far, namely *Neosartorya fennelliae* (Kwon and Kim, 1974), *N. spathulata* (Takada and Udagawa, 1985), *N. udagawae* (Horie *et al.*, 1995), *N. nishimurae* (Takada *et al.*, 2001), *Emericella heterothallica* (Kwon *et al.*, 1964; Samson *et al.*, 2007), *N. fumigata* (O'Gorman *et al.*, 2009), *N. lentulus* (Swilaiman *et al.,* 2013)], and two *Petromyces* species, *P. flavus* and *P. parasiticus* (Horn *et al.*, 2009a; Horn *et al.*, 2009b).

1.3.5.1 The importance of *Aspergillus* species

The genus *Aspergillus* contains many species that have a significant economic and scientific impact in different areas, including the agricultural, medical and industrial sectors.

Some *Aspergillus* species are being used in a beneficial way to produce enzymes and biochemical materials in the biotechnological and food industries. For example, *A. niger* is used in the manufacture of citric acid that is a very important organic acid used as a flavour in many soft drinks and food (Bennett, 2009). Many enzymes are also derived from *Aspergillus* species such as pectinases, phytase, amylase and proteinases that are commercially important in the production and improvement of bread, beer, confections, juices, wine and other foods (Magnuson and Lasure, 2004; Bentley and Bennett, 2007). Moreover, there are several species that are important in food industry, which are used in fungal fermentations due to their ability to secrete enzymes into a substrate resulting in new food or drink products e.g. *A. sojae* and *A. oryzae* that

can make soy sauce, miso (soy bean paste) and sake (rice wine); these species well known 'koji molds' in Japan (Takamine, 1894). Some *Aspergillus* species also have health benefits such as *A. terreus,* which produces a fungal secondary metabolite called Lovastatin that has been used to treat hypercholesterolemia (Albers-Schonberg *et al.*, 1980; Alberts, 1998). Additionally, *A. nidulans* is a valuable laboratory model organism that was first selected by Pontecorvo and others many years ago (Pontecorovo *et al.*, 1994). This species is being used in many fundamental investigations such as studying gene expression, analysis of the mitotic cycle, and action of cellular motors (Keller *et al.*, 2005; Goldman and Osmani, 2008). This species has two separate names: *Aspergillus nidulans* as an anamorphic name and *Emericella nidulans* as a teleomorphic state (Bennett, 2009).

On the other hand, the aspergilli include many species that have a detrimental effect. Some species can cause serious plant diseases and food spoilage e.g. *Aspergillus parasiticus* and *Aspergillus flavus*. The latter species produces 'aflatoxins', which are carcinogenic chemicals causing harmful effects to animal and human health (Eaton *et al.*, 1994). These species contaminate several agricultural crops due to their ability to grow widely on various ranges of crops and cereals. A further species also known as a crop contaminant is *A. clavatus*, which can cause health problems to malt farmers due to allergic reactions (Grant *et al.*, 1976; Blyth, 1978; Latgé, 1999). In addition, some species of *Aspergillus* have the ability to cause a range of serious diseases in humans and animals. Perhaps the best-known and most dangerous species is *A. fumigatus,* which can cause opportunistic infections that are generally termed 'aspergillosis'. There are three different types of this diseases recognised in humans: allergic bronchopulmonary aspergillosis, aspergilloma or fungus ball and invasive aspergillosis (Vanden Bossche *et al.*, 1988; Calderone *et al.*, 2002). It is also can attack the respiratory system of birds and dogs, causing pulmonary *Aspergillus* infection (Tell, 2005). In addition to *A*. *fumigatus,* there are two other species of aspergilli that are commonly identified as medical pathogens; *A. flavus* which causes an eye

infection (Hedayati *et al.*, 2007), and *A. niger* that causes an ear infection as it is commonly found on ear wax (Ozcan *et al.*, 2003).

Given their medical and economic importance, *Aspergillus* species were amongst the first filamentous fungi to be genome sequenced with analysis of the genomes of *A. nidulans, A. fumigatus* and *A. oryzae* providing important evolutionary insights into the genus (Galagan *et al.*, 2005). For example it became apparent that *A. fumigatus* and *A. oryzae* were sister taxa, whereas *A. nidulans* branched phylogenetically much earlier from them.

1.3.6 Sexual reproduction in *Aspergillus* species

The aspergilli is a valuable model group for studying the evolution and control of sexual reproduction in fungi because it includes a series of species with different modes of productivity. These include asexual ('mitosporic') species that generate only asexual spores, and also other species that can additionally form sexual spores ('meiosporic') which have either heterothallic (self-sterile) or homothallic (self-fertile) breeding systems (Dyer and O'Gorman, 2012).

The teleomorphs of the sexual species are distinguished by their cleistothecial morphology, for example the composition and colour of the cleistothecial wall (**Figure 1-8** and **Table 1-1**). (Benjamin, 1955; Geiser, 2008; Dyer and O'Gorman, 2012). The nature of ornamentation of ascospores has also been found to be a unique taxonomical character for some teleomorphic genera within aspergilli (Samson and Varga, 2010). Only in the genus *Fennellia* has a unique structure of ascogonia (female gametangium) and antheridia (male gametangia) been shown amongst the aspergilli (Geiser, 2008).

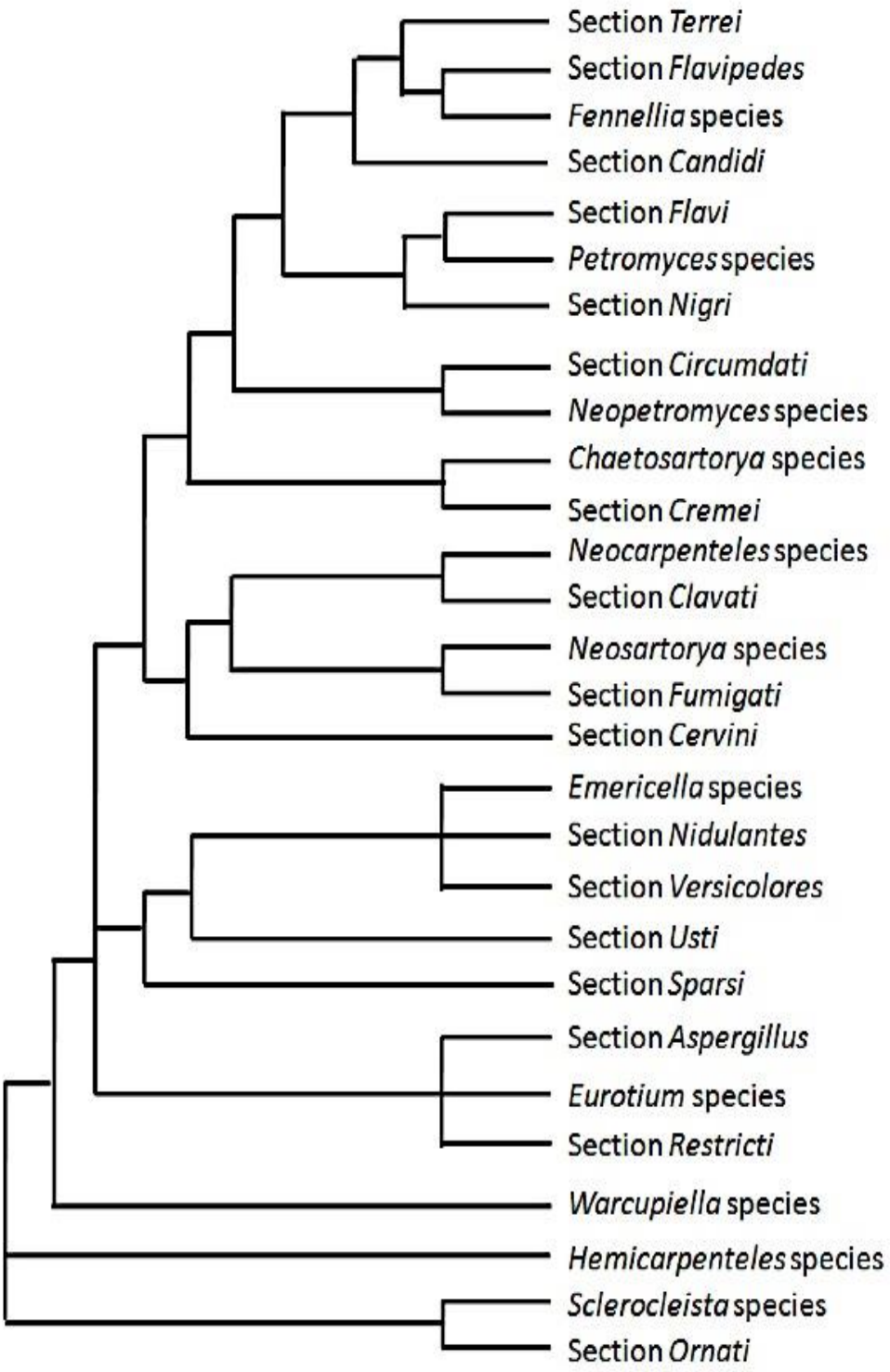

Figure 1-8 Phylogeny of the genus *Aspergillus*, with its 11 associated teleomorphic genera. [Adapted from (Frisvad and Samson, 2000; Tamura *et al.*, 2000; Varga *et al.*, 2000; Udagawa and Uchiyama, 2002; Samson *et al.*, 2007; Peterson, 2008)].

Table 1-1 Taxonomical characteristics of four representative teleomorphic genera as cited in (Dyer and O'Gorman, 2012).

Teleomorph	Description of cleistothecial wall	Example
Eurotium	A single layer of frequently yellow, large flattened cells	*Eurotium repens*
Emericella	Two layers of often dark purple flattened cells	*Emericella nidulans*
Neosartorya	A network of layers of interlocking hyphae that are normally white to light yellow in colour	*Neosartorya fischeri*
Petromyces	Irregularly dark-walled flattened cells	*Petromyces flavus*

The cleistothecia of *Aspergillus spp.* can contain up to approximately 100,000 asci with 8 ascospores in each of these. Around 80,000 viable ascospores have been estimated to be present in typical cleistothecia of *A. nidulans* (Pontecorvo *et al.*, 1953; Braus *et al.*, 2002). Ascospores are released after the peridium (outer wall) breaks down, and are then dispersed into the surrounding environment, which may be soil or decomposing organic matter. *A. athecius* is an exception of that. It is unable to form true cleistothecia. Therefore asci develop directly from ascogonial coils and borne naked on undifferentiated mycelium (Raper and Fennell, 1965).

It has become evident that nuclei from one mating partner is normally wholly involved with formation of maternal tissues such as the accessory tissues and peridium wall, whereas the compatible nucleus of the mating partner is only involved in formation of the ascogenous hyphae and

ultimately the diploid ascus mother cell (Apirion, 1963; Zonneveld, 1988a; Zonneveld, 1988b; Bruggeman *et al.*, 2003b; Todd *et al.*, 2006). However, in the case of *A. nidulans* the nuclei of both partners have infrequently been shown to contribute to the cleistothecium wall, such as in the specialised instance of sex developing from a pre-formed heterokaryotic mycelium (Bruggeman *et al.*, 2003a).

As mentioned, a variety of different sexual morphological pathways are evident in the aspergilli. Hülle cells are only present in *Emericella* species (teleomorphic of *A. nidulans*) (Raper and Fennell, 1965). Hülle cells have been termed as a 'cleistothecial nurse', having large, thick walled, globose cells that surround the cleistothecia during development. These apparently serve to provide some metabolic requirements for cleistothecial development such as the production of α-1,3 glucanase which mobilises carbon resources required for fruiting body development (Wei *et al.*, 2001). Bayram *et al.* (2010) highlighted that any decrease in a number of the Hülle cells also resulted in the generation of smaller cleistothecia. Hülle cells have been shown to present in some putatively asexual species in the sections *Nidulantes* (e.g. *A. multicolour, A. subsessilis, A. sydowii*) and *Usti* (e.g. *A. puniceus*) of the subgenus *Nidulantes* (Raper and Fennell, 1965; Klich and Voor Schimmelcultures, 2002). This might be explained in evolutionary terms by the fact that these supposedly asexual species lost their sexuality over time and these Hülle cells are now remnants of lost sexuality. Likewise, Mcalpin (2004); Samson *et al.* (2004b) state those sclerotia are produced in some putatively asexual species in sections including *Flavi* (e.g. *A. caelatus*) and *Nigri* (e.g. *A. sclerotioniger*). Production of sclerotia has previously been thought to provide an evolutionary benefit as they have the ability to survive harsh conditions. But, under the appropriate conditions sclerotia have been suggested to act as repositories for cleistothecia (Rai *et al.*, 1967; Geiser *et al.*, 1998b). Considering the morphology of sex as a whole, there are at least two different developmental pathways involved with sex. Firstly, a pathway relating to production of cleistothecia and ascospores. Secondly, one involved in generating

secondary tissues that support sexuality including Hülle cells and sclerotia (Dyer and O'Gorman, 2012). These pathways have been speculated to normally work in synchrony. However, there are exceptions e.g. *A. multicolour* and *A. sclerotioniger* which are able to independently form accessory tissues of sex, whilst certain mutants of *A. nidulans* have the capability to form high number of Hülle cells although meiosis and ascospore development are absent. Indeed, species of the genus *Petromyces* can produce single to multiple cleistothecia, each with a distinct peridium within a single sclerotium.

1.3.6.1 Genes involved with sexual development in the aspergilli

The genus *Aspergillus* provides particularly a good experimental source for studies concerning sexual reproduction because genome species of at least 14 species are now publically available (www.aspgd.org website) in addition to those initially released (Archer and Dyer, 2004; Galagan *et al.*, 2005), and therefore much genetic information is currently available. Also, there have been a series of studies into sexual reproduction providing insights into the basis of homothallism, heterothallism and asexuality in the aspergilli (Paoletti *et al.*, 2005; Paoletti *et al.*, 2007; Horn *et al.*, 2009a; Horn *et al.*, 2009b).

Various aspects of sexual reproduction of *Aspergillus* species in general have been reviewed by (Dyer, 2007; Geiser, 2008; Dyer and O'Gorman, 2011). In addition, Braus *et al.* (2002); Han *et al.* (2008); Han (2009); Han (2010) have reviewed sexual reproduction specifically in *A. nidulans*, which is a model fungal species that has been widely studied because the sexual cycle of this homothallic species can be easily induced in vitro and which has genome sequence data available and a variety of tools allowing genetic manipulation (Archer and Dyer, 2004; Galagan *et al.*, 2005; Todd *et al.*, 2007). The reader is referred to these reviews for full details of the spectrum of genes involved with control of sexual reproduction in the aspergilli. But in summary, so far around 75 'sex-related' genes have been identified as being vital for sex to occur, or important in

determining levels of sexual fertility. These genes have been identified from studies primarily involving *A. nidulans,* but more recently also from studies involving *A. fumigatus, A. flavus* and *A. parasiticus* (Dyer and O'Gorman, 2011). A schematic genetic network showing relationships between the various genes has been proposed (**Figure 1-9)** (Dyer and O'Gorman, 2012). These 'sex-related' genes include groups of genes involved with environmental sensing of suitable conditions for sex. These have two functions: activating the early stages of sexual development and upregulating genes controlling mating process (Dyer and O'Gorman, 2012). Further sets of genes involved with mating, fruit body and ascospore development have also be described (Dyer and O'Gorman, 2011). Furthermore a series of genes regulating general metabolism needed for sex have been identified including genes for arginine, histidine, tryptophan, riboflavin, phosphatidylcholine biosynthesis and sumoylation (Champe *et al.*, 1994; Todd *et al.*, 2007; Bayram *et al.*, 2010; Tao *et al.*, 2010). Finally genes involved with certain physiological processes relating to sex have been described. For example, it has been reported that a precocious sexual inducer (psi) is produced by *A. nidulans* which enhances sexual reproduction and reduces asexual reproduction (Champe and El-Zayat, 1989). This is thought to be similar to other lipoidal sexual factors (sexual morphogens) produced by the plant pathogen *Pyrenopeziza brassicae* (Ilott *et al.*, 1986; Siddiq *et al.*, 1989; Siddiq *et al.*, 1992). Much has now been learnt about the genes involved with production of Psi factors (Dyer and O'Gorman, 2011).

Although many aspects of sexual development are now understood in *Aspergillus* species, there still remain many 'mysteries of sex' to be revealed with much remaining to be discovered.

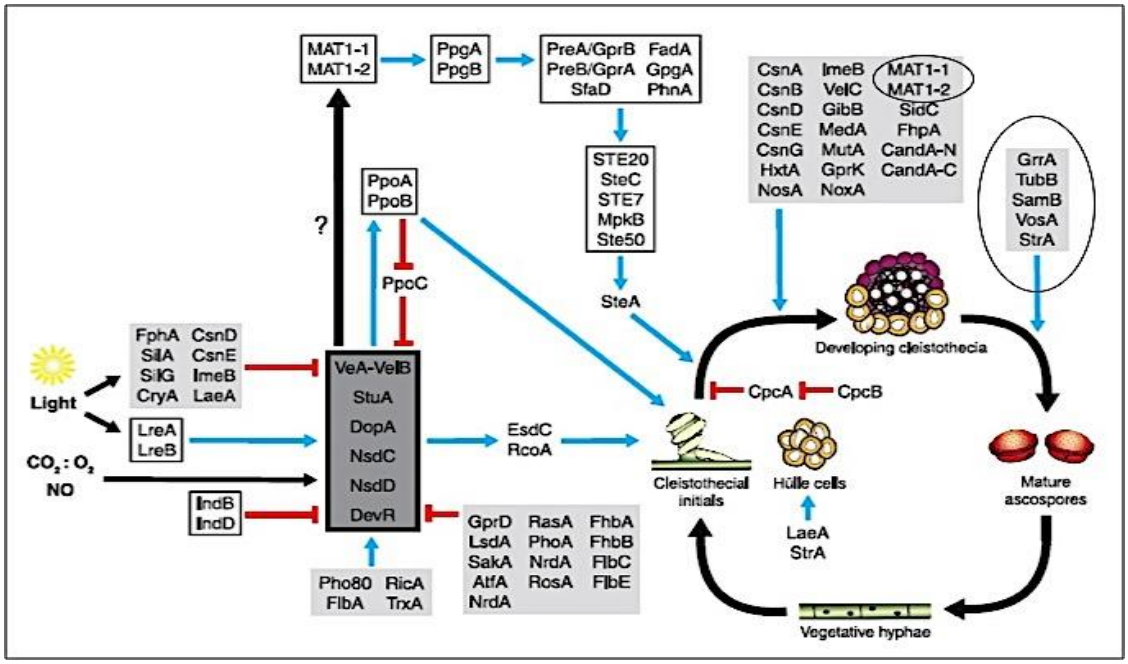

Figure 1-9 A schematic network which displays the genes which have a suggested link in orchestrating particular sections of the Aspergilli's sexual reproduction. Most studies, from which the results came, focussed on *A. nidulans*. The interacting proteins are boxed. Blue arrows: gene activation. Red inhibition lines: gene repression. [Adapted from Dyer and O'Gorman (2012)].

1.3.7 Aims of Thesis

The overall aim of this study is to investigate physiological and genetic factors that influence the evolution and control of sexual reproduction in *Aspergillus* species, in order to better understand the processes governing sexual and asexual reproduction in ascomycete fungi as a whole.

The specific objectives of this study were:

1. To establish a culture collection of suitable species and isolates for study, and to optimise growth conditions to allow reliable induction of both sexual and asexual reproduction in the target study species to facilitate later experimental work.

2. To determine the possible benefits of sexual reproduction in terms of survival of different types of spores. There is accumulating evidence that sexual ascospores exhibit greater resistance to adverse environment conditions than asexual conidia. This might explain in part why homothallic sexual reproduction is retained in otherwise asexual species.
3. To study the possible evolution of asexuality. Classical studies have suggested that sexual reproduction and fertility can be lost when sexual species are sub-cultured repeatedly in the laboratory solely by asexual transfer. But how rapidly does this occur and what genetic changes might occur as result?
4. To identify novel genes involved in regulation of sexual fertility. Ongoing genomic/molecular studies have identified over 50 genes that are essential for sexual reproduction. But over 50% of these genes are listed of 'unknown' function in genomic databases. It is therefore possible that many other so far undescribed genes might be involved with controlling sexual reproduction in ascomycete fungi. By using a series of gene deletion cassettes from a genomic resource in the USA, it was hoped to identify such new gene(s) that might be involved with sexual reproduction.

Three aspergilli, namely *N. fischeri*, *A. nidulans*, and *E. repens,* were chosen as model species for these investigations. This was because they are all amenable to laboratory culture; genomic data is available for the first two species; the classic work of Jinks (1954) and Mather and Jinks (1958) was performed with the latter two species (see section **5.1.2**); and a series of gene disruption cassettes recently became available to facilitate gene knockout in *A. nidulans.*

It was hoped, and anticipated, that results gained in the present study would give insights into the evolution of sex and asexuality in ascomycete fungi. This should provide both fundamental and applied scientific knowledge, of use for instance in further studies to induce sexuality in supposed 'asexual' species.

Chapter 2 Materials and Methods

General materials and methods used throughout the present study are described in the following chapter. Materials and methods specific only to individual chapters will be described in the relevant chapter.

2.1 Materials

2.1.1 Media

2.1.1.1 *Aspergillus* complete media (ACM) (for 1L)

10 g glucose powder (Sigma, UK), 1 g yeast extract powder (Oxoid, UK), 2 g peptone (Oxoid, UK), 1 g casamino acids (Sigma, UK), 0.075 g adenine (Sigma, UK), 10 ml *Aspergillus* vitamin solution (section **2.1.2.1**), 20 ml *Aspergillus* salt solution (section **2.1.2.2**) and 20 g agar (Oxoid) were dissolved fully into 900 ml distilled water (dH_2O) and adjusted to pH 6.5, then the solution was made up to the final volume of 1 L with distilled water and autoclaved at 117°C for 30 min. This media is as described by Paoletti *et al.* (2005).

2.1.1.2 Malt extract agar (MEA) (for 1L)

20 g malt extract powder (Sigma, UK), 1 g peptone (Oxoid, UK) and 20 g agar (Oxoid, UK) were dissolved into 900 ml distilled water and then the solution was made up to the final volume of 1 L with distilled water and autoclaved at 117°C for 30 min. MEA + 20% sucrose was used to grow *Eurotium repens* (Raper and Fennell, 1965) .

2.1.1.3 Yeast extract glucose media (YEG)

8 g of yeast extract powder (Oxoid, UK), 40 g glucose powder (Sigma, UK) were dissolved into 900 ml of distilled water. The solution was made up to the final volume of 1 L with distilled water and autoclaved at 117°C for 30 min.

2.1.1.4 Water agar media

40 g agar (Oxoid, UK) was dissolved into 900 ml distilled water. The solution was made up to the final volume of 1 L and autoclaved at 121°C for 30 min.

2.1.1.5 CaZpek dox agar (CDA)

45.5 g of caZpek dox agar was suspended in 1 L of distilled water, boiled to dissolve completely, adjusted pH to 6.8 and autoclaved at 121°C for 15 min. CaZpek dox agar + 20% sucrose was used to grow *Eurotium repens*.

2.1.1.6 Tap water agar (TWA)

20 g agar powder (Oxoid) was dissolved into 980 ml tap water and adjusted pH to 6.5. The solution was made up to the final volume of 1 L and autoclaved at 121°C for 30 min.

2.1.1.7 M40Y

400 g of sucrose (Sigma, UK), 20 g of malt extract powder (Sigma,UK), 5 g of yeast extract powder (Oxoid, UK), 20 g agar (Oxoid, UK) were dissolved into 800 ml distilled water and adjusted to pH 6.5, then the solution was made up to the final volume of 1 L with distilled water and autoclaved at 117°C for 30 min. This media was found to enhance sexual reproduction in *Eurotium spp.* (section **3.4.2.2**).

2.1.1.8 Glucose malt extract agar (GMEA)

300 g glucose powder (Sigma, UK), 20 g agar (Oxoid, UK), 20 g malt extract powder (Sigma, UK) were dissolved into 900 ml distilled water, then the solution was made up to the final volume of 1 L with distilled water and autoclaved at 117°C for 30 min (Blaser, 1975). This media was used to grow *Eurotium repens*.

2.1.2 Buffers and Solutions

2.1.2.1 *Aspergillus* vitamin solution (for 1L)

400 mg of p-Aminobenzoic acid, 50 mg of thiamine HCl (Sigma, UK), 2 mg of d-Biotin (Sigma, UK), 100 mg of nicotinic acid (Sigma, UK), 250 mg of pyridoxine hydrochloride (Sigma, UK), 1.4 g of choline chloride (Sigma, UK) and 100 mg of riboflavin (Sigma, UK) were dissolved and mixed together into 900 ml ddH_2O, and then the solution was made up to the final volume of 1 L with distilled water (Paoletti *et al.*, 2005).

2.1.2.2 *Aspergillus* salt solution (for 1L)

26 g of potassium chloride (VWR International, UK), 26 g of magnesium sulphate (Fisher, UK), 76 g of potassium di hydrogen phosphate (Fisher, UK) and 10 ml *Aspergillus* trace elements solution were dissolved and made up to the final volume of 1 L with distilled water (Paoletti *et al.*, 2005).

2.1.2.3 2M Tris

48.46 g of tris (Tris-hydroxymethyl-methylamine or TRIZMA) was dissolved into 120 ml distilled water using magnetic stirrer, and then the solution was made up to the final volume of 200 ml.

2.1.2.4 2M HCl

16.95 ml of concentrated HCl (11.8 M) was added to 83.05 ml of distilled water, mixed and then made up to the final volume of 100 ml.

2.1.2.5 pH 8.5 Tris-HCl 1 Molar

100 ml of 2 M Tris solution (section **2.1.2.3**) was added to 29.4 ml of 2 M HCl (section **2.1.2.4**), stirred thoroughly, allowed to cool down to the room temperature, then further 2 M of HCl was added with stirring using magnetic bar as required to the final pH of 8.5 (allowed to cool if necessary). The solution was diluted to the final volume of 200 ml with distilled water and autoclaved.

2.1.2.6 pH 8.0 Tris-HCl 1 Molar

100 ml of 2 M Tris solution (section **2.1.2.3**) was added to 58.4 ml of 2 M HCl (section **2.1.2.4**), stirred thoroughly, allowed to cool down to the room temperature, further 2 M of HCl was added with stirring by magnetic bar as required to the final pH of 8.0 (allowed to cool when necessary). The solution was diluted to the final volume of 200 ml with distilled water and autoclaved.

2.1.2.7 pH 7.5 Tris-HCl 1 Molar

100 ml of 2 M of Tris solution (section **2.1.2.3**) was added to 80.6 ml of 2 M HCl (section **2.1.2.4**), stirred thoroughly, allowed to cool to room temperature, further 2 M of HCl was added with stirring with magnetic bar as required to the final pH of 7.5 (allowed to cool when necessary). The solution was diluted to the final volume of 200 ml with distilled water and autoclaved.

2.1.2.8 Potassium phosphate buffer (1 Litre)

14.03 g of 1 M K_2HPO_4 was dissolved into 61.5 ml distilled water, also 6.81 g of 1 M KH_2PO_4 was separately dissolved into 50 ml of distilled water (dH_2O), then 38.5 ml from the first solution (1 M KH_2PO_4) was added to the 61.5 ml of the second solution (1 M K_2HPO_4), further 1 M KH_2PO_4 was then added dropwise until pH 7. Finally the solution was made up to the final volume of 1000 ml with distilled water and autoclaved.

2.1.2.9 Potassium phosphate buffer (1.6Litre)

22.45 g of 1 Molar K_2HPO_4 was dissolved into 98.5 ml of distilled water, also 10.89 g of 1 Molar KH_2PO_4 was dissolved into 80 ml of distilled water, then 61.6 ml from the first solution (1 M KH_2PO_4) was added to the 98.5 ml of the second solution (1 M K_2HPO_4), further 1 M KH_2PO_4 was then added dropwise until pH 7. Finally the solution was made up to 1600 ml with distilled water (dH_2O), then divided into 2 x 800 ml bottles and autoclaved.

2.1.2.10 Sodium phosphate buffer (0.1 Molar)

17.9 g of Na_2HPO_4 was dissolved into 200 ml of distilled water; also 6.9 g of NaH_2PO_4 was dissolved into a separate 200 ml of distilled water. Both of these substances were fully dissolved using a magnetic stirrer, then 24.6 ml from the first solution (Na_2HPO solution) and 175.4 ml from the second solution (NaH_2PO_4 solution) were mixed together, then 5.0 ml of concentrated Tween was also added to this solution and mixed up thoroughly. This mixture was made up to the final volume of 500 ml with distilled water.

2.1.2.11 EDTA (Disodium Ethylene Diamine Tetraaceticacid) [500 mM solution (0.5M) of pH8 solution]

37.22 g of EDTA and 4 g of NaOH pellets were dissolved completely into 160 ml distilled water using stirrer bar and stir, allowed to cool down to the room temperature, then the pH was adjusted to 8.0 by adding drops of 2 M NaOH. The solution finally was diluted to the final volume of 200 ml with distilled water and autoclaved.

2.1.2.12 TE buffer

1 ml of 1 M Tris-HCl pH 8.0 (section **2.1.2.6**) was mixed up with approximately 80 ml distilled water, also 400 µl of 250 mM EDTA pH 8 (section **2.1.2.11**) was added to this mixture, then the solution was made up to the final volume of 100 ml with distilled water and autoclaved.

2.1.2.13 DNA extraction buffer

25 ml of 1 M Tris-HCl pH 8.5 (section **2.1.2.5**), 25 ml of 1 M NaCl, 10 ml of 250 mM EDTA pH 8.0 (section **2.1.2.11**), 5 ml of 10% SDS pH 7.2 were dispensed into 35 ml distilled water, mixed up thoroughly, then made up to the final volume of 100 ml and autoclaved (Murtagh *et al.*, 1999).

2.1.2.14 DNA gel loading buffer (SBX)

2.4 g sucrose, 15 mg xylene cyanol, 15 mg bromphenol blue were dissolved thoroughly into distilled water by shaking in pre-marked bijou bottle and made up to the final volume of 6 ml.

2.1.2.15 Lambda λDNA quantity markers

Stocks of 5, 10, 20, 40, 60, 100 ng lambda λDNA in a final volume of 8 µl were made by appropriate dilutions of a concentrated lambda λDNA stock solution which was mixed with TE buffer and gel loading buffer (section **2.1.2.14**), with through mixing by pipetting pump wash and vortex mixing at each stage.

2.1.2.16 100 bp and 1 kb bp DNA marker

DNA size ladders, both 100 bp and 1 kb bp were obtained from New England Biolabs (UK) and diluted according to manufacturer's instructions prior to use in gel electrophoresis with 5.0 µl normally loaded per gel.

2.2 Methods

2.2.1 Fungal strains (freeze-dried cultures)

The strains of *Aspergillus* species used in this study were obtained from different worldwide locations. Many were received as freeze dried (lyophilised) cultures supplied in a glass ampoule containing a dried pellet of fungal material, a piece of paper with a code number, and a cotton plug. These were removed aseptically and cultured on appropriate growth media. Sometimes it was necessary to aseptically pipette a little sterile water or malt peptone solution into the opened ampoule and gently stir with a sterile needle prior to removal of the culture should the lyophilsed culture adhere strongly to the side of the ampoule wall or the pellet did not crumble.

2.2.2 Culture growth on solid media

Cultures of *A. nidulans* and *N. fischeri* were grown on ACM both in Petri dishes (9 cm and 5 cm) and also universal slope tubes, while cultures of *E. repens* were grown on M40Y in both Petri dishes and universal slopes. A sterile swab was normally used to transfer a small amount of mycelia from a pre-existing culture, a silica stock or a liquid nitrogen stock for inocula as appropriate. Petri plate and tube cultures were routinely grown under the white light (2.5 µmol m^{-2} s^{-1}) for 7 d at 28°C after which point the plates were sealed with two layers of Nescofilm (BANDO CHEMICAL IND. LTD., Kobe Japan) before being stored at 4°C.

2.2.3 Long-term storage of cultures

Fungal isolates were stored long term either on dried silica gel at 4ºC or as cryovial stocks in 10% sterile glycerol solutions in liquid nitrogen (Johnston and Booth, 1983).

2.2.4 Culture growth in liquid media

500 µl of 0.01% Tween was added to universal slopes that had fresh fungal mycelia growth, and then spores were harvested gently with a sterile loop to make spore suspension. The resulting spore suspension was mixed thoroughly and filtered through sterile Miracloth (CALBIOCHEM, USA) before quantification by haemocytometer (if required), and was then transferred to 250 ml conical flasks containing 50 ml of yeast extract glucose media (YEG) (section **2.1.1.3**). Flasks were grown in the light at 28°C with shaking (200 rpm) for 5 d, after which point the resulting mycelium was strained through sterilised Miracloth and freeze-dried overnight. Lyophilised samples were then stored at - 80°C until DNA extraction was performed.

2.2.5 DNA extraction

DNA from each fungal strain or isolate of *A. nidulans*, *N. fischeri* and *E. repens* species in this study was extracted using either a DNeasy plant Mini kit (www.qiagen.com/products/catalog/sample.../dneasy-plant-mini-

kit) or DNA extraction-phenol chloroform procedure (PLG) [modified by (Murtagh *et al.*, 1999)] as follow:

2.2.5.1 A DNeasy plant Mini kit

A freeze dried of fungal mycelium (section **2.2.1**) was thoroughly ground thoroughly under liquid nitrogen in a mortar with a pestle, and then 100 mg of ground mycelia was suspended with 800 µl DNA extraction buffer (section **2.1.2.13**) in 2 ml Eppendorf tubes at room temperature, mixed well with a pipette tip or a glass rod and gentle inversion. Tubes were incubated in water bath at 65°C for 30 min with gently mixing and shaking by hand occasionally each 10 min during the time of incubation. Tubes then were centrifuged for 1 min at 20,000xg (14,000 rpm) and the supernatant removed. 130 µl of buffer AP2 was added to the lysate, mixed and incubated for 5 min on ice. The lysate was centrifuged for 5 min at 20,000xg (14,000 rpm) and pipetted into the QIAshredder Mini spin column placed in a 2 ml collection tube, then centrifuged for 2 min at 20,000xg (14,000 rpm). The flow-through fraction from the collection tube above was transferred into a new tube without disturbing the cell-debris pellet. 1.5 volumes of buffer AP3/E were added to the cleared lysate, and mixed by pipetting. For example, to 450 µl lysate, add 675 µl buffer AP3/E. 650 µl of the mixture from the last step including any precipitate that may had already formed were pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube, then centrifuged for 1 min at 8000 rpm, and the flow-through discarded. The DNeasy Mini spin column was placed into a new 2 ml collection tube and 500 µl of buffer AW was added and centrifuged for 1 min at 8000 rpm. The flow-through was discarded and reused the collection tube in next step. 500 µl of buffer AW was again added to the DNeasy Mini spin column, and centrifuged for 2 min at 20,000xg (14,000 rpm) to dry the membrane. The DNeasy Mini spin column was transferred into a 1.5 ml or 2 ml microcentrifuge tube and 100 µl buffer AE was added and pipetted directly onto the DNeasy membrane. The tubes were incubated for 5 min at room temperature (15–25°C), and centrifuged for 1 min at 8000 rpm to elute. The DNA yield Finally was transferred into new 1.5 ml Eppendorf

tubes that had been already labelled by all the information needed and stored at 4°C to use. The yield of extracted DNA was checked by gel electrophoresis in 1.2% agarose gels in 80 ml of 10 x Tris-Acetic acid-EDTA buffer solutions using 8 µl for each product included [1 µl DNA, 5 µl distilled water and 2 µl loading buffer (section **2.1.2.14**)] with standard stocks of 5, 10, 20, 40, 60, 100 ng lambda λDNA in 8 µl final volume as a control (section **2.1.2.15**), then the gel was stained after electrophoresis was completed. Finally, the gel was immersed in water contained ethidium bromide (0.5 µg/ml) for 15-20 min at room temperature (Brown, 1991).

2.2.5.2 DNA extraction-phenol chloroform procedure (PLG)

1 x 50 ml liquid cultures from each of these isolates were prepared and inoculated in yeast extract glucose media (YEG) (section **2.1.1.3**). DNA Extraction-Phenol Chloroform Procedure (PLG) with 2 ml Phase Lock Gel (PLG) tubes was used to extract DNA from all of these putative *N. fischeri* strains [modified by (Murtagh *et al.*, 1999)]. A freeze dried mycelium (section **2.2.1**) was thoroughly ground under liquid nitrogen in a sterilised mortar with a pestle, then 50 mg from a ground mycelium suspended in 500 µl DNA extraction buffer (section **2.1.2.13**) at room temperature in 2 ml phase lock gel (PLG) tubes that had already been centrifuged in advance for 30 sec at 12,000 rpm. The mixture was entirely mixed with a pipette tip or a glass rod and gently inverted. Tubes were then incubated on ice for 15-30 min with occasion shaking by hand during the time of incubation. DNA was purified with 500 µl of ice cold phenol:chloroform:isoamylalcohol (25:24:1) and mixed thoroughly by hand with gentle inversion for 2 min in a fume hood. Goggles/gloves were also worn for safety. The solution was centrifuged at 12,000 rpm for 10 min at 4°C and the upper (aqueous) phase was removed to a fresh PLG tube taking care not to disturb the interface. The lower phenol/ PLG phase was put into a waste phenol bottle and the supernatant was removed to a new tube. 10 µl RNAse (10 mg ml^{-1} stock) was added to the upper phase and incubated for 30 min at 37^{o}C, then 500 µl of ice cold chloroform:isoamyl alcohol (24:1 v/v) was added, mixed thoroughly,

centrifuged at 12,000 rpm for 10 min at 4ºC and the upper (aqueous) phase removed to a 1.5 ml tube. A 0.54 volume of isopropanol (-20°C from the freezer) was also added, mixed well by inversion and incubated for 40 min at -20°C to precipitate the DNA, then centrifuged at 12,000 rpm for 20 min at 4ºC and the supernatant discarded carefully retaining the pellet. The DNA pellet was washed twice with a cold 70% ethanol (-20ºC) with gentle vortexing, then centrifuged at 12,000 rpm for 5 min at 4ºC and the supernatant removed. The pellet was dried in room temperature for 10 min using a yellow pipette tip to remove the last drops of liquid. Lastly, the DNA was re-suspended overnight in 50 µl TE buffer (section **2.1.2.12**) at 4°C.

2.2.6 DNA purification

A Geneflow Q-Spin gel Extraction/PCR Purification Kit (QIAGEN, Sample and Assay Technologies (www.qiagen.com) was used to purify the DNA from PCR reaction following the manufacturer's instructions.

2.2.6.1 Gel electrophoresis

The yield of extracted DNA was checked by gel electrophoresis in 1.2% agarose gels in 80 ml of 10 x Tris-Acetic acid-EDTA buffer solutions using 8 µl for each product included [1 µl DNA, 5 µl distilled water and 2 µl loading buffer. The PCR products were estimated using a 1 kbp DNA ladder (New England Biolabs Inc., UK). Electrophoresis gels were running at V=120 and I=250 for 1.5 h at room temperature, before being stained for 20 min in 1 µl/ml ethidium bromide solution (10 mg/ml), after which bands were visualised using UV light and photographed using a BioRad Chemidoc XRS (Bio-Rad Laboratories, UK).

Chapter 3 Development of Model Systems for Studying Sexual Reproduction in *Aspergillus* species

3.1 Introduction

3.1.1 Factors affect sporulation in fungi

There are many environmental factors including temperature, pH, and nutrient availability which, have an effect on sporulation in fungi (Moore-Landecker, 1996). These factors can influence whether sexual or asexual reproduction occurs (Dyer *et al.*, 1992). Generally, asexual reproduction occurs over a wider range of conditions than sexual reproduction. However, the specific conditions for sexual development vary considerably between species, possibly related to the specific ecology of the species involved (Dyer and O'Gorman, 2012). Factors which affect sporulation in fungi can be divided into biotic and abiotic types (**Figure 3-1**). A brief overview will now be provided of these factors, with an especial mention on their impact on sporulation in *Aspergillus* species, the focus of the present study.

3.1.1.1 Biotic (physiological) factors

3.1.1.1.1 Pheromone and hormone effects

Sex hormones, including pheromones, can trigger the differentiation from an asexual or vegetative growth form to a sexual state (Gooday, 1983) For example, a lipid 'precocious sexual inducer' (psi factor) has been described from *Aspergillus nidulans,* which inhibits asexual reproduction and causes the premature development of fruiting bodies (Champe *et al.*, 1987). Similarly, secreted pheromones can inhibit asexual reproduction and trigger, for example, chemotroric growth of trichogynes linked to sexual morphogenesis. These pheromones, and their cognate receptors, have been detected in both heterothallic and homothallic species and are

normally under the control of *MAT* genes (Pöggeler and Kück, 2001; Lee *et al.*, 2008).

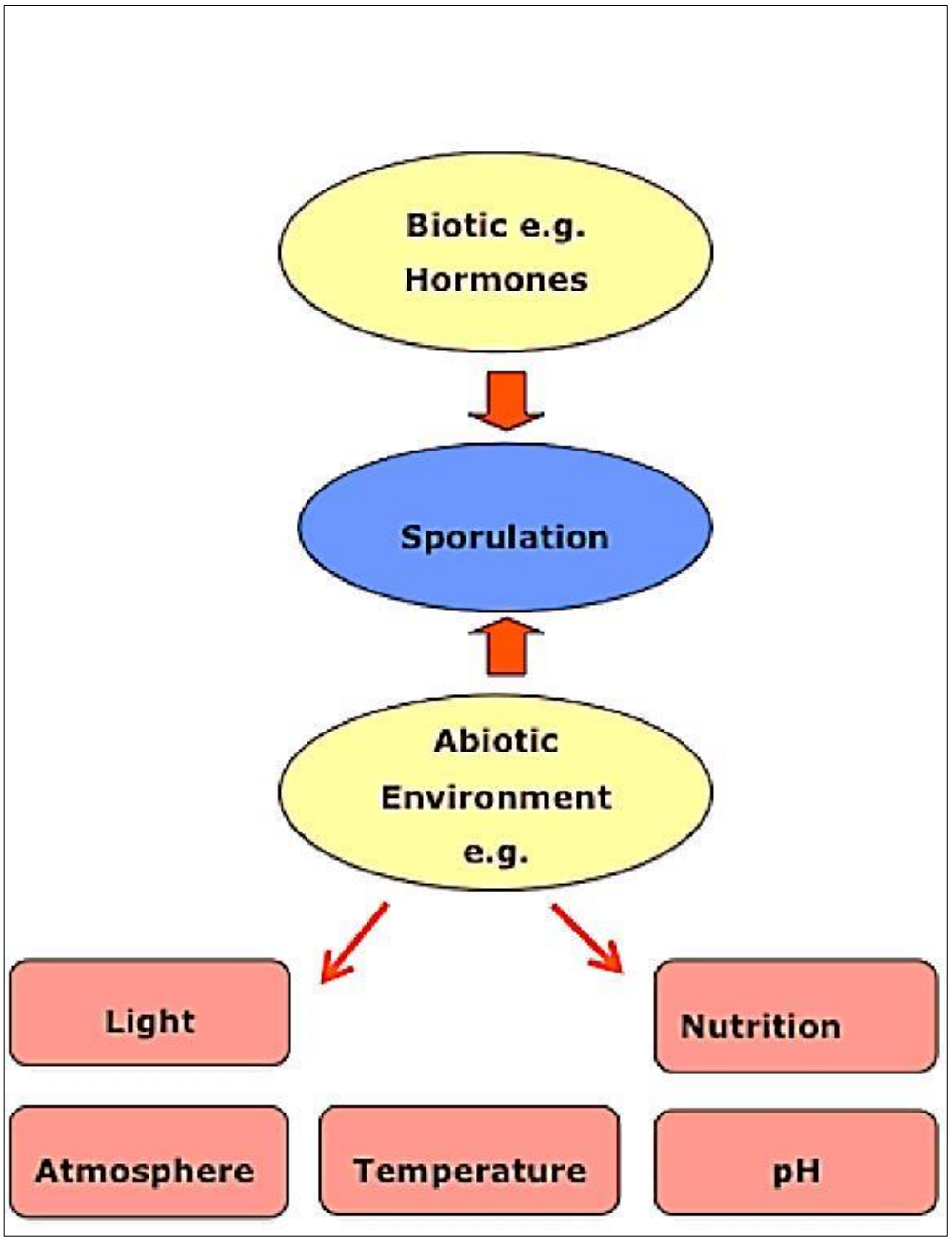

Figure 3-1 Factors affecting sporulation of fungi.

3.1.1.1.2 Linoleic acid

Changes in endogenous levels of linoleic acid, a C18:2 fatty acid, have been linked to development of fruiting bodies in a number of ascomycete species. For example, levels of free linoleic acid increased two-fold during the formation of perithecia in *Nectria haematococca*, and supplementation of linoleic acid resulted in a significant increase in the numbers of perithecia produced (Dyer *et al.,* 1993). Similarly, levels of free linoleic acid were observed to increase during perithecial development in *N. crassa*, and supplementation with external linoleic acid again increased perithecial production (Nukina *et al.,* 1981). Linoleic acid has also been shown to stimulate fruit body formation in Ophiostoma species (Dalpe and Neumann, 1976; Marshall *et al.,* 1982; Brasier, 1988) and have a significant effect on the formation of cleistothecia in *A. nidulans* and sclerotia in *A. flavus* (Calvo *et al.,* 1999; Brown *et al.,* 2008). Data overall suggests that this might be related to a dual role for linoleic acid as an energy reserve and also as a precursor for oxylipin biosynthesis (Dyer *et al.,* 1993; Brodhun and Feussner, 2011).

3.1.1.2 Spore concentration

The density of spore inoculum, and related total number of spores, used to establish Petri dish cultures has been found to impact on formation of sclerotia in both *A. flavus* and *A. parasiticus*. Relatively low spore concentrations within the range 10^1-10^3 spores per plate formed the highest numbers of sclerotia. This might be related to quorum-sensing oxylipin signalling (Brown *et al.*, 2008; Brown *et al.*, 2009).

3.1.1.3 Abiotic (environmental) factors

Abiotic factors include nutrient availability, atmospheric conditions, pH and also other physical factors such as light, temperature and UV. These factors can have either a positive or negative effect on sporulation of fungi, depending on their availability, time and dosage of shock, length of exposure and other parameters.

3.1.1.3.1 Nutritional effects

In general, sexual sporulation in fungi is more likely to occur when nutrients have been exhausted in the growth medium, with the consequent starvation due to lack of carbon and/or nitrogen sources triggering an initiation of sexual development in several fungal species e.g. meiosis is induced in *S. cerevisae* when transferred from a nutrient-rich to nutrient-poor medium (Dyer *et al.*, 1992; Elliot, 1994; Moore-Landecker, 1996). However, there are exceptions. For example, although pheromone production in *Neurospora crassa* is triggered by a lack of nitrogen (Bobrowicz *et al.*, 2002; Kim *et al.*, 2002a) no such effect is seen in *Gibberella zeae* (Lee *et al.*, 2008). Similarly, sexual reproduction in *Aspergillus nidulans* requires "well nourished" rather than "harsh" conditions (Kim *et al.*, 2009). Sexual development of ascomycete species such *as Aspergillus* species in general occurs under a more restricted, narrow set of conditions than asexual reproduction (Kwon-Chung and Sugui, 2009).

Considering specific nutrients, a low concentration of glucose generally promotes sex, whereas higher concentrations of lactose and sucrose are required where these are the primary sugar source (Elliot, 1994). In addition to a carbon source, a nitrogen source is also required for both vegetative growth and sexual development. Turian (1978) asserted that the "Carbon:Nitrogen" ratio has a fundamental role in sexual reproduction in some species, and showed this to be an essential factor controling sex in *N. crassa*. Similarly, minimum levels of certain amino acids are required for sex in *A. nidulans*, with cleistothecial development blocked at the micro-cleistothecial stage when cultivated under amino acid starvation (Bussink and Osmani, 1998; Hoffmann *et al.*, 2000).

There are also some specific nutritional requirements for sex in certain species, such as vitamins and mineral elements (including iron, manganese and zinc), and traces of fatty acids and caffeine (Moore-Landecker, 1992). To conclude, each fungal species has particular nutrient requirements to trigger sexual reproduction.

The depth and water availability of various media also have dramatic influences on sclerotial generation in *A. flavus* (Okuda *et al.*, 2000; Nesci *et al.*, 2007).

3.1.1.3.2 pH

Fungal species have different optimum pH values for sexual development, which occurs in general within a more narrow pH range than vegetative growth (Hawker, 1966). For example, the production of cleistothecia in *A. nidulans* is greatly influenced by the pH of the growth medium, with highest numbers of cleistothecia formed at a neutral pH (Rai *et al.*, 1967). Similarly, the development of sclerotia of *A. ochraceus* was significantly affected by both the nutrient source and the pH of the growth medium (Paster and Chet, 1980). The pH of media also has a great effect on the formation of sclerotia in some species of black aspergilli (Rai *et al.*, 1967).

3.1.1.3.3 Light and darkness

Light and darkness can have either an inhibitory or stimulatory role in sporulation. Moore-Landecker (1982); (1996) classified fungi into five main groups depending on their response to light including those which: are indifferent to light, exhibit inhibited or decreased sexual sporulation in light, require alternating light/dark cycles for sporulation, produce asci in darkness but prefer light, and finally those which require light to produce sexual structures.

Both the duration of exposure to light and the light quality (e.g. wavelength and intensity) often have important effects on sexual sporulation, with certain development phases of species shown to be light or dark dependent (Dyer and O'Gorman, 2012). For example, blue light was shown to initiate sexual development directly in *N. crassa,* involving detection by the White Collar Photoreceptor system (Corrochano, 2007), whereas the related species *Gelasinospora reticulospora*, needs around 30 hr of darkness to trigger sexual state before exposure to a blue light source (Inoue and Furuya, 1974). Conversely a red light sensitive

photosystem linked to the FphA protein is present in *A. nidulans* which represses sex in the light. Zonneveld (1977) and Geiser (2008) reported that a period of darkness in the first 24 hr after inoculation was necessary to produce cleistothecia. Exposure to blue light also inhibits sexuality whereas far-red light can induce the process (Blumenstein *et al.*, 2005; Bayram *et al.*, 2008b).

Meanwhile, light also influences the production of sclerotia in the genus *Petromyces*. Sclerotial production for several species, including *A. flavus* and *A. parasiticus,* generally increases in darkness (Rai *et al.*, 1967; Bennett *et al.*, 1978; Calvo *et al.*, 2004; Duran *et al.*, 2007). On the other hand, the same or even greater numbers of sclerotia have been formed by some strains of *A. flavus* and black aspergilli in the light and therefore the impact is strain dependent (Rai *et al.*, 1967; Calvo *et al.*, 1999). The overall response of *Aspergillus* species to light might relate to their ecology given that the majority of species are soil-borne (Klich, 2002).

3.1.1.3.4 Temperature

Temperature is a vital environmental factor affecting sporulation in fungi. Species have been classified into three major groups depending on their reaction to temperature. These are namely: mesophilic, including the majority of fungi, with their optimal temperature of sporulation at approximately 25^{o}C (Moore-Landecker, 1996). Secondly, psychrophilic, referring to a minority of fungi that have the ability to grow and germinate at lower temperatures, down to less than zero. Thirdly and finally, thermophiles or thermotolerant species which can grow and reproduce at higher temperature levels up to a maximum of 50^{o}C (Carlile, 2001).

In general, fungal sexual reproduction occurs over a more limited temperature range than vegetative growth (Moore, 1998). Temperatures from 20^{o}C to 24^{o}C are optimal for sexual development of most fungal species, but a minority of fungi can reproduce sexually at temperatures below 10ºC and over 30^{o}C (Moore-Landecker, 1992). Considering the

aspergili, the sexual cycle of *A. nidulans* is traditionally induced at 37°C (Pontecorvo *et al.*, 1953). However, trial experiments have suggested that increased numbers of cleistothecia are formed at lower incubation temperatures (Dyer, 2007). Sexual development in *A. fumigatus* is also temperature dependent with ascocarps only observed at 30°C among various incubation temperatures assayed (O'Gorman *et al.*, 2009). Likewise, highest numbers of cleistothecia in *Eurotium* species were formed between 27°C and 33°C (Blaser, 1975). Furthermore, the production of sclerotia in *A. flavus* was also observed to be a temperature dependent (Rai *et al.*, 1967).

3.1.1.3.5 Atmospheric conditions (e.g. O_2 and CO_2)

Aeration can have a major impact on sexual reproduction of fungi, with many species demonstrating a requirement for elevated levels of CO_2 and/or O_2 for sex (Moore-Landecker, 1982). For example, Carlile (2001) reported that sexual reproduction of *Chaetomium globosum* required a relatively high level of atmospheric CO_2, and this is also suggested to be the case for *A. nidulans* (Dyer and O'Gorman, 2012). Meanwhile, some species need a switch between aerobic to anaerobic conditions to induce sex e.g. sexual reproduction of *S. cerevisiae* requires a shift from aerobic to anaerobic respiration (Moore-Landecker, 1996). In parallel, high levels of oxygen are essential for the formation of fruiting bodies in some species that generally require more oxidative metabolism during sexual development. Moreover, sclerotial formation of some strains of *A. flavus* can be prevented by plate sealing (Okuda *et al.*, 2000).

Both sclerotial and cleistothecial formation of some aspergilli have been shown to be extremely influenced by relative humidity (RH) of the atmosphere with highest numbers of cleistothecia produced at 100% RH (Rai *et al.*, 1967). Furthermore, it has also been recently shown that the formation of cleistothecia in *A. nidulans* can be enhanced by exposure to nitric oxide (Marcos *et al.,* 2011).

3.1.2 Overview of model *Aspergillus* species used in the present study

As previously mentioned, the genus *Aspergillus* is one of the most studied genera of fungi. It comprises approximately 250 species of which ca. 70 species having a teleomorph (sexual state) (sections **1.3.5** and **1.3.6**). Species of this genus produce conidia during asexual reproduction and ascospores during sexual reproduction (Raper and Fennell, 1965). Species exhibit either homothallic (self fertile) or heterothallic (self sterile) breeding systems. For the purposes of the present study three homothallic species, namely *A. nidulans*, *N. fischeri* and *E. repens*, were chosen as model species. This was due to factors such as their amenability to laboratory culture, the availability of genome sequence data (for *A. nidulans* and *N. fischeri*), and their use in previous studies of the evolution of sexuality in the aspergilli. Some brief background details of these species will now be provided.

3.1.2.1 *Aspergillus nidulans*

Aspergillus nidulans has been widely used as a model filamentous fungus for studies of cell biology, gene regulation and genetic analysis. It is an obligate aerobe, which grows rapidly and produces a high amount of biomass in few days (Todd *et al.*, 2007). *A. nidulans* has been genome sequenced, revealing a genome size of ca. 30 Mb (Galagan *et al.,* 2005). *A. nidulans* is a homothallic sexual species, which produces dark red or purple-globose cleistothecia, surrounded by Hülle cells, within which red to purple ascospores with smooth walls are formed (**Figure 3-2**) (Frisvad and Samson, 2000). The sexual teleomorph state is named *Emericella nidulans*, although the anamorph name *A. nidulans* is used most widely, even in regard to studies of sexuality. *A. nidulans* forms green conidiospores when undergoing asexual reproduction (**Figure 3-2**) and can also reproduce parasexually (section **1.3.2** and **Figure 1-2)**. This species is found worldwide in soils and tropical origins, and also in potatoes and some grains such as, rice, cotton, wheat, corn and oats (Raper and Fennell, 1965).

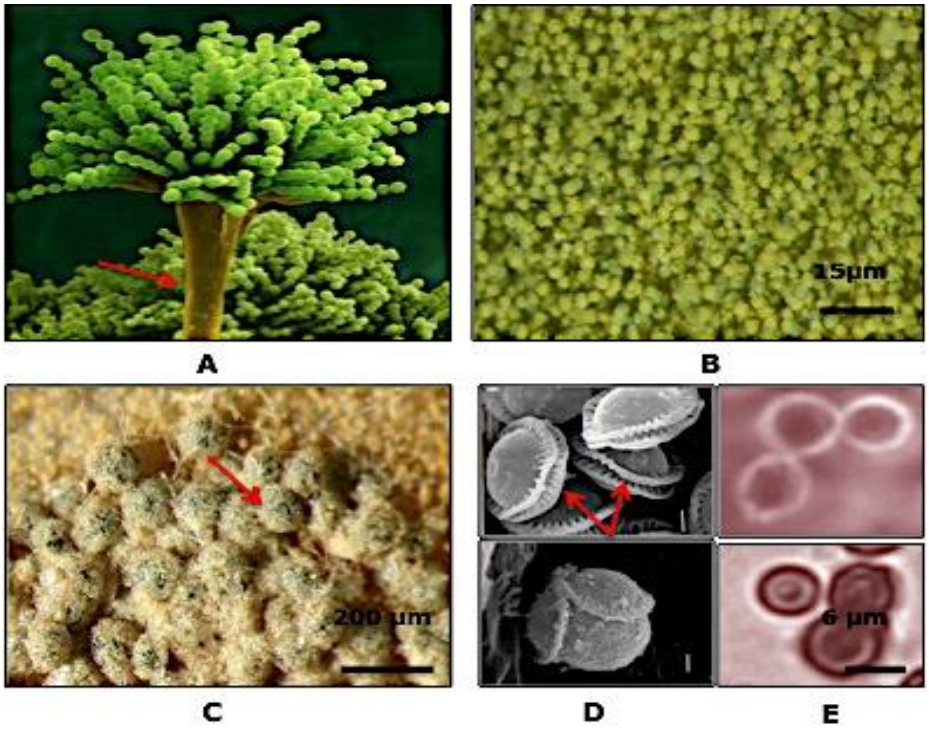

Figure 3-2 Asexual and sexual reproduction of *Aspergillus nidulans*: Arrows point to (A,B) conidiospores chain (conidial head); (C) Mature cleistothecia surrounded by Hülle cells on ACM plate incubated in the dark at 32ºC for 4 weeks; (D) SEM of ascospores; (E) brown pigmented ascospores. (Images A taking by scanning electron microscopy adopted from: http://www.visualsunlimited.com/image/I0000jsmfHhjZ71E. B taking with Nikon Coolpix 4500 camera on dissecting microscopy (Nikon OPTIPHOT- P1000, Japan), C, D and E courtesy of PS Dyer).

3.1.2.2 *Neosartorya fischeri*

Neosartorya fischeri is a homothallic species that is common in soils worldwide (Girardin *et al.,* 1995). Isolates obtained from the wild normally exhibit abundant sexual reproduction with little asexual sporulation (Raper and Fennell, 1965). The species has recently been genome sequenced, revealing a genome size of ca. 32.5 Mb (Wortman *et al.,* 2006). It is of particular interest to the bioemedical community because it is closely related to the opportunistic pathogen *Aspergillus fumigatus*, but rarely causes infection despite the genetic relatedness. *N. fischeri* typically produces green to blue conidia during asexual

reproduction (the anamorph being termed *A. fischerianus*) and white to cream cleistothecia during sexual development (**Figure 3-3**) (Raper and Fennell, 1965). Ascospores of *N. fischeri* are highly resistant to heat shock and have been found to be viable in some fruit products even after pasteurisation treatments (Samson, 1988).

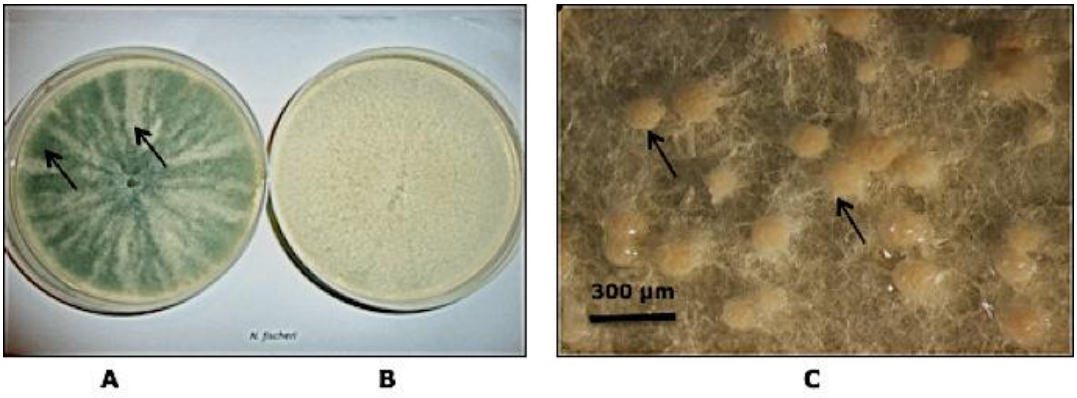

Figure 3-3 Asexual and sexual reproduction of *N. fischeri*: Arrows refer to (A) Mycelia covered by green creamy conidia; (B) White or cream mature cleistothecia (C) cleistothecia formed abundantly on surface of ACM at 28°C as viewed by Stereo microscopy.

3.1.2.3 *Eurotium repens*

Eurotium repens is a homothallic species, which is widespread in the environment. It is mainly distributed in tropical and subtropical areas, but also found in temperate areas (Kozakiewicz, 1995). It is able to grow on high osmolarity media such as 40% glucose and as a xerophilic species often found on dry or concentrated products as a contaminant of foodstuffs with high sugar content such as jellies, jams, nuts, fruit-cake etc. (Raper and Fennell, 1965; Kozakiewicz, 1995). When undergoing sexual reproduction the mould produces bright yellow-globose to subglobose cleistothecia on yellow to orange-red hyphae (**Figure 3-3**). The cleistothecia contain many ascospores that have convex surfaces with equatorial ridges. *Eurotium repens* can also reproduce asexually, producing greyish yellow to yellow orange conidia on CY20S medium and dull green to dull blue conidial heads on CYA and MEA media (the anamorphic state is termed *A. repens* or *A. reptans*) (Raper and Fennell,

1965; Samson and Pitt, 1985; Pitt, 1986). There is current debate over whether *E. repens* should be considered a synonym for *E. herbariorum* (R Samson & J Houbraken pers. comm).

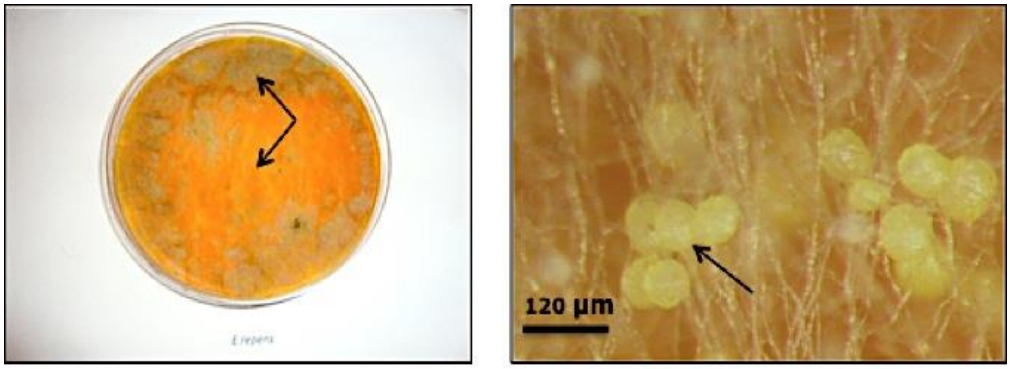

Figure 3-4 Asexual and sexual reproduction of *E. repens*: Arrows refer to (A) yellow to orange hyphae covered by cleistothecia; (B) yellow small cleistothecia (arrows) formed abundantly on M40Y medium at 28ºC after 7 d incubation.

3.2 Aims of this chapter

The species *Aspergillus nidulans*, *Neosartorya fischeri* and *Eurotium repens* were used in the present study to investigate the evolution of sexuality and asexuality in the aspergilli. It was therefore necessary at the onset of studies to fulfill the following objectives to facilitate later experimental work.

1. Establish a culture collection of suitable isolates of *A. nidulans*, *N. fischeri* and *E. repens* for study and confirm their identify and genotype where necessary.
2. Determine suitable conditions to reliably induce asexual reproduction of the target study species
3. Determine conditions to reliably and reproducibly induce sexual reproduction in the target study species. In particular detailed studies were undertaken with *A. nidulans* to assess the effects of the following factors on sexual development:
    - **A.** Time of sealing of plates after inoculation.

B. The use of different number of plates in 'stacks' when placed in an incubator.

C. Incubation at different temperatures (32°C and 37°C).

3.3 Materials and Methods

3.3.1 Identification, DNA extraction, and PCR of *Aspergillus* species

Isolates of *Aspergillus nidulans*, *Neosartorya fischeri* and *Eurotium repens* were obtained from different worldwide locations from either culture collections or as gifts from researchers. In order to confirm species identity isolates were first sub-cultured on either ACM (*A. nidulans* and *N. fischeri*) or M40Y (*E. repens*) and examined morphologically by microscopy using a WILD M3 optical light microscope (HEERBRUGG, Switzerland). Where necessary to confirm species identity, DNA was extracted (section **2.2.5**) and target genes were amplified using specific procedures, primers and enzymes for each species as detailed below. After identification, each strain was given a new number according to the 'Nottingham University BDUN collection'. All isolates were then stored safely in liquid nitrogen as long-term stocks (section **2.2.3**). Specific methods for each species are as follows.

3.3.1.1 *A. nidulans* isolates and strains

Nine known isolates or strains of *Aspergillus nidulans* were obtained from different worldwide locations (2-137, 2-139, 2-224, 2-227, 2-232, 2-250, 2-154, 2-155 and 2-149 (**Table 3-1**). DNA from each strain was extracted using a DNeasy plant Mini kit as described before (section **2.2.5.1**). In the specific case of *A. nidulans* it was important to confirm that strains were of the *veA* wild type genotype. Many laboratory strains contain a *veA1ku* mutation which alters the natural sexual fertility of isolates and thus might yield confounding results (Dyer 2007). The *veA* gene was amplified by PCR using forward (*veA* F) and reverse (*veA* R) primers designed in the present study (**Appendix 1**) and FastStart High

Fidelity Enzyme. Each 25 µl reaction contained ~50 ng genomic DNA, 2.5 µl of 10X PCR buffer, 0.2 µl (25 mM each) dNTPs, 1.4 µl (35 pmol/ml) of forward primer, 1.4 µl (35 pmol/ml) of reverse primer, 0.25 µl of FastStart High Fidelity Enzyme and ~ 18.25 µl of ultra pure water (ddH_2O) (Sigma, UK). The amplification of fragments was performed on a Techne Genius thermal cycler (Techne ltd, UK) using the following cycling parameters: initial denaturation step at 95°C for 2 min; 35 cycles consisting of denaturation 30 sec at 95°C, annealing 30 sec at 58°C and extension (elongation) for 30 sec at 72°C; followed by a final extension step at 72°C for 5 min; all steps were at a ramp rate of 60°C/min, and then held for an unlimited cooling time at 4°C. 7 µl of each PCR resulting products from amplification of VeA regions were analysed via gel electrophoresis in 1.2% agarose gels containing 1% TBE buffer (section **1.2.1.6)**. Fragments of size approximately 500-600 bp were purified using a Geneflow Q-Spin gel Extraction/PCR Purification Kit (section **2.2.5**). Subsequent DNA concentration was measured by using Nanodrop software and diluted by TE buffer (section **2.1.2.12**) to a concentration of 5 ng/µl - 20 ng/µl (500-1000 bp PCR product) before being submitted for DNA sequencing at the Queens Medical Centre (QMC) sequencing facility at the University of Nottingham. Finally, the programme MacVector 11.1 was used to edit DNA sequences.

Table 3-1 information on worldwide *Aspergillus nidulans* strains/isolates

Number of strain/isolate	Source Location	Isolation Year	Genetic Markers
2-137	Birmingham (UK)	1960	Wild-type
2-139	Birmingham (UK)	1960	Wild-type
2-149*	Glasgow (UK)	n/a	pyrG89, pyroA4, nkuA::argB, veA1
2-154*	Glasgow (UK)	n/a	yA1, paba A1, nii A4,nkuA::bar, veA1
2-155*	Glasgow (UK)	n/a	pyrG89, pyro A4, nkuA::bar, veA1
2-224	Gold coast**	1982	Wild-type
2-227	California (USA)**	1984	Wild-type
2-232	Barbados**	1987	Wild-type
2-250	Cardiganshire (UK)**	1992	Wild-type

*Laboratory strains derived from original Glasgow strain G00.
**Gift from R. Hoekstra.

3.3.1.2 *N. fischeri* isolates

Nineteen putative wild-type isolates of *N. fischeri* (47-133, 47-166, 47-167, 47-176, 47-177, 47-179, 47-182, 47-183, 47-184, 47-185, 47-201, 47-202, 47-203, 47-204, 47-205, 47-206, 47-207, 47-210 and 47-221) were obtained from various worldwide locations (**Table 3-2**) and DNA extracted as described before (section **2.2.5.2**). Gel electrophoresis with 1.2% agarose was used to check the extracted DNA yields (section **1.2.6.1**). A fragment of the beta-tublin gene was amplified by PCR using

primers BT2a and BT2b (**Appendix 1**) and FastStart High Fidelity Enzyme. The amplification of these fragments was performed on a Techne Genius thermal cycler using specific cycling parameters, then the Products were resolved via gel electrophoresis then bands were visualised via UV light and photographed using a BioRad Chemidoc XRS as previously stated (section **3.3.1.1**). PCR purification was performed (section **2.2.5**) and the PCR products were sequenced at the QMC sequencing facility at the University of Nottingham.

3.3.1.3 *Eurotium repens* isolates

Four wild-type isolates of *E. repens* (DTO 123-D8, DTO 89-F5, DTO 117-F9 and CBS.117336) were obtained from the Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre (the Netherlands). DNA was extracted as described previously (section **2.2.5.2**). RAPD-PCR DNA finger printing was then performed to confirm that these were independent non-clonal isolates, according to (Murtagh *et al.*, 1999) using Dynazyme II Polymerase (Finnzymes, Finland) and four different primes including RC08, R151, UBC90 and OPAX16 (**Appendix 1**) in 25 µl reaction volumes containing ~1 ng genomic DNA. Amplification products were resolved by electrophoresis in 1.5% agarose gels containing TBE buffer. Gels were stained with ethidium bromide (1 µl/ml) after which bands were visualised via UV light and photographed using a BioRad Chemidoc XRS (Bio-Rad Laboratories, UK).

3.3.2 Determination of suitable conditions to reliably induce asexual and sexual reproduction of target species

3.3.2.1 Asexual reproduction in *N. fischeri*

N. fischeri normally reproduces sexually in abundance (section **3.3.1.2**), but for later experimental work conidia were required (see **chapter 5**). Therefore representative isolates of *N. fischeri* were grown under a range of conditions to attempt to reliably induce asexual sporulation. An inoculating loop was used to spread hyphae over 9 cm diameter Petri dishes containing either ACM (section **2.1.1.1**), MEA (section **2.1.1.2**), CDA (section **2.1.1.5**) or TWA (section **2.1.1.6**), then plates were

incubated at a range of temperatures at 15ºC, 25ºC, 28ºC, 32ºC and 37ºC under white light (2.5 – 5.5 µmol m^{-2} s^{-1}) for 7 d, plates were not sealed. After seven days cultures were inspected visually for the most asexual sporulation using an estimate of the percentage area of the plates covered in which asexual sporulation was occurring in abundance (judged by optical light microscope).

3.3.2.2 Asexual reproduction in *E. repens*

E. repens normally reproduces sexually in abundance, producing numerous relatively small cleistothecia (section **3.1.2.3**), but for later experimental work conidia were **required** (see **chapters 4** and **5**). Therefore representative isolates of *E. repens* (DTO 123-D8, DTO 89-F5, DTO 117-F9) were grown under a range of conditions to attempt to reliably induce asexual sporulation. Isolates were grown on four different media, using sterile loops to spread hyphae over the surface of 9 cm diameter Petri dishes containing either GMEA (Blaser, 1975) (section **2.1.1.8**), MEA+20% sucrose, CDA+20% sucrose or M40Y medium (section **2.1.1.7**) (Raper and Fennell, 1965). Sets of plates were either sealed with two layers of Nescofilm, or left unsealed and then were incubated for 7 d at 25ºC and 28ºC under white light (2.5 – 5.0 µmol m^{-2} s^{-1}) and dark. After seven days cultures were inspected visually for asexual sporulation using an estimate of the percentage area of the plates covered in which asexual sporulation was occurring in abundance (judged by optical light microscope).

3.3.3 Determining conditions to reliably and reproducibly induce sexual reproduction in *A. nidulans*

Although *A. nidulans* has long been used as an experimental organism, there have been ongoing reliability issues about the ability to consistently induce similar levels of sexual reproduction in replicate experimental plates (PS Dyer & G Braus, pers comm.). Therefore, a series of initial experiments were performed to determine conditions to reliably and consistently induce the sexual state to facilitate later experimental work

[NB. this was not required for asexual reproduction which was easily induced under a variety of conditions].

3.3.3.1 The influence of time of sealing plates on sexual reproduction of *A. nidulans*

To induce sexual reproduction in *A. nidulans*, cultures are traditionally inoculated and left unsealed for 24 hr, at which point plates are sealed with an appropriated sealing method (e.g. Parafilm, Nescofilm or even cling film) and then left for a further period of incubation for cleistothecia to form. It is thought that this plate sealing results in a build up of CO_2 levels which promotes sexual reproduction (Dyer, 2007). However, there has apparently not been any systematic study of the time of plate sealing and sexual fertility of plates can vary considerably possibly due to early inoculation effects (PS Dyer and G Braus, pers comm.). Therefore, four representative isolates of *A. nidulans* (2-137, 2-139, 2-154 and 2-155) comprising both *veA* and *veA1ku* genotypes were identified (section **3.3.1.1**). Then sexual cycle plates were set up, which involved inoculating six replicate 5 cm diameter of ACM Petri dishes (section **2.1.1.1**) for each isolate. A spore suspension of 1 x 10^5 conidia in 50 µl was made by harvesting spores with sterile 0.01% Tween and filtering through sterile Miracloth (using a haemocytometer method to adjust spore concentration as appropriate), and spores were spread over the entire surface of plates using a sterile spreader. Plates were then incubated in a stack of six plates in the dark at 32ºC. To promote sexual reproduction in *A. nidulans*, plates were sealed with two layers of Nescofilm at either 0 hr, 12 hr, 15 hr, 18 hr, 21 hr and 24 hr after inoculation and placed in different parts of the incubator. Cultures were left for a further 4 weeks and then inspected for the formation of mature cleistothecia, as defined by presence of a characteristic dark purple peridium and size greater than ca. 100 µm diameter. Plates were scored visually and by microscopy for numbers of cleistothecia and conidia formed, counting numbers of cleistothecia formed in replicate viewing areas of known size (75.36 mm^2), with arising data converted to numbers of cleistothecia formed per 100 mm^2.

3.3.4 The influence of depth of stacks on sexual reproduction of *A. nidulans*

In preliminary experiments aforementioned (section **3.3.3.1**) it became apparent that the way in which plates were stacked in the incubator seemed to influence the degree of formation of cleistothecia. Therefore this experiment was performed to assess this effect. Sexual cycle plates were set up as described above (section **3.3.3.1**). Plates (6 replicate for each strain) were then incubated in the dark at 32ºC in three different patterns; in a single layer depth (i.e. individually) and also in different stacks (piles), including a stack of 2×3 plates high and a stack of 3×2 plates high. All plates were subsequently sealed at 15 hr after inoculation with two layers of Nescofilm and placed in different parts of the incubator. Cultures were left for a further 4 weeks in the incubator then inspected for the formation of mature cleistothecia. The number of cleistothecia formed was finally scored under the microscope to determine whether the number of plates incubated in different stacks influences the sexual fertility.

3.3.5 The effect of incubation at 32°C and 37°C on sexual reproduction of *A. nidulans*

Standard experimental procedures to induce sexual reproduction in *A. nidulans* involve incubation at 37°C (Pontecorvo *et al.*, 1953; Calvo *et al.*, 1999; Bruggeman *et al.*, 2003b). However, no systematic investigation has been conducted to find the optimum temperature triggering the formation of cleistothecia in *A. nidulans.* Consequently, an experiment was carried out to find the desirable temperature to induce sexual fertility in this species given that previous unpublished data (PS Dyer, pers. comm.) had suggested a lower temperature optimum. Spores from two wild-type strains (2-137, 2-139) and two *ku* mutant strains (2-149, 2-154) of *A. nidulans* were obtained. Sexual cycle plates were set up as described above (section **3.3.3.1**) using 8 replicates per isolate/strain. Plates were incubated individually rather than in stacks (section **3.4.3.2**) in the dark at 32ºC and 37ºC. After 15 hr of inoculation, plates were

sealed with two layers of Nescofilm. Plates were then regularly inspected for the formation of cleistothecia and scored every four days from 8 to 20 d after inoculation (i.e. at 8 d, 12 d, 16 d and 20 d) by light microscopy (x40 lens). Simultaneously, numbers of ascospore formed at each time point were also determined at both 32ºC and 37ºC. This was achieved by cutting out 8 agar plugs with cleistothecia from each replicate plate using a sterilised cork borer (15 mm) (**Figure 3-5**). Each plug was then suspended in a 2.0 ml Eppendorf tube containing 500 µl of 0.01% Tween, then cleistothecia were harvested by using a sterilised disposable loop to carefully remove cleistothecia, which were then crushed against the wall of the Eppendorf tube using a sterilised wire to release ascospores. Suspensions were vortexed thoroughly to ensure that the ascospores were evenly distributed through the mixture. Eventually, tubes were centrifuged at 12,000 rpm for 10 min at 4ºC, and then new sterile disposable loops were used to disrupt gently the structures of remains cleistothecia at the bottom of Eppendorf tubes to ensure that entire cleistothecia were broken down and ascospores released. Tubes were vortexed again to distribute ascospores evenly within suspension. A haemocytometer method was then used to calculate the ascospore concentration (using an improved Neubauer haemocytometer).

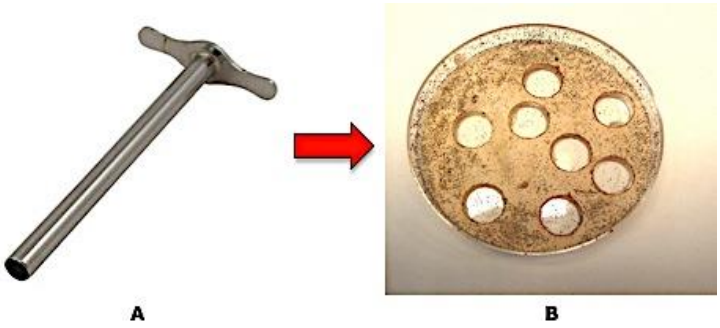

Figure 3-5 Shows (A) Cork borer (15 mm); (B) ACM after plugs covered in cleistothecia removed of *A. nidulans* grown at 32ºC in the dark.

3.3.6 Statistical Analysis

Statistics software (SPSS, version 21) for Macintosh (Mac OS X version 10.6.8) was used. ANOVA (analysis of variance) was used to analyse the

data and to determine whether the results were significant at a 5% confidence level according to Barnard *et al.* (2007).

3.4 Results

3.4.1 Establishing a collection of *Aspergillus* species

3.4.1.1 Identification of *A. nidulans* isolates and strains

DNA was successfully extracted from all nine isolates and strains of *A. nidulans* (**Table 3-1**) using a DNeasy plant kit and the *veA* gene was amplified, producing fragments approximately 500-600 bp in size which were sequenced. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and subsequent sequence analysis identified that the six wild-type isolates (2-137, 2-139, 2-224, 2-227, 2-232, 2-250) were all of *veA* genotype, whilst the three laboratory strains (2-154, 2-155 and 2-149) exhibited the *veA1ku* genotype, with a G to T mutation in the initiation codon of the wild-type ORF resulting in production of a shortened polypeptide chain (data not shown) (Kim *et al.*, 2002b).

3.4.1.2 Identification and characterisation of *Neosartorya* isolates

The 19 putative isolates of *Neosartorya fischeri* all produced white to cream cleistothecia and green to blue conidia characteristic of homothallic *Neosartorya* species. However, to verify that isolates were indeed *N. fischeri* rather than close relatives such as *N. spinosa* (Raper and Fennell, 1965; Samson *et al.,* 2007) it was necessary to undertake molecular characterisation. DNA was successfully extracted from all putative strains using the PLG-phenol/chloroform procedure and then beta-tublin gene was amplified from these isolates producing DNA fragments approximately 600 bp in size which were sequenced. BLAST searching (both nucleotide BLAST and megabBLAST) was used to identify homologous sequences for all 19 test isolates aforementioned. Four isolates (47-166, 47-167, 47-185, 47-221) from these cultures were identified as *N. fischeri* based on DNA similarity of >99%, whilst the rest

of isolates were instead found to be more closely related to other *Neosartorya* species including *N. spinosa, N. paulistensis* and *N. laciniosa*. The former strains of *N. fischeri* (47-166, 47-167, 47-185 and 47-221) were then given new numbers, becoming 53-13, 53-16, 53-14 and 53-15 respectively in the 'Nottingham University BDUN collection' (**Table 3-2**). Three of these strains with an existing wild-type isolate held in the BDUN collection (53-2) were adopted for further work in the current project.

Table 3-2 Identification of *Neosartorya spp*. (*N. fischeri* **). DNA similarity values provided from BLAST analysis. All isolates obtained as gift from G. Szakacs.

Putative strains	Old Number	Nottingham Number	Location	Identification	DNA similarity Value
47-133	TUBF-1430		Soil, South Africa, Natal	*N. spinosa*	97%
47-166	TUBF-1768	53-13	Soil, Lima, Peru, Canete, San Vicente, " Zona EL Perdal"	*N. fischeri* **	99%
47-167	TUBF-1769	53-16	Soil, Lima, Peru, Canete, San Vicente, " Zona EL Perdal"	*N. fischeri***	98%
47-176	TUBF-1820		Soil, Malaysia Pulau, Penang	*N. spinosa*	98%
47-177	TUBF-1821		Soil, Malaysia Pulau, Penang	*N. spinosa*	98%
47-179	TUBF-1823		Soil, Malaysia Pulau, Penang	*N. spinosa*	98%
47-182	TUBF-1841		Red soil, Peru, Dist.Yorimagoas	*N. spinosa*	100%
47-183	TUBF-1844		Red soil, Peru, Dist.Yorimagoas	*N. spinosa*	100%
47-184	TUBF-1845		Red soil, Peru, Dist.Yorimagoas	*N. spinosa*	100%

47-185	TUBF-1846	53-14	Garden soil, Sri Lanka, Ceylon	*N. fischeri***	99%
47-201	TUBF-1883		Soil, Malaysia Pulau, Penang	*N. spinosa*	98%
47-202	TUBF-1893		Field soil, Sri Lanka	*N. paulistensis*	100%
47-203	TUBF-1986		Park soil, Sentosa Singapore	*N. paulistensis*	97%
47-204	TUBF-1987		Forest soil in Jungle, Malaysia, PulauPayar island.	*N. spinosa*	99%
47-205	TUBF-1988		Forest soil in Jungle, Malaysia, Pulav Payar	*N. laciniosa*	99%
47-206	TUBF-1989		Forest soil in Jungle, Malaysia, Pulau island Payar	*N. spinosa*	99%
47-207	TUBF-1990		Forest soil in Jungle, Malaysia, Pulau Payar island	*N. spinosa*	99%
47-210	TUBF-2003		Forest soil in Jungle, Malaysia, Pulau Payar island	*N. spinosa*	98%
47-221	TUBF-2442	53-15	Soil, Fiji Island	*N. fischeri***	100%

3.4.1.3 Characterisation of *Eurotium repens* isolates

The four isolates of *E. repens* were all found to produce characteristic abundant, relatively small bright yellow to orange cleistothecia when grown on M40Y media at 28ºC for 7 d. All four isolates had been obtained from environmental sampling in the Netherlands (**Table 3-3**) and appeared morphologically very similar when grown on both ACM and M40Y. Therefore it was considered necessary to confirm that these were independent, rather than clonal, isolates. RAPD-PCR DNA finger printing revealed that the four isolates were clearly different and independent as each isolate yielded different fingerprint patterns, particularly with primer RC08 primer (**Figure 3-6**), which generated notably different bands for each isolate in terms of size, number and position. The four isolates DTO 123-D8, DTO 89-F5, DTO 117-F9 and CBS 117336 were given new numbers 51-3, 51-4, 51-5 and 51-6 respectively in the 'Nottingham University BDUN collection' (**Table 3-3**).

3.4.2 Determination of suitable conditions to induce asexual reproduction in *N. fischeri* and *E. repens*

Both of the homothallic species *N. fischeri* and *E. repens* were found to be highly sexual fertile in that they produced cleistothecia in abundance, consistent with previous reports (Raper and Fennell, 1965). However, preliminary observations revealed that conidia were generally produced in low quantities. Given that conidia were required for further investigations to be undertaken in the current study, attempts were therefore made to find reliable conditions which might enhance asexual development.

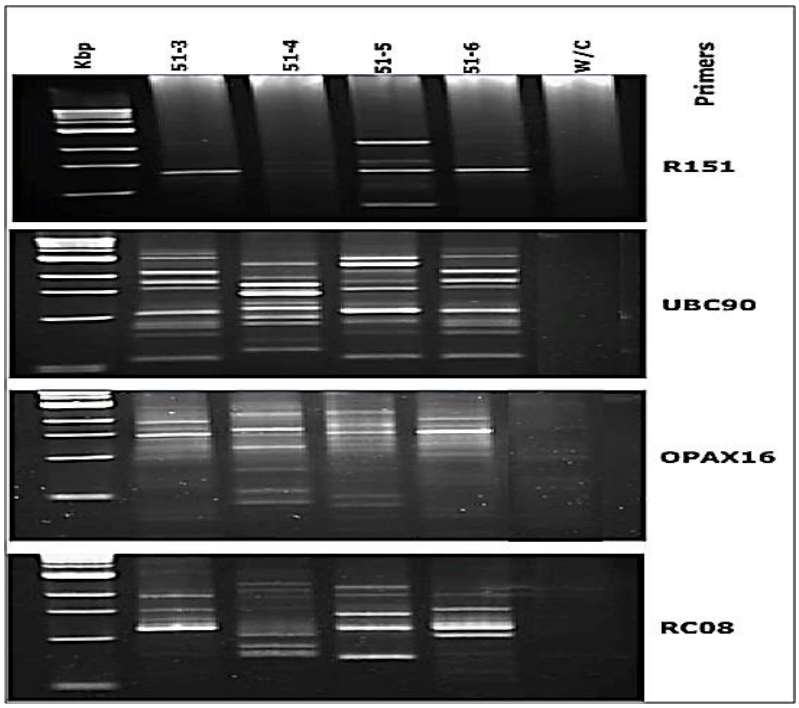

Figure 3-6 RAPD-PCR fingerprint bands of *Eurotium repens* following amplification with four primers (as labelled to the right hand side) produced by four isolates 51-3, 51-4, 51-5 and 51-6 (as labelled above). Kbp = DNA size marker, W/C = water control.

Table 3-3 Four strains of *E. repens* from different locations in the Netherlands (obtained as gifts from R. Samsom and G. Houbraken).

Old number	New number	Location	Isolation date
DTO 123-D8	51-3	Ceiling canteen, Zutphen	February 2010
DTO 89-F5	51-4	Air of living room, Eindhoven	January 2009
DTO 117-F9	51-5	Archive, Hoornaar	January 2010
CBS. 117336	51-6	Inner part of a chocolate truffle	January 2005

3.4.2.1 Asexual reproduction in *N. fischeri*

Five strains of *N. fischeri* (53-2, 53-13, 53-14, 53-15 and 53-16) were grown on four different media (ACM, MEA, CDA and TWA) at five different temperatures (15ºC, 25ºC, 28ºC, 32ºC and 37ºC) in the light in an attempt to promote asexual reproduction (section **3.3.2.1**). There was found to be a marked difference in the amount of visible spore colonies observed between the different media and temperatures (**Figure 3-7 and Figure 3-8**). Highest numbers of conidia (as judged by percentage coverage of the plate) were produced by all isolates on ACM and MEA between 28ºC and 37ºC. Conversely, lowest numbers of conidia were observed on CDA and TWA, particularly at the lower temperatures 15ºC and 25ºC. Due to the subjective nature of the scoring of sporulation it was considered inappropriate to attempt any further statistical analysis.

Representative data is shown from isolate 53-13 (**Figure 3-7**). Here, highest numbers of conidia were formed on ACM and were fairly constant between the different temperatures. High numbers of conidia were also formed on MEA, but only at higher temperatures between 28ºC and 37ºC **Figure 3-7**). A very similar pattern of conidiation on the different growth media and at different temperatures was observed for isolates 53-14, 53-15 and 53-16 (data not shown).

The only exception was isolate 53-2. This produced the lowest number of asexual colonies among all the five isolates investigated. Most conidia were produced on ACM and MEA, but in this case the production of conidia even on ACM was temperature dependent, with maximum numbers forming only at 32ºC and 37ºC. Although not quantified, it was noted that isolate 53-2 appeared to be the most sexually fertile isolate in terms of number of cleistothecia produced (**Figure 3-8**).

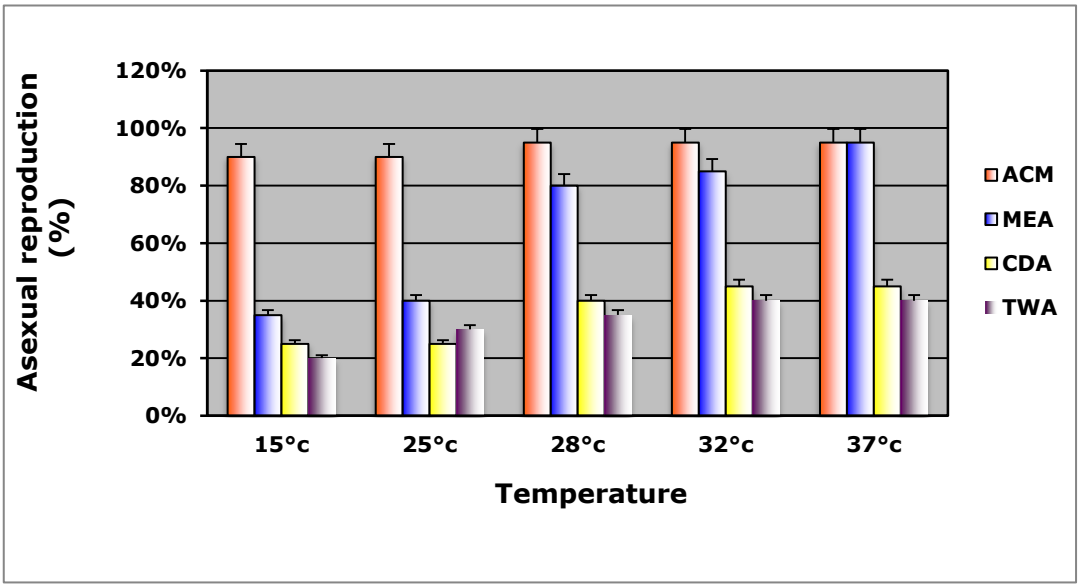

Figure 3-7 Effects of temperature (15ºC, 25ºC, 28ºC, 32ºC and 37ºC) and growth media type (ACM, MEA, CDA and TWA) on asexual reproduction of *N. fischeri* strain 53-13 as judged by percentage of plate surface exhibiting asexual conidiation. Error bar shows ± SEM.

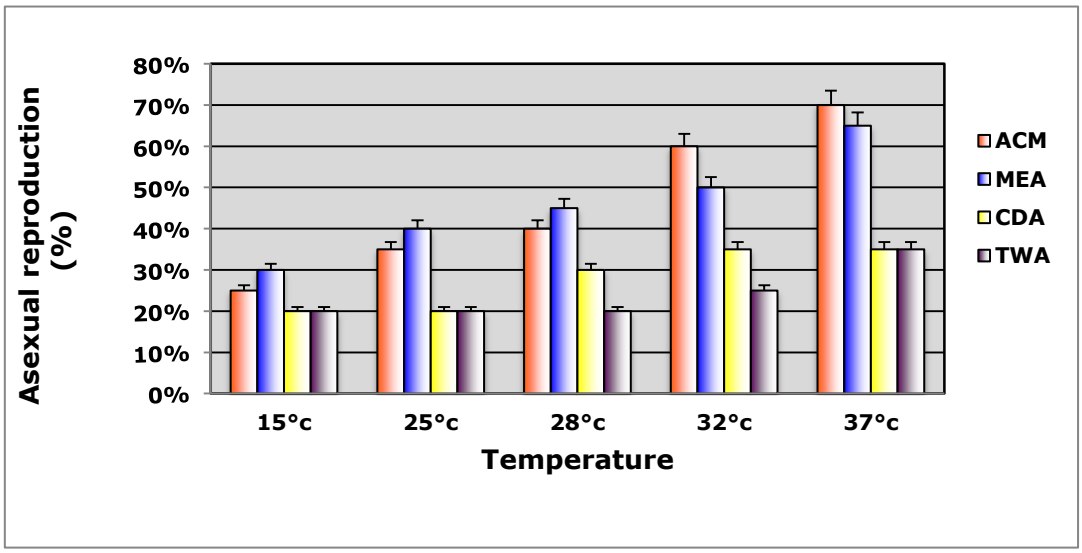

Figure 3-8 Effects of temperature (15ºC, 25ºC, 28ºC, 32ºC and 37ºC) and growth media type (ACM, MEA, CDA and TWA) on asexual reproduction of *N. fischeri* isolate 53-2 as judged by percentage of plate surface exhibiting asexual conidiation. Error bar shows ± SEM.

3.4.2.2 Asexual reproduction in *E. repens*

The four isolates of *Eurotium repens* (51-3, 51-4, 51-5 and 51-6) were inoculated onto four different media (M40Y, GMEA, MEA+20% sucrose and CDA+20% sucrose) at two different temperatures (25ºC and 28ºC) in either unsealed or sealed plates, both in the dark or light, and were incubated for 7 d (section **3.3.2.2**). All isolates were found to grow in a similar fashion to each other across the different media and temperature conditions. In unsealed plates, all isolates grew most rapidly on M40Y and GMEA on which 80-100% of the surface showed evidence of conidial formation at both 25ºC and 28ºC. However, all plates were also totally covered by huge numbers of cleistothecia. Slower growth was evident on the other two media (MEA+20% sucrose and CDA+20% sucrose), which showed a lower degree of asexual sporulation (approximately 40-60% coverage) and reduced level of cleistothecial formation. There was no clear difference between plates incubated in the dark and light. However, interestingly and unexpectedly sexual fertility (as judged by eye) decreased dramatically when plates were sealed; this applied to all media incubated in darkness at both 25ºC and 28ºC (section **3.1.1.3.5**). Asexual reproduction of all isolates however remained higher at those conditions. Representative data is shown below for isolate 51-3 (**Figure 3-9**). Due to the subjective nature of the scoring of sporulation it was considered inappropriate to attempt any further statistical analysis.

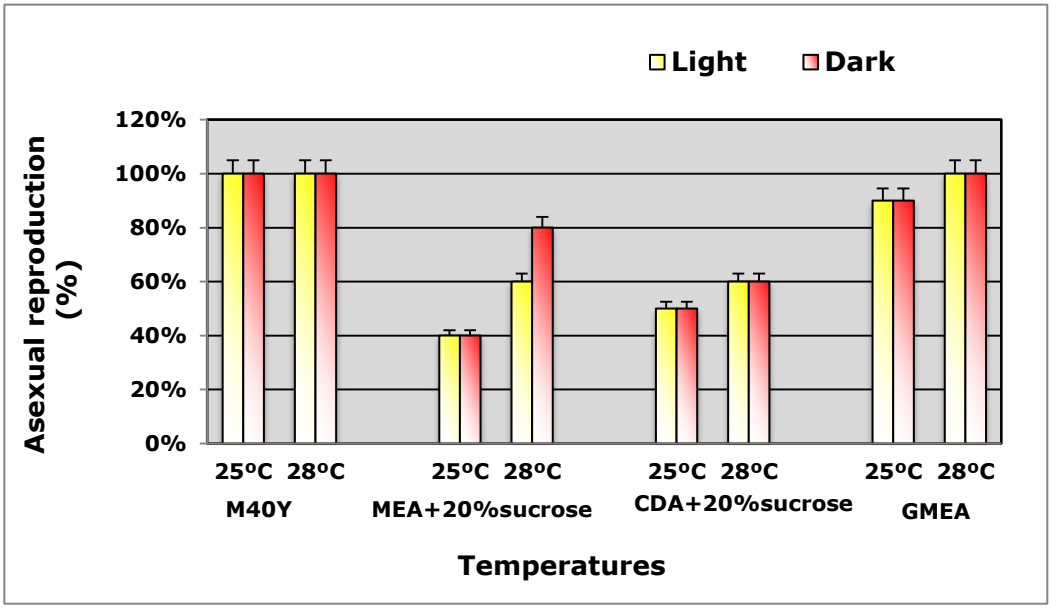

Figure 3-9 Effects of temperature (25ºC and 28ºC) and growth media type (M40Y, MEA, CDA and GMEA) in the light or dark on asexual reproduction of *E. repens* isolate 51-3 as judged by percentage of plate surface exhibiting asexual conidiation. Error bar shows ± SEM.

3.4.3 Determination of conditions to reliably and reproducibly induce sexual reproduction in *A. nidulans*

3.4.3.1 Influence of time of sealing plates on sexual reproduction of *A. nidulans*

Sexual plates were inoculated for four isolates of *A. nidulans* (2-137, 2-139, 2-154 and 2-155) and were incubated in stacks of 6 plates in the dark at 32ºC. Plates were then sealed with two layers of Nescofilm at 0 hr, 12 hr, 15 hr, 18 hr, 21 hr and 24 hr and were inspected for the number of mature cleistothecia formed after four weeks of incubation (section **3.3.3.1**). A two-way ANOVA between all strains (VeA + VeA1) revealed that there was a significant overall effect of sealing time on production of cleistothecia [F (df) = 5.320 (5, 120); p <0.001], a significant effect of strain on production of cleistothecia [F (df) = 24.783 (3, 15); p <0.001], and also a significant interaction between strain and time of sealing on production of cleistothecia [F (df) = 4.428 (15, 120); p

<0.001]. However, there was no overall consistent pattern when comparing isolates, which each responded differently to these conditions (**Figure 3-10** to **Figure 3-13**). Furthermore, most isolates showed no clear trend over time, with rather erratic peaks of formation of cleistothecia evident. Despite this, it could be seen that the most reliable time for sealing for most isolates was between 12 hr and 18 hr after inoculation.

Considering specific isolates there was no significant effect of sealing time on production of cleistothecia in 2-137 (VeA) [A one-way ANOVA F (df) = 1.575 (5, 30); p = 0.197]. Highest numbers of cleistothecia were produced when plates were sealed at 12 hr after inoculation, thereafter a gradual decrease in fertility was evident up to 24 hr (**Figure 3-10**).

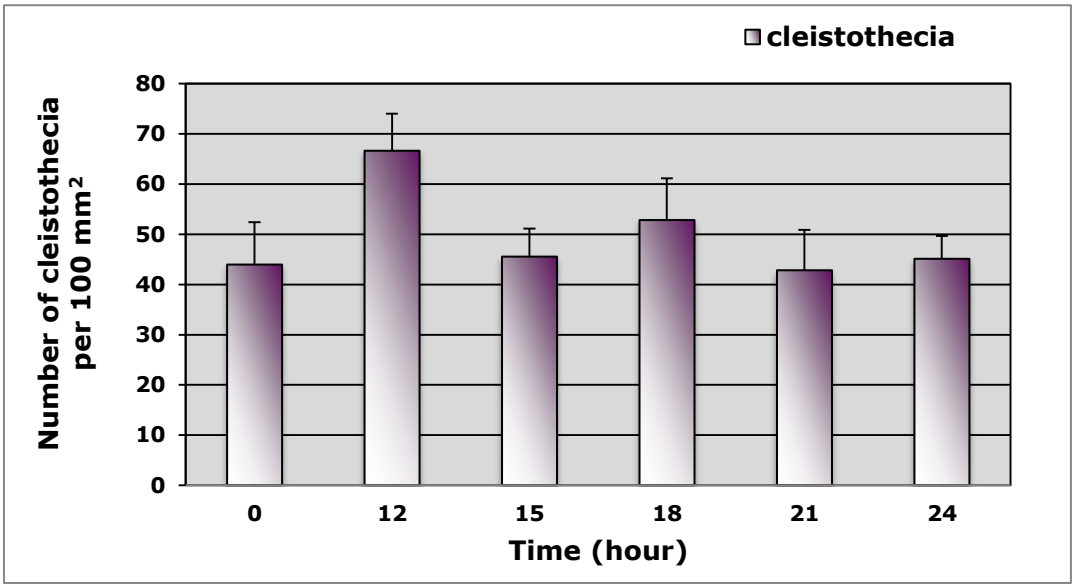

Figure 3-10 Number of cleistothecia formed per 100 mm² in *A. nidulans* isolate 2-137 grown on ACM for 4 weeks in the dark at 32ºC after plates were sealed at 0 hr, 12 hr, 15 hr, 18 hr, 21 hr and 24 hr after inoculation. Error bar shows ± SEM.

For isolate 2-139 (*ve*A) there was a significant difference in production of cleistothecia according to sealing time (**Figure 3-11**) [A one-way ANOVA: F (df) = 6.212 (5, 30); p <0.001]. There was a general trend of increased cleistothecial production up to a maximum at 18 hr and 24 hr,

although there was an unexpected drop in numbers at 21 hr. Maximum numbers of cleistothecia were also formed at 18 hr by the *ku* mutant 2-154 (*ve*A1), although differences in numbers were marginally below the significance threshold (**Figure 3-12**) [A one-way ANOVA: F (df) = 2.315 (5, 30); p = 0.068)].

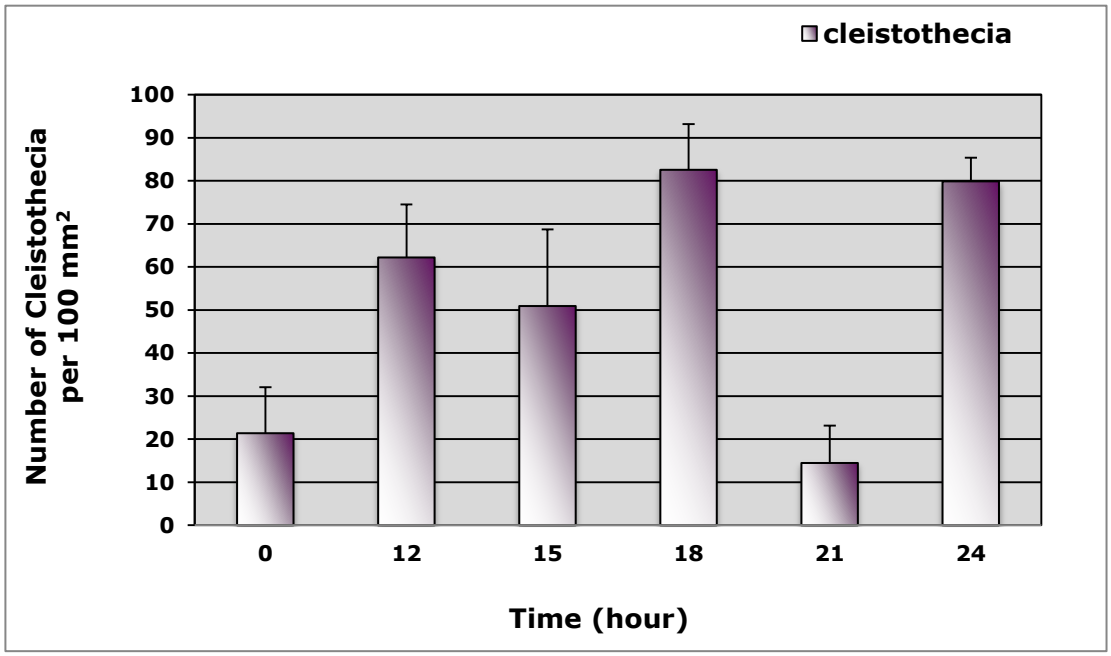

Figure 3-11 Number of cleistothecia formed per 100 mm² in *A. nidulans* isolate 2-139 grown on ACM for 4 weeks in the dark at 32ºC after plates were sealed at 0 hr, 12 hr, 15 hr, 18 hr, 21 hr and 24 hr after inoculation. Error bar shows ± SEM.

Finally, there was no significant difference between sealing time and production of cleistothecia of the *ku* mutant 2-155 (VeA1), which formed similar numbers of cleistothecia throughout the time range (**Figure 3-13**) [A one-way ANOVA: F (df) = 0.662 (5.30); p = 0.684]. However, it was observed that this strain was noticeably less fertile than all the other isolates and strains examined. Whereas 2-137, 2-139 and 2-154 formed a maximum 60-85 cleistothecia per 100 mm^2, 2-155 exhibited a maximum of only around 20 cleistothecia per 100 mm^2.

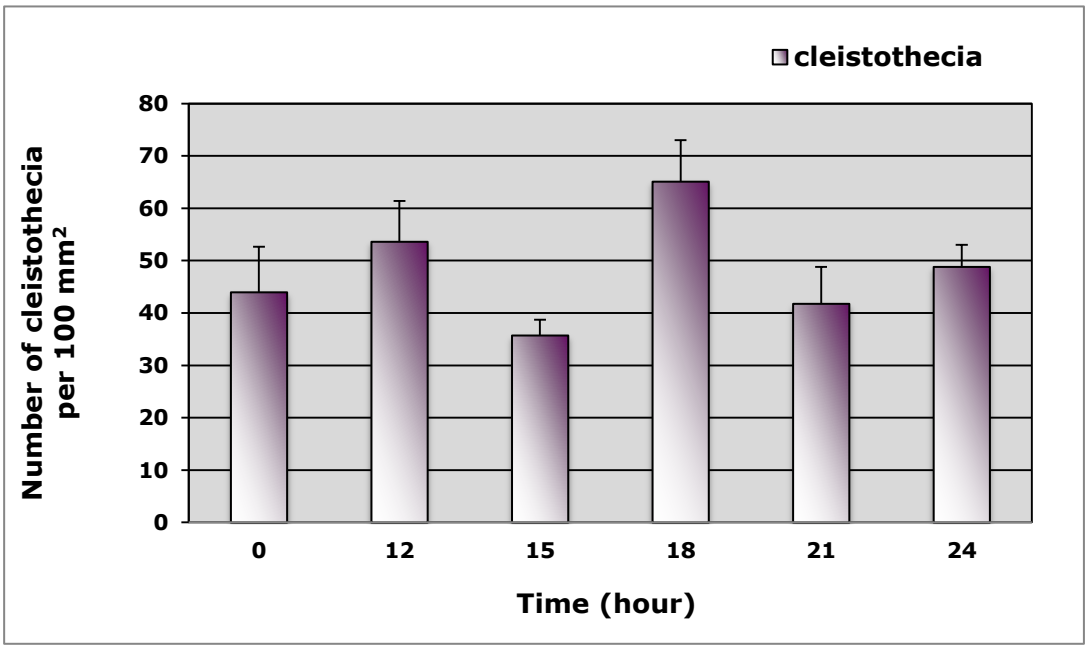

Figure 3-12 Number of cleistothecia formed per 100 mm² in *A. nidulans* strain 2-154 grown on ACM for 4 weeks in the dark at 32°C after plates were sealed at 0 hr, 12 hr, 15 hr, 18 hr, 21 hr and 24 hr after inoculation. Error bar shows ± SEM.

NB. The number of conidia formed by all four isolates/strains was also counted using a haemocytometer method. However, this number was found to be highly variable and was not considered reliable. This was most likely to the fact that plates were incubated in stacks of 6 Petri dishes, whereas later results were to show that more reliable results were obtained when plates were incubated as a single layer depth (section **3.3.4**).

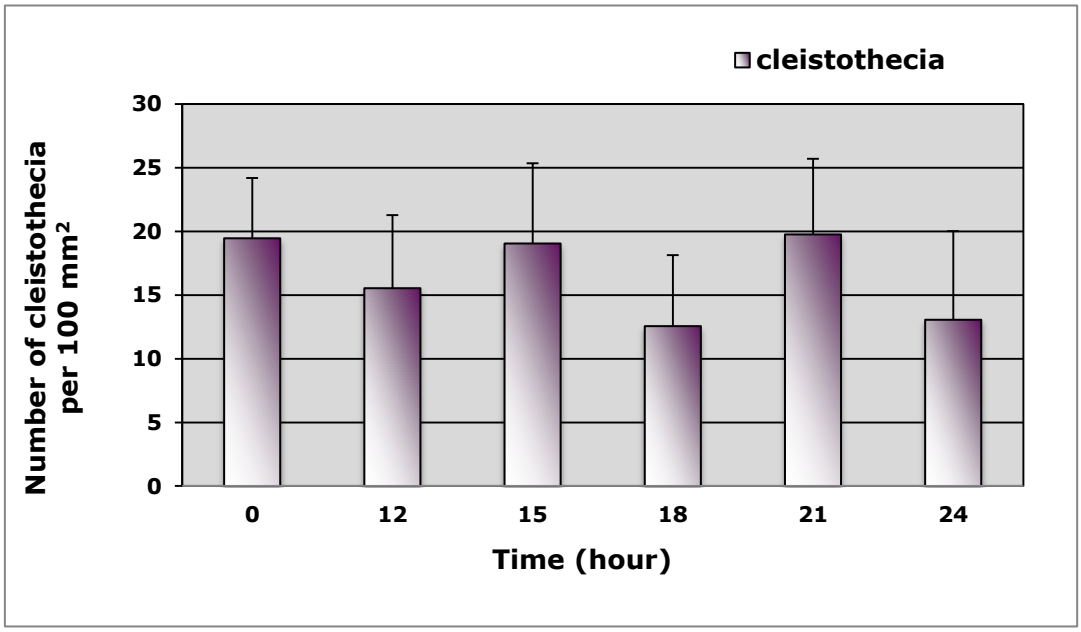

Figure 3-13 Number of cleistothecia formed per 100 mm² in *A. nidulans* strain 2-155 grown on ACM for 4 weeks in the dark at 32ºC after plates were sealed at 0 hr, 12 hr, 15 hr, 18 hr, 21 hr and 24 hr after inoculation. Error bar shows ± SEM.

3.4.3.2 Influence of incubation in stacks on sexual reproduction of *A. nidulans*

Sexual plates of five isolates/strains of *A. nidulans* [2-137 and 2-139 (VeA), and 2-149, 2-154 and 2-155 (VeA1)] were set up to investigate the effect of manner of stacking plates. Inoculated plates were sealed after 15 hr of inoculation and incubated in the dark for four weeks in three different patterns: individually, a stack of 2 high and a stack of 3 high at 32ºC. A two-way ANOVA between all strains (VeA+VeA1) revealed that there was a significant overall effect of incubation in different stacks on production of cleistothecia [F (df) = 33.765 (2, 75); p <0.001], a significant effect of strain on production of cleistothecia [F (df) = 16.021 (4, 75); p <0.001] and also a significant interaction between strains and stacks on production of cleistothecia [F (df) = 2.552 (8, 75); p = 0.016]. All isolates/strains produced maximum numbers of cleistothecia when incubated individually, and showed a general trend of

decreased numbers of cleistothecia when plates were incubated in stacks of two or three Petri dishes.

Considering individual isolates, 2-137 formed maximum numbers of cleistothecia when plates were incubated in a single layer and numbers decreased in stacks of two and three plates, although differences were marginally outside of the level of significance (**Figure 3-14**) [A one-way ANOVA: F (df) = 3.146 (2, 15); p = 0.072). Similar results were obtained in isolate 2-139 which produced greatest levels of cleistothecia in plates incubated in a single layer depth, with the sexual fertility dropping sharply when cultures were kept in stack of 2 and 3 high respectively e.g. from 90 down to 20 cleistothecia per 100 mm^2 (**Figure 3-15**). In this case there was a significant difference between incubation in stacks and production of cleistothecia [A one-way ANOVA; F (df) = 9.686 (2,15); p <0.002].

Regarding the *veA1ku* mutant strains, 2-149 showed a dramatic *ca.* 75% decrease in number of cleistothecia formed in plates incubated in stacks of 2 and 3 compared to single plates (**Figure 3-16**).

Statistical analysis revealed strongly significant differences in production of cleistothecia according to manner of incubation in stacks (A one-way ANOVA: F (df) = 16.595 (2, 15); p <0.001). A similar pattern was evident in the *veA1ku* mutants 2-154 and 2-155 (**Figure 3-17** and **Figure 3-18**). Sexual development decreased considerably when plates were incubated in piles of 2 and 3 high, forming less than 20 cleistothecia per 100 mm^2 in stacks of 3 compared to approximately 50 cleistothecia per 100 mm^2 in plates grown in an individual layer. A one-way ANOVA revealed that there was a significant effect of incubation in stacks on production of cleistothecia of both 2-154 [F (df) = 44.111 (2, 15); p <0.001] and 2-155 [F (df) = 5.630 (2, 15); p = 0.015].

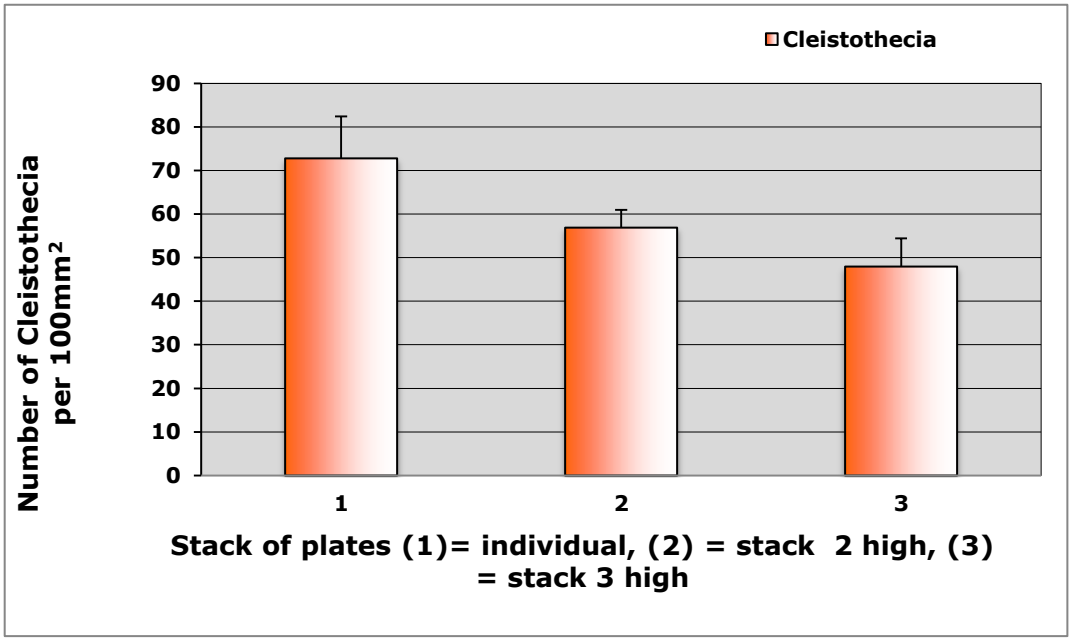

Figure 3-14 Effect of incubation in stacks on production of cleistothecia in *A. nidulans* isolate 2-137 (*ve*A). Values shown are means across six plates in replicate stacks. Error bar shows ± SEM.

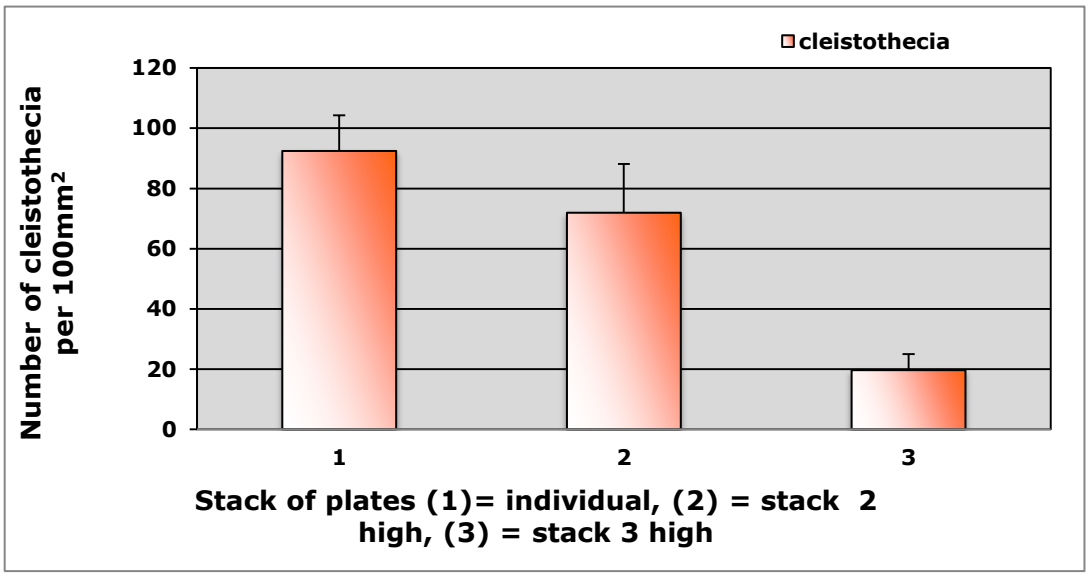

Figure 3-15 Effect of incubation in stacks on production of cleistothecia in *A. nidulans* isolate 2-139 (*ve*A). Values shown are means across six plates in replicate stacks. Error bar shows ± SEM.

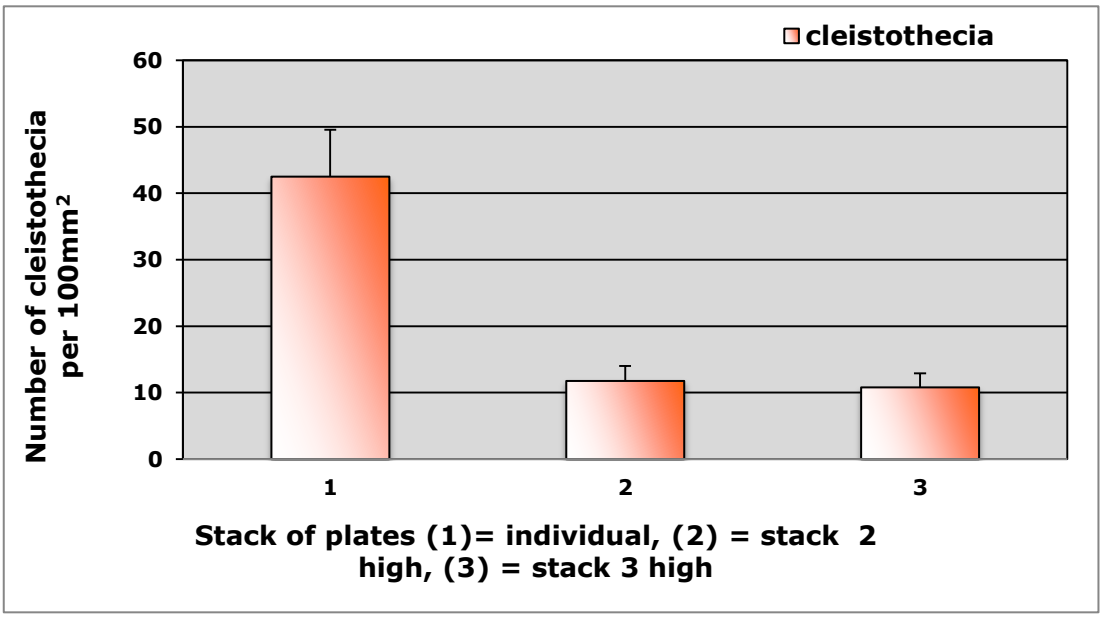

Figure 3-16 Effect of incubation in stacks on production of cleistothecia in *A. nidulans* strain 2-149 (*ve*A1). Values shown are means across six plates in replicate stacks. Error bar shows ± SEM

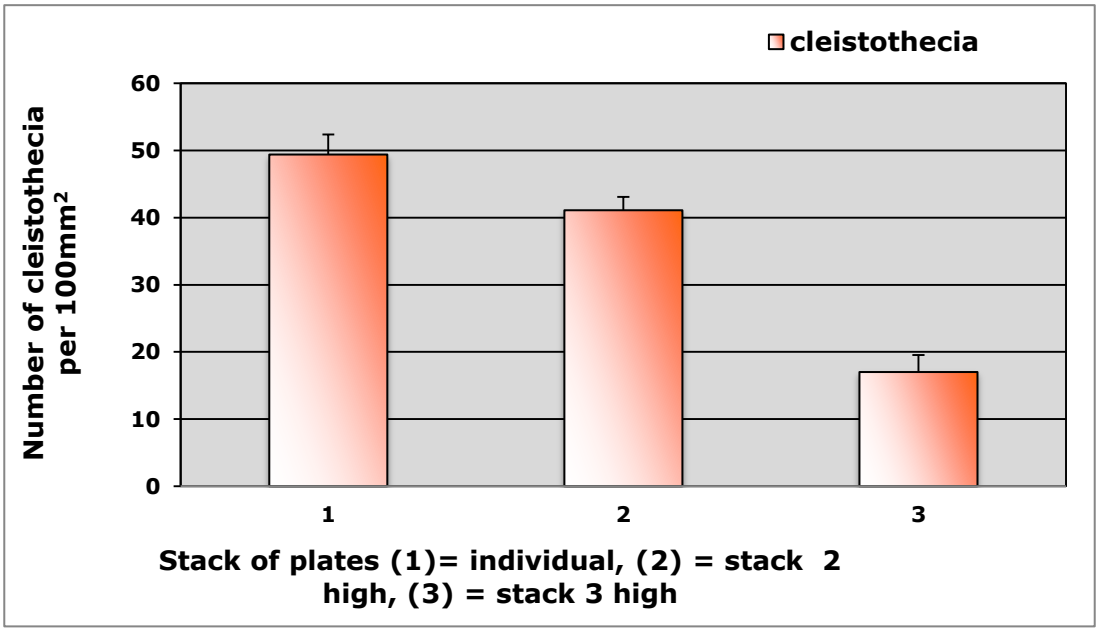

Figure 3-17 Effect of incubation in stacks on production of cleistothecia in *A. nidulans* strain 2-154 (*ve*A1). Values shown are means across six plates in replicate stacks. Error bar shows ± SEM.

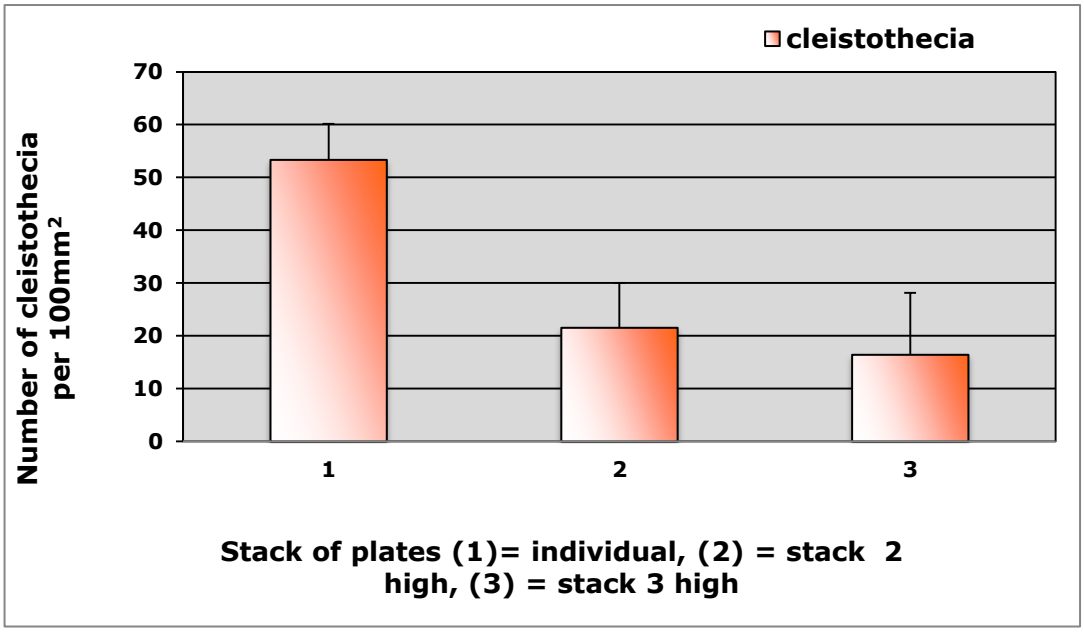

Figure 3-18 Effect of incubation in stacks on production of cleistothecia in *A. nidulans* strain 2-155 (*ve*A1). Values shown are means across six plates in replicate stacks. Error bar shows ± SEM.

3.4.4 Effect of incubation at 32°C or 37°C on sexual reproduction of *A. nidulans*

Sexual plates were scored at 8, 12, 16 and 20 d after inoculation for the presence of cleistothecia and ascospores. It was found that cleistothecia containing ascospores were present from 8 d onward at both 32ºC and 37 ºC, but not before this point. Numbers of cleistothecia and ascospores then showed a steady increase until day 20. Therefore a full statistical analysis was performed on the dataset from the endpoint day 20 (data from 8, 12 and 16 d not shown). Incubation temperature was found to have a significant influence on both numbers of cleistothecia and ascospores produced in all four isolates/strains of *A. nidulans* [2-137 and 2-139 (VeA), 2-149 and 2-154 (VeA1)] (**Figure 3-19** and **Figure 3-20**). A two-way ANOVA between all four strains revealed that there was a significant effect of incubation at 32°C versus 37°C on production of cleistothecia [F (df) = 83.242 (1, 248); p <0.001] and ascospores [F (df) = 19.700 (1, 248); <0.001], a significant effect of isolates/strains on production of cleistothecia [F (df) = 3.673 (3, 248); p = 0.013] and

ascospores [F (df) = 7.371 (3, 248); p <0.001], and also a significant interaction between isolates/strains and incubation temperature on production of cleistothecia [F (df) = 2.984 (3, 248); p = 0.032] and ascospores [F (df) = 4.370 (3, 248); p = 0.005]. Results as a whole showed that highest numbers of cleistothecia and ascospores were formed at 32°C in all strains (VeA and VeA1) with a dramatic decline in numbers at 37°C for all isolates/strains investigated (**Figure 3-19** and **Figure 3-20**). Slightly higher levels of sexual fertility were evident in the wild-type VeA isolates (2-137 and 2-139) compared to the *veA1ku* mutant strains (2-149 and 2-154) e.g. between 30-35 cleistothecia compared to 15-25 cleistothecia per 100 mm^2, respectively (**Figure 3-19**).

In more depth, a two-way ANOVA of the data for the individual isolates/strains revealed that there was a significant effect on incubation at different temperatures (32°C and 37°C) on production of cleistothecia in all strains, with statistical analysis of each strain showing the following values: 2.137 [F (df) = 32.807 (1, 62); p <0.001], 2-139 [F (df) = 18.894 (1, 62); p <0.001], 2-149 [F (df) = 18.277 (1, 62); p <0.001] and 2-154 [F (df) = 21.346 (1, 62); p <0.001].

Meanwhile, a two-way ANOVA of data from each individual isolate/strain revealed that there was a significant effect of incubation at either 32°C or 37°C on production of ascospores in some but not all strains. In detail, the wild-type strain 2-137 and the *ku* mutant 2-149 showed significant differences according to the ANOVA analysis, with the following values: 2-137 [F (df) = 33.804 (1, 62); p <0.001], 2-149 [F (df) = 8.379 (1, 62); p =0.005]. However, the wild-type strain 2-139 and the *ku* mutant 2-154 showed no significant differences [F (df) = 2.802 (1, 62); p = 0.099; and F (df) = 0.484 (1, 62); p =0.489, respectively].

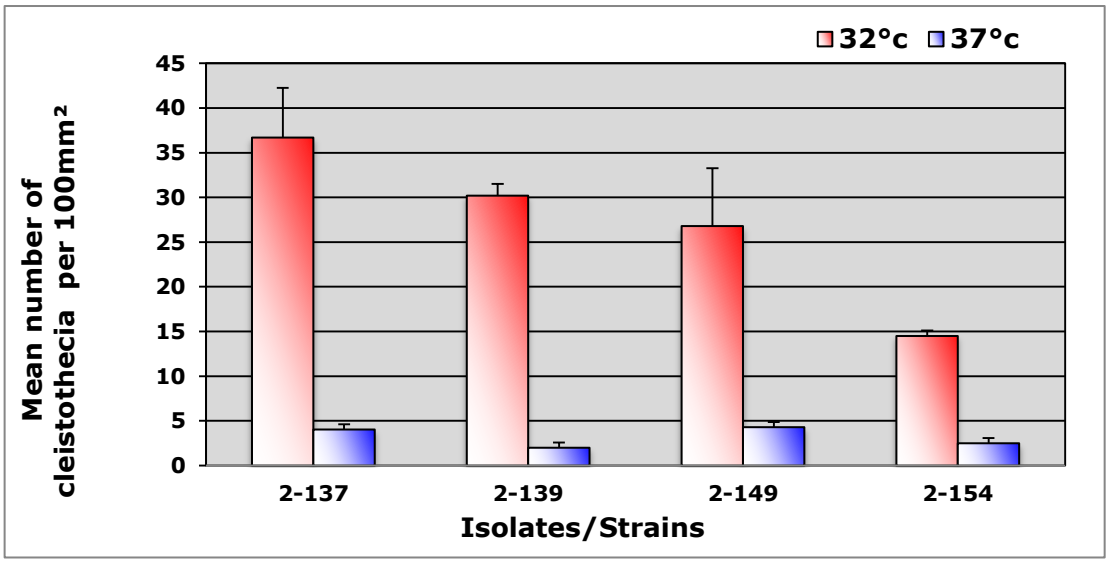

Figure 3-19 Effect of incubation temperature (32°C or 37°C) on number of cleistothecia formed in isolates/strains of *A. nidulans* (2-137 and 2-139 *ve*A; 2-149 and 2-154 *ve*A1). Error bars indicate ±SEM.

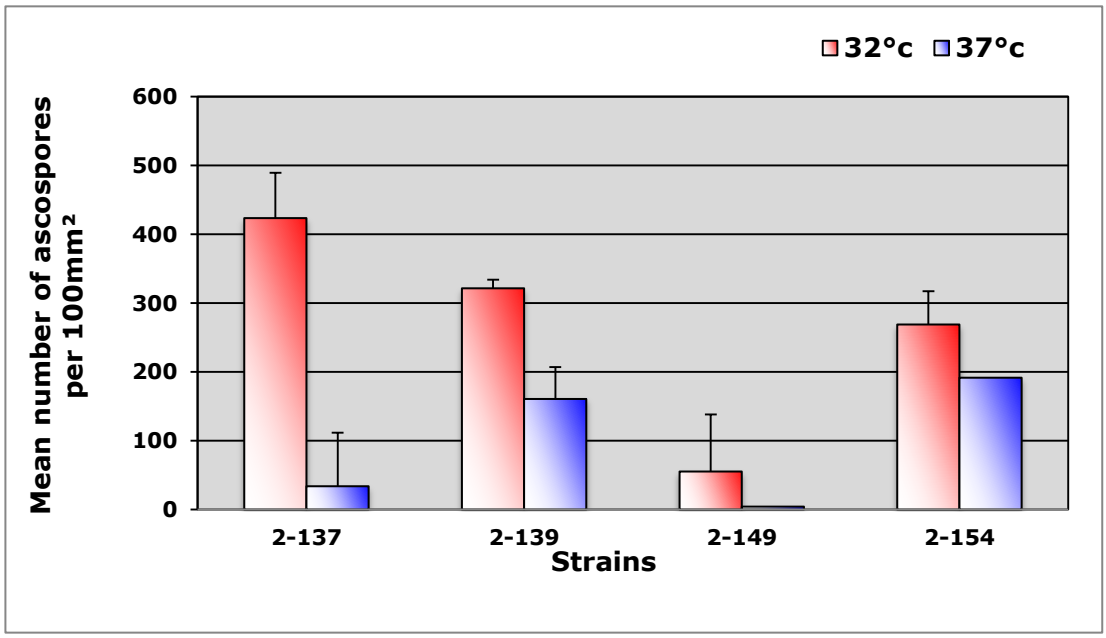

Figure 3-20 Effect of incubation temperature (32°C or 37°C) on number of ascospores formed in isolates/strains of *A. nidulans* (2-137, and 2-139 *ve*A; 2-149 and 2-154 *ve*A1). Error bars indicate ±SEM.

3.5 Discussion

The overall aim of the current study is to investigate genetic and physiological factors influencing sexual and asexual development in the genus *Aspergillus,* and how different modes of reproduction might evolve. In future work it is planned to assess whether asexuality might arise from continued vegetative propagation (**chapter 5**) and compare fertility of gene knockout strains (**chapter 6**). Therefore it was first necessary to establish conditions under which asexual and sexual reproduction could be reliably and consistently induced *in vitro* for all of the study species.

The experimental aims of this chapter were three-fold. Firstly, to identify suitable isolates/strains for study. Secondly, to determine suitable conditions to induce asexual reproduction of various isolates of *N. fischeri* and *E. repens* investigating the effect of different incubation conditions such as temperature, media, darkness and light. Thirdly, to identify conditions to reliably induce sexual reproduction in *A. nidulans,* assessing the effects of various factors including sealing plates at different time points, incubating plates in three different stacking arrangements, and incubation at either 32^{o}C or 37^{o}C.

3.5.1 *Neosartorya fischeri*

It has previously been reported that *N. fischeri* readily undergoes sexual reproduction *in vitro*, for example forming abundant cream to white cleistothecia after 14 d incubation on MEA (Raper and Fennell, 1965; Klich and Voor Schimmelcultures, 2002).

3.5.1.1 Identification of *N. fischeri* strains

Nineteen putative isolates of *N. fischeri* were obtained from worldwide sources. However, only four of these isolates (53-13, 53-16, 53-14 and 53-15) were finally shown to be *N. fischeri* following morphological and molecular analysis. This was not totally surprising given the morphological similarity of a number of homothallic *Neosartorya* species (Hong *et al.*, 2008). A series of new species have recently been isolated and described including *N. denticulate* sp. nov. (CBS 652.73) from soil in

Suriname; *N. assulata* sp. nov. (KACC 41691) from soil in a tomato field in North Korea; and *N. galapagensis* sp. nov. (CBS 117522) from soil, Galapagos Islands. All of those species were described using a polyphasic approach based on macro and micromorphology, extrolite production and partial β-tubulin, calmodulin and actin gene sequences (Hong *et al.*, 2008). Moreover, four new taxa have also been proposed based on this same approach, namely *N. australensis* (CBS 112.55T), *N. ferenczii (*CBS 121594T) and *N. warcupii* (NRRL 35723T), which all were isolated from soil in Adelaide (Australia), and in addition *N. papuaensis* (CBS 841.96) obtained from Papua New Guinea (Samson *et al.*, 2007). Additionally, Hong *et al.* (2006) have re-examined *N. spinosa, N. glabra* and other related taxa in a new survey of soil-borne *Aspergillus* and *Penicillium* in Korea, using β-tubulin and calmodulin gene sequences for the phylogenetic analyses. Consequently, two novel taxa were detected including *Neosartorya coreana* sp. nov. (CBS 117059T=NRRL 35590T=KACC 41659T) and also *Neosartorya laciniosa* sp. nov (CBS 117721T=NRRL 35589T=KACC 41657T). Beta-tubulin sequence was used to analyses the DNA sequence of members of the *A. fumigatus* sensu latu clade. Currently, 23 *Neosartorya* species including 7 new taxa and also 10 *Aspergillus* species (anamorphic) have been described from the section *Fumigati* giving a total of 33 species have been described (Horie *et al.*, 2003; Hong *et al.*, 2005; 2006; 2008; Samson *et al.*, 2007). The data presented in the current study adds to information about the geographic distribution of certain of these species. For instance, this is the first reported isolation of *N. laciniosa* from Malaysia (from jungle forest soil), this species previously having been reported from South Korea, the U.S.A., Pakistan, the Netherlands, Suriname, the Dominican Republic and Kenya (Samson *et al.*, 2007). This indicates that *N. laciniosa* is a geographically widespread species. Meanwhile, *N. spinosa* was isolated for the first time from South Africa, Malaysia and Peru. The species had previously been reported from Nicaragua, Kenya, Denmark, the Dominican Republic, the U.S.A., Belgium, Sudan, Japan, India, Pakistan, and South Korea (Samson *et al.*, 2007). This also indicates that *N. spinosa* is a widespread species. Finally, *N. paulistensis* was isolated

from Sri Lanka and Singapore (from field soil) for the first time, having been previously found only in soil in Brazil (Hong *et al.*, 2006). This indicates that the species has a wider than formally known geographical range.

3.5.1.2 Determining suitable conditions for asexual reproduction of *N. fischeri*

Neosartorya fischeri normally reproduces sexually in abundance, whereas conidia are formed in lower numbers (Raper and Fennell, 1965). When a series of incubation conditions were assayed it was found that highest numbers of conidia were produced by all five strains (53-2, 53-13, 53-16, 53-14 and 53-15) on ACM and MEA at temperatures between 28ºC and 37ºC with a remarkable decrease of asexual development on the two other media CDA and TWA assayed and at lower temperatures for some isolates. Therefore, to favour asexual sporulation it was decided to incubate cultures of *N. fischeri* unsealed under white light (2.5 µmol m^{-2} s^{-1}) at 28 ^{o}C on ACM.

The fact that ACM and MEA contain a relatively high concentration of glucose is consistent with other reports that have found that the presence of glucose in media is conducive to vegetative growth and asexual sporulation in preference to sexual development (Moore-Landecker, 1992; Elliot, 1994). Thus, the lack of sporulation on the two other media assayed could be as a result of the absence of glucose in CDA (section **2.1.1.5**) and TWA (section **2.1.1.6**). Considering the effect of temperature, the vegetative growth of fungi normally occurs over a wider range of temperatures than sexual reproduction (Moore, 1998), with sex generally induced at temperatures less than 25^{o}C (Moore-Landecker, 1992). However, *N. fischeri* is unusually thermotolerant (Beuchat, 1986). Therefore, although the results obtained in this study showed that higher temperatures of 28ºC and 37ºC were more conducive to asexual reproduction of *N. fischeri*, there was nevertheless still the possibility for sexual reproduction at these temperatures as described for other *Neosartorya* species (O'Gorman *et al.,* 2009). Meanwhile, the fact

that asexual sporulation was favoured in the light in unsealed plates is consistent with studies involving *A. nidulans,* in which sex requires darkness whilst light enhances vegetative growth and conidiation (Zonneveld, 1977). Also sealing plates can trigger sexual development in *A. nidulans* by limiting the air exchange whereas asexual development is promoted in unsealed plates (Zonneveld, 1977; Zonneveld, 1988a).

In other parallel work Hong *et al.* (2008) used four different media [Czapek yeast autolysate (CYA), Malt extract agar (MEA), CZ agar and Oatmeal agar (OA) (Samson *et al.*, 2004a)] and two different temperatures (25ºC and 37ºC) to cultivate and make macro-morphological observations with three new species of *Neosartorya*: *N. denticulate, N. assulata, N. galapagensis* and also *Aspergillus turcosus* (new *Aspergillus* of section Fumigati). It was found that all species rapidly generated a high amount of asexual colonies on MEA and CYA with different sizes and colours of conidial heads depending on the species. The same media (except for OA) were also used for growth of two novel taxa; *Neosartorya laciniosa* sp. and *Neosartorya coreana* sp. nov., which were isolated from soil in Korea (Hong *et al.*, 2006). Both of these latter species produced cleistothecia in abundance but few conidia. Thus, many homothallic *Neosartorya* species appear to show a bias towards sexual reproduction *in vitro*; this contrasts with heterothallic *Neosartorya* species which can show prolific asexual reproduction *in vitro* (e.g. *A. fumigatus*), although some species show less conidiation (e.g. *A. lentulus*, *A. fumigatiaffinis* and *A. novofumigatus*) (Hong *et al.*, 2005). The reasons for the apparent preference towards sex in homothallic species is not known, but might be related to better environmental resistance of ascospores than conidia in *Neosartorya* species (Dyer and O'Gorman, 2012).

3.5.2 *Eurotium repens*

Four isolates of *Eurotium repens* were obtained (51-3, 51-4, 51-5 and 51-6), which were first ascertained to be independent isolates by RAPD-PCR DNA fingerprinting, as used elsewhere in population studies of *Aspergillus* species (e.g. O'Gorman *et al.,* 2009).

3.5.2.1 Determining suitable conditions for asexual reproduction of *E. repens*

Conditions have been described to induce abundant sexual reproduction *in vitro* for *E. repens* (e.g. Raper and Fennel, 1965) but to our knowledge no dedicated studies have been performed to find conditions to favour asexual sporulation. Therefore different media (GMEA, MEA+20% sucrose, CDA+20% sucrose and M40Y), temperatures (25ºC and 28ºC), and light and darkness were assayed for their effect on asexual sporulation in unsealed plates.

The sugar rich media M40Y (containing 400 g of sucrose per litre) and GMEA (containing 300 g glucose per litre) were found to be the best media for growth of all the strains of *E. repens* assayed in this study, with a significant number of cleistothecia and conidia produced at both 25ºC and 28ºC in both the light and dark. By contrast, MEA+20% sucrose and CDA+20% sucrose formed fewer cleistothecia and conidia under similar conditions. These findings are in accordance with a previous study that investigated the effect of temperature and osmotic pressure of the medium on sporulation and cleistothecial formation in three *Eurotium* species*: E. herbariorum, E. amstelodami* and *E. echinulatum*. In this study Glucose Malt Extract Agar (GMEA) was found to be the best medium to promote cleistothecial formation when plates were incubated at 33°C in the dark for 21 d (Blaser, 1975). A series of other studies have also found that sporulation of *Eurotium* species is favoured on media of high osmotic potential containing glucose. For example, Splittstoesser *et al.* (1989) observed that *E. herbariorum* grew slowly on standard laboratory growth media such as potato dextrose agar but grew rapidly in media containing 10% or more sucrose, whilst media containing sucrose and glucose were found to promote growth of *E. amstelodami* and *E. rubrum* in preference to those with NaCl or glycerol of the same a_w (Wheeler and Hocking, 1988; Wheeler and Hocking, 1993; Pitt and

Hocking, 2009). This contrasts with other aspergilli such as *A. nidulans,* for which high glucose levels can inhibit sex (Han *et al.*, 2003).

With respect to temperature, both asexual and sexual sporulation occurred at 25ºC and 28ºC for of all isolates of *E. repens.* This was in agreement with Butinar *et al.* (2006) who found that *E. repens, E. rubrum* and *E. herbariorum* grew poorly or even had no growth at 37ºC, while *E. amstelodami* and *E. chevalieri* grew well at the same temperature (Blaser, 1975; Klich and Voor Schimmelcultures, 2002). Those observations indicate that *E. repens* is a mesophilic organism, preferring medium temperatures between 25ºC and 30ºC to grow and germinate, consistent with observations that members of the genus *Eurotium* were the most frequently isolated species collected from 30 samples of Egyptian date fruits on 50% sucrose–Czapek's agar medium incubated at 27ºC (Gherbawy, 2001). Meanwhile, incubation in the light or dark had no significant impact on asexual and/or sexual spores in *E. repens* at the assayed temperatures of 25ºC and 28ºC. This is consistent with previous reports that *E. repens* is among those fungi indifferent to the light (Moore-Landecker, 1982).

In previous studies of *Aspergillus* species it was found that sealing plates could significantly increase the production of cleistothecia, for example as observed for *A. nidulans* (Zonneveld, 1977; Zonneveld, 1988a) and *A. fumigatus* (O'Gorman *et al.*, 2009). By contrast *E. repens* was unexpectedly completely different because the production of cleistothecia was remarkably inhibited when plates were sealed by two layers of Nescofilm and incubated at given temperatures in the dark, whereas unsealed plates generated cleistothecia in all investigated strains (NB. the effect of sealing plates in the light on sex was not tested). This could reflect the different ecological niches of each *Aspergillus* genus, but more investigations must be considered to clarify this effect. Therefore, to favour asexual sporulation it was decided to incubate cultures of *E. repens* under white light (2.5 µmol m^{-2} s^{-1}) at 28ºC on M40Y.

3.5.3 *Aspergillus nidulans*

A. nidulans is widely used as a model fungal species for a wide range of laboratory studies including those investigating sexual development (Todd *et al.*, 2007; Dyer and O'Gorman, 2012). Since the pioneering work of Pontecorvo (1953) sexual reproduction in *A. nidulans* has routinely been induced at 37ºC, with plates traditionally sealed after 24 hr to promote the formation of cleistothecia (Braus *et al.,* 2002). However, there have been few systematic studies on the impact of certain environmental factors on sexual fertility, and production of cleistothecia in this species can be erratic in replicate plates. Therefore, the present study was carried out to determine the conditions to reliably induce sexual reproduction considering parameters such as temperature, sealing of plates and stacking of plates in different patterns. To enable this work a series of either wild-type VeA isolates or VeA1 mutant strains were obtained to represent *A. nidulans* from nature and in common laboratory usage. The genotype of these *A. nidulans* was determined using a molecular diagnostic developed in the present study.

3.5.3.1 Determination of conditions to reliably induce sexual reproduction in *A. nidulans*

Various factors were assessed for their impact on sexual fertility as follows:

Effect of time of sealing plates after inoculation:

Plates were sealed over a 24 hr time range to assay the impact on cleistothecial development. Although results were erratic, overall it was found that sexual fertility was highest in plates that were sealed 12-18 hr after inoculation at 32ºC in the dark. This differs from the conventional time for sexual plate sealing of 24 h after inoculation for *A. nidulans* (Braus *et al.*, 2002; Dyer, 2007; Todd *et al.*, 2007) and this finding should be of great value to the *A. nidulans* community as results applied to both *veA* and *veA1ku* genotypes. Plate sealing in *A. nidulans* is proposed to result in a build up of carbon dioxide which is thought to

trigger a developmental response favouring sexual reproduction (Braus *et al.,* 2002; Dyer 2007). This might be related to the importance of carbon dioxide for synthesising and breaking down alpha-1,3-glucan (Zonneveld, 1988a). Another function of sealing plates might be to cause physiological stress arising from partial blockage of the electron transport system from lowered levels of O_2 (Han *et al.*, 2003), which would again induce a sexual response. Similarly, the sexual cycle of *A. fumigatus* could be induced when paired MAT1-1 and MAT1-2 isolates were grown on Parafilm-sealed Oatmeal agar plates and incubated 6 months at 30^{o}C in the dark (O'Gorman *et al.*, 2009).

Effect of placing plates in different 'stacking' patterns:

Plates were placed on shelves within the growth incubator in different stack patterns, which has not to our knowledge been previously studied. This revealed the dramatic result that the number of cleistothecia formed differed very significantly according to the number of plates stacked; with maximum numbers of cleistothecia formed when plates were incubated individually rather than being stacked on top of each other. This effect was particularly clear in the *veA1ku* mutant strains 2-149 and 2-155 (**Figure 3-16** and **Figure 3-18**). Thus, although inconvenient in terms of space occupied in the incubator, this observation should be of value to the *A. nidulans* community to obtain reliable results for laboratory VeA1 strains. This was consistent with earlier observations that when up to six plates were stacked on top of each other, that highest amounts of cleistothecia were formed at the top of the stack, with lower numbers or absence of cleistothecia in the lower parts of the stack (data not formally recorded).

There are various possible explanations for this result. Firstly, some fungal strains are highly oxygen demanding during incubation and incubating in a single layer might permit better diffusion of oxygen into plates through the semi-permeable Parafilm. Secondly, the release of heat, arising from metabolism within cultures placed on the top of others in a stack, might somehow affect the development of cleistothecia in

plates at the bottom. Thirdly, plates on top of a stack might dry out faster than plates kept underneath, with the differential stress affecting sexual development.

In parallel studies it has been reported that growth of various *Penicillium* and *Aspergillus* species can be affected by factors such as wrapping plates, putting them in a box during incubation, half-sealing with tape, incubation upside down, incubation in darkness but with opening of the incubator door occasionally and finally incubation in complete darkness (Samson and Pitt, 2000). In the present study all cultures were incubated in the dark as it has been well established that light has a significant inhibitory effect on the number of cleistothecia formed by *Aspergillus* species, with sexual development favoured in the dark and requiring at least 24 hr of darkness at the start of reproduction (Zonneveld, 1977). Various genes encoding proteins responding to light have been identified in *A. nidulans* such as *lreA* and *lreB* (Purschwitz *et al.,* 2008), *fphA* (Blumenstein *et al.*, 2005), and *cryA* (Bayram *et al.*, 2008a). These are thought to interact with the 'velvet' complex which plays an important role in both light and dark response (Bayram *et al.*, 2010).

Effect of incubation temperature:

Surprisingly, 32°C rather than the 'classical' 37ºC was found to be the optimum temperature for sexual development of all strains of *A. nidulans* in regards to both the production of cleistothecia and ascospores. For example, up to seven times as many cleistothecia were formed by isolate 2-137 at 32°C rather than 37ºC (**Figure 3-19**). This finding should again be of value to the *A. nidulans* community who in contrast routinely incubate sex plates at 37ºC e.g. the wild-type strain JC256 *veA*$^{+}$ of *A. nidulans* isolated from a cereal field soil in Hungary was found to produce sexual spores after 7 d of incubation on a complete growth medium (Bruggeman *et al.*, 2003b), and out-crosses of *A. nidulans* are routinely set up with the two parental strains incubated at 37ºC in sealed plates (Todd *et al.*, 2007). Elsewhere Zonneveld (1977) reported that *A. nidulans* could form cleistothecia at temperatures as low as 25^{o}C.

It is noted that it is conceivable that although fewer cleistothecia might be formed at 37ºC, that ascospore development might nevertheless occur more rapidly at this temperature than at 32ºC. Indeed cleistothecia development from 50 hr onwards and ascospore development within ca. 150 hr has been claimed for some strains of *A. nidulans* (**Figure 3-21**). Thus, incubation at 37ºC might still be beneficial. However, in the present study there was no clear difference in the timing of appearance of cleistothecia, with these being visible only from 8 d onward at both 32ºC and 37ºC. Thus, it appeared that ascospores did not develop more rapidly at 37ºC than 32ºC.

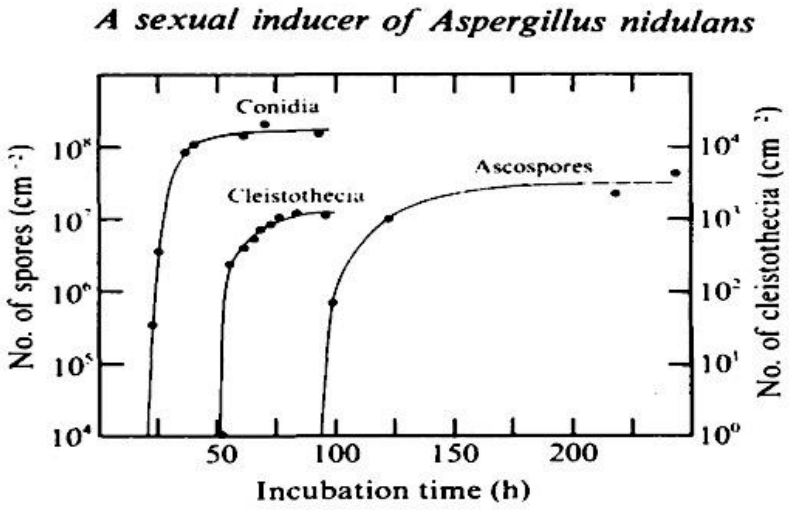

Figure 3-21 The developmental stages of *A. nidulans*. Conidia of strain WIM-64 were spread on the surface of an agar plate which was incubated at 37ºC. Adopted from (Yager *et al.*, 1982; Hermann *et al.*, 1983; Champe *et al.*, 1987)

3.6 Conclusions

Suitable conditions for asexual and sexual reproduction have been shown to vary between species of *Aspergillus* and even between strains within the same species. The three target species of *Aspergillus* used in the present study *N. fischeri*, *E. repens* and *A. nidulans* were each found to have specific conditions of light, dark, temperature, media, aeration (O_2 and CO_2) and time of incubation favouring asexual and sexual development. This allowed selection of the following conditions to favour asexual or sexual development.

Neosartorya fischeri Incubation of plates unsealed under white light (2.5 µmol m^{-2} s^{-1}) at 28ºC on ACM to favour asexual reproduction.

Eurotium repens Incubation of plates unsealed under white light (2.5 µmol m^{-2} s^{-1}) at 28ºC on M40Y to favour asexual reproduction.

Aspergillus nidulans Incubation of plates individually (rather than in stacks) at 32ºC in the dark, with plates sealed with two layers of Nescofilm between 12 hr and 18 hr after inoculation to favour sexual reproduction .

Chapter 4 Differential Spore Survival (Conidia vs. Ascospores)

4.1 Introduction

Two methods of sporulation occur in the aspergilli: asexual (mitotic) and sexual (meiotic). Asexual reproduction involves the production of clusters of conidia from characteristic conidiogenous cells on specialised branches of hyphae termed conidiophores (Moore-Landecker, 1996). The sexual cycle involves either homothallic (self fertile) or heterothallic (self sterile) breeding systems dependent on the species in question (Casselton and Zolan, 2002). Sexual reproduction involves the production of ascospores, with eight ascospores normally produced in each ascus. These asci form within an ascocarp, with various forms of closed structure cleistothecium produced in the aspergilli (**Figure 1-4**). In *Aspergillus nidulans* cleistothecia might contain up to 100,000 asci (Lee *et al.,* 2010). Both asci and the cleistothecial wall break down naturally in their habitat to release ascospores (Dyer and O'Gorman, 2012). The structure and physiology of ascospores are different to conidia, which can result in increased resistance to adverse conditions such as physical and chemical effect, particularly heat shock, in certain *Aspergillus* species (Coluccio *et al.*, 2008) as will now be described. This provides one evolutionary explanation why ascospore production might be retained in homothallic species that are otherwise able to produce enormous numbers of conidia by asexual reproduction.

4.1.1 Structural features of conidia and ascospores of *Aspergillus* species

It is thought in certain *Aspergillus* species that structural differences present between conidia and ascospores, e.g. (**Figure 4-1**) might explain differences in their dormancy and ability to resist harsh environmental stresses including oxidation, heat shock, and chemical entry into spores, which potentially could cause damage to DNA (Sussman and Douthit,

1973). In the case of *Byssochlamys fulva* at least three different components are thought to be responsible for the increased resistance of ascospores, including the presence of a thick cell wall, a large intermediate space, and a relatively dense cytoplasm (Partsch *et al.*, 1969). In particular, the ascospore cell wall is much thicker than the cell wall of the conidium, which is thought to confer increased resistance to heat shock and other stresses (Partsch *et al.*, 1969). Many other aspergilli are also found to produce ascospores with relatively thick cell walls, which might again explain their superior ability to withstand adverse environmental conditions such as temperature and desiccation compared to conidia (Baggerman and Samson, 1988; Dijksterhuis, 2007; Dyer and O'Gorman, 2012). Furthermore, there is a positive relationship between the age of fungal culture and spore resilience (Conner and Beuchat, 1987). It has been found that older ascospores of *Neosartorya fischeri* are more heat resistant than those from younger cultures, which is thought to be related to differences in the structure of ascospores such as an alteration in the inner cell wall ridge. Other structural factors which might explain the increased environmental resistance of ascospores over conidia include the potential presence of multiple layers of plasma membrane (Dijksterhuis, 2007) and the occurrence of two nuclei in ascospores versus one nucleus in conidia (Elliott, 1960).

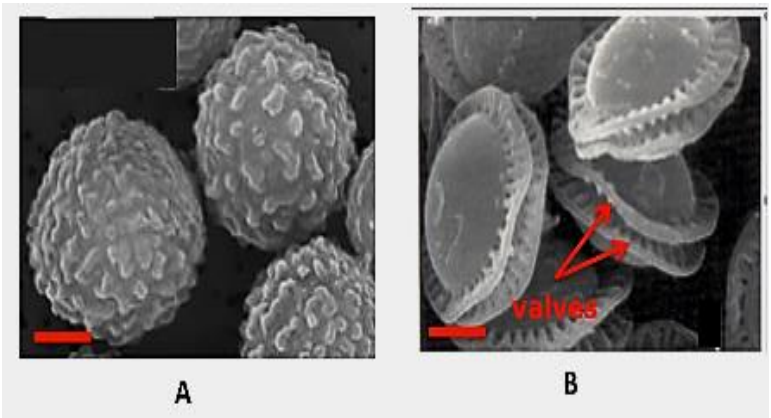

Figure 4-1 Scanning electron micrographs of *A. nidulans* spores. (A) conidia and (B) ascospores. Scale bars indicate 1 µm [Adapted from (Rossomando and Alexander, 1992)].

4.1.1.1 Physiological features of conidia and ascospores of *Aspergillus* species

In addition to structural features, there are various physiological differences between ascospores and conidia that might explain differences in their resistance to environmental stresses. Firstly, ascospores may contain increased levels of heat shock proteins (HSP), such as HSP60 and HSP70, which are molecular chaperones that help to maintain native protein structures (Dijksterhuis, 2007). Secondly, water activity (a_w) has a considerable effect on heat resistance of fungal spores (Lubieniecki and Heiss, 1975; Beuchat, 1981). For example, the heat tolerance of ascospores of *T. flavus* is increased in apple juice with sugars after heating to reduce the water activity up to 0.96 and it is well known that ascospores of several other moulds are more resistant in media of low water activity (Corry, 1987). Ascospores might contain elevated levels of sugars such as trehalose that are linked to this heat resistance. For example, increased levels of trehalose were observed in older (114 d), more heat resistant, ascospores of *N. fischeri* than those from younger (42 d) cultures, whereas no clear change in lipid or fatty acid composition was apparent (Conner and Beuchat (1987). Similarly, ascospores of *T. flavus* showed a higher heat resistance with age (Beuchat, 1988a). Dijksterhuis and Teunissen (2003) also found that ascospores of *T. macrosporus* harvested from cultures between 20 and 40 d increased in levels of heat resistance by 2.4-fold, and that older cultures between 40 and 67 d exhibited an additional increase in heat resistance levels. However, the age of cultures might have less influence on the resistance of ascospores of *E. herbariorum* (Splittstoesser *et al.*, 1989).

Also related to the presence of internal solutes is the phenomenon of "Biological glass", referring to an amorphous phase characterised by very low speed of movement of the molecules inside the cytoplasm (Buitink, 2000). A sudden lowering of temperature, reduction of water content, plunging of cells in liquid nitrogen and also controlled drying to below 3% water may introduce such a glass transition inside fungal cells, and be a

feature of ascospores of some species helping to explain their resistance to environmental stress whilst dormant (Dijksterhuis and Samson, 2006).

4.1.2 Use of *D*-value and *z*-value terms to describe heat resistance in fungi

Two main parameters that are used to describe the heat resistance of fungal cells are the so-called '*D*'-value and '*z*'-value. The *D*-value is "the decimal reduction time, usually cited in minutes, that is needed to inactivate 90% of the cells of a microorganism at a given temperature" (Put and Jong, 1982; Splittstoesser and King, 1984; King and Halbrook, 1987; Dijksterhuis and Samson, 2007). Meanwhile, the z-value refers to the increase or decrease in temperature that is needed to decrease or increase, respectively, the *D*-value by a factor 10. For example, conidia of *A. niger* have a D_{59} value of 3.3 min, meaning that 90% of conidia are heat inactivated by 3.3 min exposure to a temperature of 59ºC. Meanwhile, conidia of *A. niger* have a *z*-value of 4.9°C, meaning that a decrease in the heat shock temperature from to 59°C to 54ºC will result in a D_{54} value of around 33 min (Baggerman and Samson, 1988; Dijksterhuis and Samson, 2007). Normally one *D*-value alone is not sufficient to describe entirely the heat resistance of a spore suspension, and *D*-values are often obtained for a range of temperatures.

Some fungi are known to produce highly heat-resistant ascospores, which therefore have relatively high *D*-values. These are mostly soil-borne fungi or other fungi that affect fruits grown close to the soil, such as strawberries and pineapple. Well known species include *Talaromyces flavus* (Fravel and Adams, 1986), *Byssochlamys nivea* (Oliver and Rendle, 1934), *Neosartorya fischeri* (Kavanagh *et al.*, 1963), *Talaromyces trachyspermus* (Enigl *et al.*, 1993), *Talaromyces helices* and *Talaromyces stipitatus* (Dijksterhuis and Samson, 2006). Scholte *et al.* (2004) and Dijksterhuis and Samson (2006) reported the *D*-values of several heat-resistant fungal species noting spore survival at up to 90°C for 1.5–11 min, with for example the ascospores of *T. macrosporus* having a D_{85}-value of 100 min. Many of these species are known as

contaminants of processed fruit, canned food and vegetable products. The *D*-values of ascospores of a large number of yeast strains from soft drinks and fruits have also been investigated and they were higher than comparable conidia and vegetative cells, e.g. the D_{60}-values of ascospores of *Saccharomyces cerevisiae*, *S. chevalieri* and *Zygosaccharomyces bailii* respectively were 22.5, 13 and 10 min (Put and Jong, 1982). Ascospores of filamentous fungi are generally more heat resistant than ascospores of yeasts (Scholte *et al.*, 2004; Dijksterhuis and Samson, 2006)

4.1.3 Influence of external factors on resistance of spores to environmental stress

It is noted that a series of external factors can influence the resistance of spores to environmental stresses such as heat shock and chemical resistance. Therefore these factors must be carefully controlled where possible when assaying for resistance of spores to environmental stress. For example, the pH of external medium, the presence of organic acids, the composition (e.g. presence of sugars) and type (e.g. solid or liquid) of media can all affect the heat resistance of the majority of fungi (Splittstoesser and Splittstoesser, 1977; Beuchat, 1988b; King and Whitehand, 1990). Thus, Baggerman and Samson (1988) observed elevated *D*-values for *Hansenula anomala, Saccharomyces bisporus, Zygosaccharomyces bailii* and *Z. rouxii* after heating in a buffer solution (pH 5,5 supplied with 400 g/l sucrose) versus water alone at 56°C, 55°C, 54°C and 50°C respectively. Similar results were obtained when yeast cells were heated in grapefruit serum (Parish, 1991). In parallel, *T. macrosporus* exhibited greater heat resistance on a solid than liquid medium, and *N. fischeri, B. nivea*, *T. macrosporus* and *T. flavus* all exhibited variable heat resistance according to the type of growth media used e.g. oatmeal agar versus malt extract agar (Bayne and Michener, 1979; Beuchat, 1986; King and Whitehand, 1990). Availability of salt in media may also increase the heat resistance of fungi at lower temperatures, but not at higher temperature (Panagou *et al.*, 2002). Several organic acids such as sorbic, benzoic and fumaric acid can also

modulate the heat resistance of ascospores of some species including *Eurotium herbariorum*, *Byssochlamys nivea, N. fischeri* and *T. flavus* (Beuchat, 1981; Beuchat, 1988a; Rajashekhara *et al.*, 1998). The effect of pH on ascospore resistance to heat shock was investigated by Bayne and Michener (1979), who studied seven different strains of *Byssochlamys fluvus* grown at either pH 3.6 or 5.0. Five of the strains were found to exhibit much higher heat resistant at pH 5.0 than 3.6, while the two other strains had similar resistance at both pH values. In addition, ascospores of *E. herbariorum* were found to exhibit higher heat resistance when sugar levels were higher in the external medium e.g. a D_{70} value of 2.5 min in 5º Brix juice versus a value of 5.0 min in 65º Brix, and *z*-value of 9.1°C in 5º Brix versus 7.1°C in 65º Brix juice, indicating a protective effect of sugar (the degrees Brix value refers to the sugar content of an aqueous solution)(Splittstoesser *et al.,* 1989). Finally, sulphur dioxide may influence the stability and heat resistance of spores. For example the survival of ascospores of *Byssochlamys* species were decreased after heating in a solvent contaminated by trace levels of sulphur dioxide (Gillespy, 1938).

4.1.4 Relationship between heat resistance and requirement for external activation of spore germination

Although the production of heat-resistant ascospores can confer evolutionary advantages in terms of resistance to external environmental stress in some species, there may nevertheless be some detrimental aspects. Thus, some heat resistant ascospores require a heat shock or high pressure to break their constitutive dormancy. Constitutive dormancy can be defined as "a condition in which development is delayed as an innate property (of spores) due to factors such as a barrier to the penetration of nutrients or water, a metabolic block or as a result of the action of a self-inhibitory compound" (Dijksterhuis and Samson, 2007). Thus, thickened cell walls might impede the entry of nutrients or water and prevent germination. For example, a short heat treatment was required to activate spores to germinate (with the number of viable colonies often increasing over several log cycles) in species such as *E.*

herbariorum (15 min, 60°C) (Splittstoesser *et al.,* 1989), *N. fischeri* (Beuchat, 1986; Gomez *et al.*, 1989), *T. flavus* (80°C and 85°C) (Beuchat, 1986; King and Halbrook, 1987), *Neurospora tetrasperma* (5 min, 65°C) (Yates *et al.,* 1968) and *T. macrosporus* (7-10 min, 85°C) (Dijksterhuis and Teunissen, 2003). A study by Van Assche *et al.* (1972) also illustrated that dormant spores of the zygomycete *Phycomyces blakesleeanus* required 3 min to activate at 50°C.

In addition to a heat shock, some fungi require high-pressure activation for germination of heat resistant ascospores. It is thought that such strong shock causing damage and changes in structure of the cell wall of these spores, which then allows germination (Dijksterhuis and Teunissen, 2003). Studies by Reyns *et al.* (2003) and Dijksterhuis and Teunissen (2003) revealed that ascospores of *T. macrosporus* were activated by a high-pressure treatment even at moderate heat levels. Ascospores could germinate after short treatment at very high pressures from 400 to 800 MP_a (Dijksterhuis and Teunissen, 2003). Ascospores of *E. herbariorum* were activated from dormancy after 60 min exposure to a pressure value of 200 MP_a (Eicher and Ludwig, 2002). Entire activation of *T. macrosporus* ascospores was found after a short heat shock and exposure to 800 MP_a (Dijksterhuis and Teunissen, 2003). Indeed, there is a relationship between pressure and temperature on ascospore activation. Spores of *T. macrosporus* that were exposed to a pressure shock between 100 and 250 MP_a required a decrease in the temperature of activation by 9°C, although spores were entirely killed when at pressures greater than 250 MP_a (Dijksterhuis and Teunissen, 2003).

As described above (section **4.1.3**), the composition of the external medium can not only influence spore survival but also spore germination. Thus, Dijksterhuis and Samson (2007) observed that ascospores of *T. macrosporus* were entirely activated between 81°C and 82.5°C within 100 sec exposure at pH 6.5, but required 120 sec at pH 6.8. Meanwhile, storing food products under dry conditions ($_{aw}$=0.23) for more than 30 months allowed an increased percentage of ascospores of *T. macrosporus* to germinate without any heat treatment (Gomez *et al.*, 1989; Gomez *et*

al., 1993; Dijksterhuis and Samson, 2007). It has been suggested that a medium contains a higher level of soluble ingredients might enhance the heat activation of ascospores of *Eurotium* species given the fact that this genus is xerophilic (Splittstoesser *et al.*, 1970).

4.1.5 Evolutionary significance of ascospore formation in homothallic species

A key aim of the present thesis is to better understand the processes governing the evolution of sexual and asexual reproduction in the aspergilli. In the case of heterothallic species there are clear evolutionary benefits to sex arising from the generation of novel genetic variation through recombination during the sexual cycle (Lee *et al.,* 2010), and the spread of advantageous genes at a faster rate than in asexual reproduction (Crow, 1994). Outcrossing is also possible in homothallic *Aspergillus* species, but is likely to be much less frequent so in general there is very rarely any increased genetic variation in the ascospore offspring. Therefore, given that homothallic species are also able to reproduce asexually, an outstanding question is why is sexual reproduction maintained in the majority of homothallic aspergilli? This is particularly mysterious because there are extra metabolic costs associated with sexual compared to asexual reproduction, sexual spore formation requires a longer period of time, and a lower number of sexual than asexual spores are ultimately produced. Such factors have been termed the "cost of sex" (Bell, 1982). It is apparent that many homothallic species have evolved from previously heterothallic fungi, and that they maintain the "sexual machinery" relating to heterothallic mating (Dyer *et al.*, 2003; Paoletti *et al.*, 2007). Another reason for maintenance of sex in homothallic species might be to retain the fitness of nuclei as proposed in the 'selection arena' hypothesis of Bruggeman *et al.* (2004). One key factor that could explain the maintenance of sexuality in homothallic species is that sexual ascospores might have increased resistance to environmental stresses compared to asexual conidia, and thus would contribute to the survival of a species under harsh conditions. However, traditionally for species such as *A. nidulans* it is considered that

conidia and ascospores are equally resistant to environmental stresses (e.g. Braus *et al.,* 2003). However, to our knowledge a systematic investigation of the resistance to environmental stresses such as high temperature and UV stress has, surprisingly, not been performed for *A. nidulans* given its pre-eminence as a model species for fungal biology. Similarly, no systematic studies have been performed for the food contaminant *E. repens*. These therefore offer perfect experimental models for the assessment of differential spore survival in homothallic species.

4.2 Aims of this chapter

The principal aim of this chapter is to assess whether asexual and sexual spores of the homothallic species *A. nidulans* and *E. repens* have differential resistance to environmental stresses such as heat and UV. The hypothesis is that such differential resistance might explain why homothallic sexual reproduction has been maintained in species that are otherwise able to reproduce asexually in abundance. Particular aims are:

A. To determine the effect of heat shock for 30 min at different temperatures (50°C, 55°C, 60°C, 65°C, 70°C, 75°C) on spore viability (both conidia and ascospores) of a range of *A. nidulans* and *E. repens* isolates/strains.

B. To determine the effect of exposure to UV light (at a wavelength of 254 nm) over a time course (10–60 min) on spore viability (both conidia and ascospores) of a range of *A. nidulans* isolates/strains.

C. Based on results from (A), to determine whether it is possible to use a heat shock treatment of ascospores within entire cleistothecia to obtain pure ascospore suspensions of *A. nidulans* free of contaminating conidia, given that current methods of ascospore isolation are prone to contamination from other *A. nidulans* tissues.

4.3 Materials and Methods

4.3.1 Isolates and strains of *A. nidulans* and *E. repens*

Six representative wild-type (VeA) isolates (2-137, 2-139, 2-224, 2-227, 2-232 and 2-250) and three mutant (*veA1ku*) strains (2-149, 2-154 and 2-155) of *A. nidulans* (sections **3.3.1.1, 3.4.1.1** and **Table 3-1**) together with the four available isolates of *E. repens* (51-3, 51-4, 51-5 and 51-6; section **3.3.1.3** and **Table 3-3**) were selected for study. To ensure against any confounding effect of age of cultures all spores were harvested from cultures of known age, as described below, in which conidia and ascospores were expected to have reached maturity in terms of development of their cell walls. Also, given that pH can have a significant effect on spore survival during heat shock (Baggerman and Samson, 1988), all spore suspensions were made up in a pH 6 phosphate buffered 0.01 M Tween 20 solution (sections **2.1.2.8** and **2.1.2.9**), consistent with other *A. nidulans* experimental work (Han *et al.*, 2003).

4.3.1.1 Harvesting and heat treatment of asexual spores

Conidia of *A. nidulans* were harvested from 7 d old cultures grown on ACM in the light at 28ºC (section **2.2.2**), whilst conidia of *E. repens* were harvested from 7 d old cultures grown on M40Y medium in the light at 28ºC (section **3.3.2.2**). With a pipette, 3-5 ml of pH 6 phosphate buffered 0.01M Tween 20 solution was squirted onto the surface of the fungi and the end of pipette or a disposable plastic loop was used to agitate the surface and mix the spores into the liquid. The resulting suspension was passed through a Miracloth filter (to remove hyphal and agar debris) and the spore suspension collected in a 30 ml sterile universal tube. The resulting spore concentration was determined using an Improved Neubauer haemocytometer, ensuring that suspensions were vortexed thoroughly to ensure an even distribution of spores. This initial spore suspension was then diluted appropriately with further buffered Tween solution to a final concentration of 625 spores ml^{-1}.

For heat shock work, 190 µl of the final diluted spore suspension was aliquoted into a series of four replicate sterile PCR tubes. These were then placed in PCR machines programmed to have a constant holding temperature of 50°C, 55°C, 60°C, 65°C, 70°C or 75°C. Four replicate tubes were also left without heating at room temperature to act as a control. Where practical, all isolates/strains were heated in tandem. After 30 min of heating, the samples were removed and vortex mixed and then 40 µl aliquots from each tube pipetted onto each of four 9.0 cm ACM plates (i.e. a total of 160 µl from each tube) and the suspension was distributed across the agar surface with a sterilised spreader. Plates were left for 3 d at a constant temperature of 28°C in the light to allow the spores to form visible colonies, and were then stored at 4ºC to prevent any further growth. Visible colonies were then counted and the total number of the spores per replicate treatment summed from the four individual ACM pseudo-replicate plates. Finally, survival data at each individual temperature was expressed as a percentage of number of spores germinating in the room temperature control.

4.3.1.2 Harvesting and heat treatment of sexual ascospores

To induce the formation of sexual ascospores in *A. nidulans*, 1 x 10^5 conidiospores were spread over the surface of a 5.0 cm ACM agar plate (Paoletti *et al.*, 2007). Petri dishes were incubated in dark at 32^{o}C for 15 hr and then plates sealed with two layers of Nescofilm and returned back to the incubator at 32^{o}C and left for a further 30 d to ensure maturation of cleistothecia (section **3.3.3.1**). To induce the formation of sexual ascospores in *E. repens* isolates, 1 x 10^5 conidiospores were spread over the surface of a 5.0 cm unsealed M40Y agar plate and incubated at 28ºC for 7 d in the light.

For both *A. nidulans* and *E. repens* ascospores then had to be isolated from encasing cleistothecia, which were themselves coated in mycelium and contaminating conidia. In order to clean the cleistothecia, groups of cleistothecia were teased apart (as they were often clustered) and then an individual cleistothecium was rolled over the surface of 4% tap water agar plates to remove any mycelium, conidia and other fungal debris

(Todd *et al.*, 2007) (section **4.3.4**). Cleistothecia were then suspended in 100 µl of the pH 6 tween buffer solution. It was expected that each cleistothecium would contain thousands of ascospores (Bennett, 2010). However, in order to ensure enough sexual spores were present, approximately 50-100 cleistothecia were collected. A needle point was then used to carefully burst the structures releasing the ascospores, using gentle vortexing to mix the mixture evenly. An additional 400 µl of pH 6 tween buffer was then added to the spore suspension, followed by vortexing. The spore concentration was then measured using an Improved Neubauer haemocytometer, ensuring that suspensions were vortexed thoroughly to ensure an even distribution of ascospores. Ascospores were finally diluted down to 625 ascospores ml^{-1} in pH 6 tween buffer solution. Heat treatment was then performed in exactly the same way as described above for the heat shock of conidiospores at room temperature, 50°C, 55°C, 60°C, 65°C, 70°C and 75°C for 30 min (section **4.3.1.1**).

4.3.2 Determination of *D*-values of conidia and ascospores of *A. nidulans* relating to heat shock

As mentioned, the *D*-value (decimal reduction time) is the time (usually in minutes) that is needed to inactivate 90% of the microorganisms at a given temperature. Data is often drawn as a linear curve, plotting the number of surviving microorganisms on a logarithmic scale versus the time of heating, although non-linear death-rate kinetics can sometimes be observed (Put and Jong, 1982; Splittstoesser and King, 1984; King and Halbrook, 1987; Dijksterhuis and Samson, 2007).

To date no data is available in literature describing the *D*-values of both conidia and ascospores of *A. nidulans*. Therefore as part of the current study the first measurements of *D*-values relating to heat shock were made for this species. During initial heat shock work it became apparent that temperatures around 55°C appeared to provide the most reliable conditions to achieve *ca.* 90% kill of conidia and ascospores. Therefore, this temperature was selected to be the main representative temperature

used to determine the *D*-value of asexual and sexual spores of *A. nidulans.* Spore suspensions (conidia and ascospores) of the nine isolates/strains of *A. nidulans* [2-137, 2-139, 2-224, 2-227, 2-232, 2-250, 2-149, 2-154 and 2-155) (section **3.3.1.1** and **Table 3-1**) were made exactly as described for the former heat shock experiment (section **4.3.1.1**). 190 µl of the spore suspensions from each isolate/strain were subjected to heat shock at 55°C over a time course of 40 min, 55 min, 70 min, 85 min and 105 min, with a separate 190 µl aliquot of the spore suspension left at room temperature without heating to act as a control. Once the treatment was completed, aliquots of the spore suspensions were spread across each sets of four 9.0 cm ACM petri dishes per temperature as described above (section **4.3.1.1**). All the plates were then incubated at a constant temperature of 28°C for 3 d and visible colonies scored (section **4.3.1.1**). The data was then plotted on a logarithmic scale as the number of surviving spores on the vertical axis versus the heating times on the horizontal axis, resulting in a linear relationship for *D*-value calculation. Precise values were obtained using the equation editor on Microsoft Excel, using the Y= mx + c equation form.

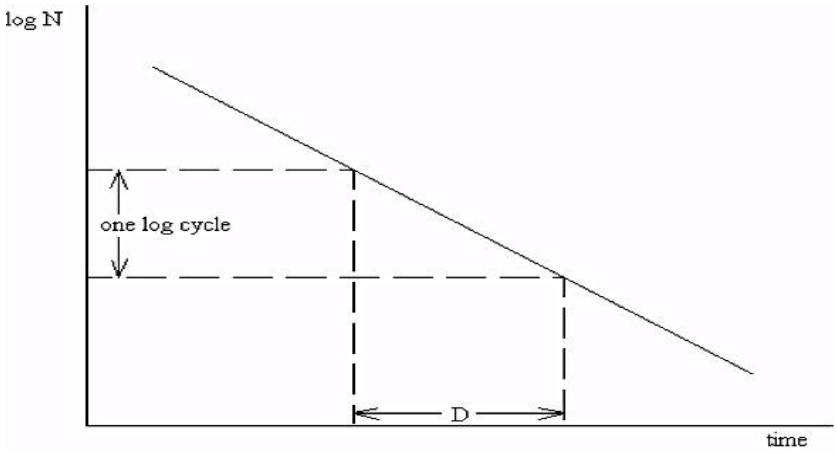

Figure 4-2 A linear curve shows the number of surviving microorganisms on the vertical axis (log N) versus the time of heating on the horizontal axis.http://sstweb.tees.ac.uk/external/U0000504/Notes/ProcessPrinciples/Sterilisation/Sterilisation.html.

4.3.3 The effect of UV radiation on spore viability of *A. nidulans*

4.3.3.1 Isolates and strains of *A. nidulans* and UV measurement

To investigate possible differences in resistance between asexual and sexual spores in *A. nidulans*, both conidia and ascospores had to be subjected to UV radiation for a certain time and at a known wavelength. Six wild-type isolates (2-137, 2-139, 2-224, 2-227, 2-232 and 2-250) and two laboratory *veA1ku* mutant strains (2-149, 2-154) of *A. nidulans* were selected for study. A Sylvania G15T8 germicidal UV-C source emitting at a wavelength of 254 nm was used for UV treatment. The intensity of UV exposure was determined using a J-225 Blak-Ray UV Meter from Ultra-Violet Products (San Gabriel, California, USA).

4.3.3.2 Effect of UV exposure on survival of asexual conidia and sexual ascospores

Conidia of *A. nidulans* were harvested from 7 d old cultures grown on ACM in the light at 28ºC as previously described (section **2.2.2**). Meanwhile ascospores were harvested from approximately 100 mature cleistothecia that had been already grown at 32^{o}C in the dark for 4 weeks as previously described (section **4.3.1.2**). Conidial and ascospore suspensions of 1 x 10^5 spores ml^{-1} were then made using pH 6 phosphate buffered 0.01M Tween 20 solution as described above (section **4.3.1.1**). To provide a sufficient volume of spore suspension for UV exposure, 3 ml of the spore suspension was measured into individual sterilised 9.0 cm Petri dishes that contained a sterilised stir bar on the bottom. Petri dishes were then placed into a sterile UV cabinet. In initial work a UV intensity of 100 µW cm^{-2} (corresponding to 0.0001 Joule s^{-1}) was found to provide a suitable kill rate, which was achieved by adjusting the height of the Petri dish relative to the UV lamp (35 cm between lamp and Petri dish, see **Figure 4-3B**; data not shown). The lid of the Petri dish was taken

off, then the magnetic stirrer and UV lamp were turned on and spore suspension was subjected to the UV radiation at different times of 10 min, 20 min, 30 min, 40 min, 50 min and 60 min (**Figure 4-3**). The 9.0 cm Petri dishes were shifted out carefully at each 10 min interval and 4 replicates of 190 µl aliquots from the treated spore suspension were taken out (a similar aliquot was also removed from each plate at 0 min to act as a control) and stored in sterile Eppendorf tubes. Finally, 40 µl aliquots were spread over four 9.0 cm ACM plates as described above (section **4.3.1.1** and **Figure 4-3**). All cultures including the control plates were then incubated for 3 d at a constant temperature of 28°C to allow the spores to form visible colonies. Finally, these colonies were counted and the survival of the spores for each time from 10 to 60 min expressed as a percentage of the number of spores germinating in the 0 min control.

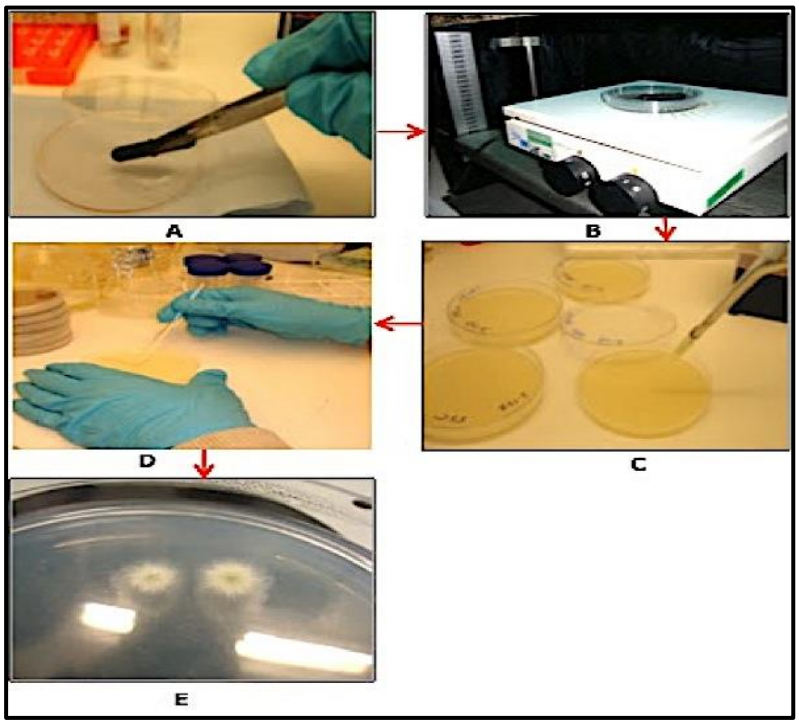

Figure 4-3 Procedure for UV exposure of spores of *A. nidulans.* (A) spore suspension were placed in a sterilised 9.0 cm Petri dish with a stirrer bar. (B) The Petri dish on a magnetic stirrer under the UV light. (C) Pipetting 40 µl onto each of the 4 plates. (D) Distributing over agar surface with a sterilised spreader. (E) Visible colonies after 3 d of incubation.

4.3.3.3 Determination of *D*-values of conidia and ascospores of *A. nidulans* relating to UV exposure

D-values for spore survival of the various isolates/strains of *A. nidulans* were calculated by plotting data on a logarithmic scale as the number of surviving spores on the vertical axis versus the UV exposure time on the horizontal axis, resulting in a linear relationship for calculation as described above for the heat shock work (section **4.1.2** and **Figure 4-2).** Precise values were obtained using the equation editor on Microsoft Excel, using the Y= mx + c equation form.

4.3.4 Influence of heating entire cleistothecia on isolation of ascospores of *A. nidulans*

Ascospores arise within asci that are surrounded by cleistothecial wall tissues, which themselves may be coated in vegetative mycelia and conidia. Therefore, ascospores must be cleaned very carefully to remove any fungal debris and also adhering conidia. This is traditionally achieved by rolling cleistothecia on TWA plates to remover contaminating hyphae and conidia prior to crushing of cleistothecia to obtain ascospores (Todd *et al.*, 2007). However, this method is still prone to contamination, especially from cleistothecial wall tissues and ascogenous hyphae. It has recently been found that ascospores of certain other *Aspergillus* species have significantly increased resistance to heat shock compared to conidia when incubated within cleistothecia, presumably because the cleistothecial wall tissues act to buffer changes in temperature and so confer extra protection to ascospores (Swilaiman *et al.*, 2013). In addition, preliminary results from the present chapter suggested that ascospores of *A. nidulans* might exhibit slightly greater resistance to thermal stress than conidia. Therefore an experiment was performed to determine whether this might provide an effective way to isolate pure ascospores suspensions of *A. nidulans*, noting the dogma that conidia and ascospores of *A. nidulans* are said to have very similar resistances to environmental stress such as temperature (Braus *et al.,* 2002).

Cleistothecia from two wild-type isolates (2-137, 2-139) and two *veA1ku* mutant strains (2-149, 2-154) of *A. nidulans* were obtained from 4 wk old cultures and then individual cleistothecia were rolled gently over the surface of 4% TWA plate, before being suspended in 500 µl of pH 6 phosphate buffered 0.01M Tween 20 solution as described above (section **4.3.1.1**). To ensure sufficient ascospores were within the suspension, around 50-70 cleistothecia were picked up and cleaned using the point of a sterile wire. Whole cleistothecia were then heated for 30 min at different temperatures (65°C, 68°C, 70°C and 72°C) before being ruptured as previously described (section **4.3.1.1**). Then an additional 500 µl of phosphate buffer was added separately to each Eppendorf tube containing the spore suspension and vortexed twice to distribute spores evenly into the mixture. Tubes were then centrifuged at 12,000 rpm for 10 min at 4ºC. To ensure that all cleistothecia within the suspension were entirely broken down, an additional sterilised disposable loop was used to gently burst any remnant cleistothecia. Again, tubes were vortexed twice to distribute ascospores evenly within suspension. The spore concentration was then determined using an improved Neubauer haemocytometer. The spore suspension was then diluted to 625 spores ml^{-1} and 40 µl aliquots were pipetted onto 9.0 cm ACM plates (using 4 replicates per strain per temperature) and distributed across the agar surface with a sterilised spreader. Plates were then left for 3 d at a constant temperature of 28°C to allow the spores to grow and form visible colonies. These colonies were then counted and the total number of visible colonies established for each temperature. Meanwhile, for comparison, conidia from the same isolates/strains aforementioned were subjected to the same range of temperatures (65°C, 68°C, 70°C and 72°C) to determine whether there was differential resistance of conidia and ascospores under these heat shock regimes. In order to form a spore suspension, conidia were extracted from 7 d old cultures. A 1 x 10^5 spores ml^{-1} solution was prepared by harvesting spores through Miracloth filters in pH6 phosphate buffered 0.01M Tween 20 solution as described above (section **4.3.1.1).** A 1 x 10^5 spores method was used to calculate the spore concentration (utilising an improved Neubauer

haemocytometer). Then an additional 500 µl of phosphate buffer was added separately to each Eppendorf tube of the suspension and vortexed twice gently to mix the spores evenly. Then 40 µl aliquots were pipetted onto ACM plates (using 4 replicates per strain per temperature) and distributed across the agar surface with a sterilised spreader. The plates were then left for 3 d at a constant temperature of 28°C to allow the spores to form visible colonies. These colonies were then counted under light microscope and data analysed statistically for each temperature. In each experiment, a tube of either ascospores or conidia was also left at room temperature for 30 min to act as a control and to provide a success rate with which the other temperatures could be compared.

4.3.5 Statistical Analysis

Statistics software (SPSS, version 21) for Macintosh (Mac OS X version 10.6.8) was used. ANOVA (analysis of variance) was used to analyse the original data (i.e. before conversion to percentages) and to determine whether the results were significant at a 5% confidence level according to Barnard *et al.* (2007).

4.1 Results

4.1.1 Heat treatment and survival of asexual and sexual spores of *A. nidulans* and *E. repens*

Both conidia and ascospores of nine *A. nidulans* and four *E. repens* isolates/strains were obtained and subjected to different heat shock treatments with results as follows.

4.1.1.1 Effect of heat shock on viability of conidia and ascospores of *A. nidulans*

Figure 4-4 to **Figure 4-12** clearly demonstrate that the heat shock had a significant effect on spore viability (both conidia and ascospores) of *A. nidulans* in both the wild-type isolates (2-137, 2-139, 2-224, 2-227, 2-232, 2-250; VeA) and the *veA1ku* mutant strains (2-149, 2-154 and 2-

155; VeA1) when spores were subjected to the different temperatures 50°C, 55°C, 60°C, 65°C, 70°C, and 75°C for 30 min (sections **4.3.1.1**).

Considering the results as a whole, ascospores were found to have a slightly greater level of heat resistance than conidia in all strains. Spore viability (as determined by germination rate relative to the room temperature control) decreased gradually as the applied temperature was increased. Generally, both ascospores and conidia were entirely inactivated at 70-75^{o}C in most isolates/strains with exception of some wild-type isolates that showed very minor survival rate after 70^{o}C. Two-way ANOVA encompassing data for either conidia or ascospores of all isolates/strains revealed that there was a significant overall effect of temperature on survival of both conidia [F (df) = 489.824 (6, 189); p <0.001], and ascospores [F (df) = 891.726 (6, 189); p <0.001], a significant effect of strain on conidia [F (df) = 31.873 (8, 189); p <0.001] and ascospores [F (df) = 103.296 (8, 189); p <0.001], and also a significant interaction between temperature and strain on both conidia [F (df) = 7.593 (48, 189); p <0.001] and ascospores [F (df) = 27.372 (48, 189) ; p <0.001]. Meanwhile, a separate two-way ANOVA incorporating data for all isolates/strains and survival of ascospore versus conidia in the same analysis revealed that there was a significant difference in survival of ascospores versus conidia [F (df) = 6.2 (125,1); p = 0.014], a significant effect of temperature on spore survival [F (df) = 17.5 (125,6); p <0.001], but no significant interaction between spore type and temperature [F (df) = 1.6 (125,6); p =0.16].

Wild-type isolates: Regarding individual isolates/strains, the wild-type 2-137 and 2-139 showed very similar results.

Figure 4-4 shows that there was a significant effect of temperature on isolate 2-137 on both survival of conidia [A one-way ANOVA: F (df) = 195.989 (5, 18); p <0.001), and ascospores (F (df) = 207.218 (5, 18); p <0.001). Similarly, **Figure 4-5** shows that there was a significant effect of temperature on strain 2-139 of both conidia [A one-way ANOVA: F (df) = 161.296 (5, 18); p <0.001), and ascospores [F (df) = 311.081 (5, 72);

p <0.001]. Survival of both conidia and ascospores of 2-137 and 2-139 was high at 50°C compared to the control (*ca.* 90% survival). However, the number of conidia surviving in both isolates decreased sharply at 55°C, whereas higher numbers of ascospores survived e.g. for 2-137 only 8.8% of conidia survived compared to 86.8% of ascospores (**Figure 4-4**), whilst 14.4% of conidia survived compared to 84.0% of ascospores at 55°C and then dropped sharply at 60°C giving a survival rate of 6.9% **Figure 4-5**). Above this temperature both ascospores and conidia showed low survival, with no spore germination after heat treatment at 65°C.

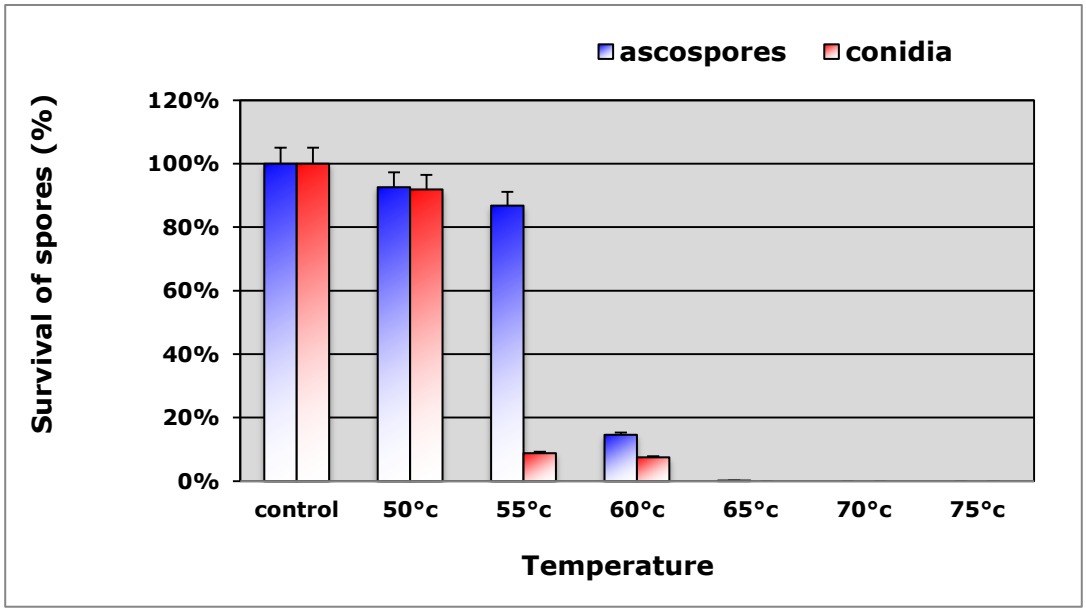

Figure 4-4 Effect of heat shock on spore viability of *A. nidulans* isolate 2-137 (wild-type). Spore survival is expressed relative to room temperature controls. Error bars indicate ± SEM.

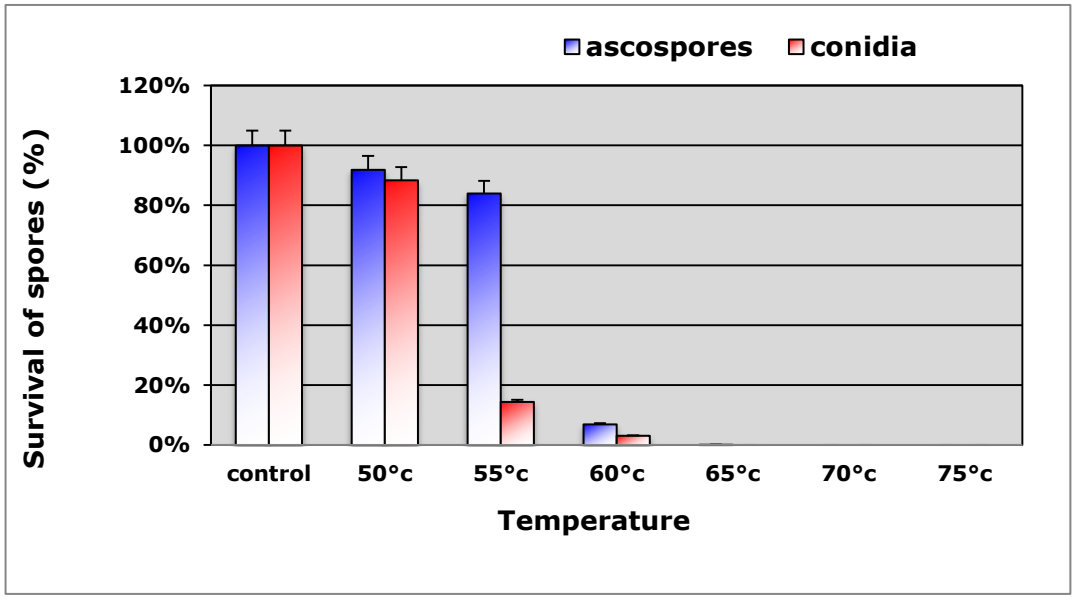

Figure 4-5 Effect of heat shock on spore viability of *A. nidulans* isolate 2-139 (wild-type). Spore survival is expressed relative to room temperature controls. Error bars indicate ± SEM.

The other four wild-type isolates 2-224, 2-227, 2-232 and 2-250 yielded slightly different results, but showed the same overall pattern as 2-137 and 2-139. Both conidia and ascospores showed good survival (ca. 90% or greater) at 50ºC, but then exhibited differential survival at 55ºC, with ascospores showing higher percentage survival values (**Figure 4-6** to **Figure 4-9**). However, unlike 2-137 and 2-139, a small percentage of ascospores continued to show survival even up to 75°C, and in the case of 2-250 some conidia survived at 60ºC for 30 min.

Specifically, **Figure 4-6** illustrates that there was a significant effect of temperature on isolate 2-224 of survival of both conidia [A one-way ANOVA: F (df) = 227.030 (6, 21); p <0.001], and ascospores [F (df) = 44.698 (6, 21); p <0.001]. 80.0% of ascospores survived heat treatment at 55°C whereas only 28.0% of conidia survived. Similarly, **Figure 4-7** illustrates that there was a significant effect of temperature on isolate 2-227 of survival of both conidia [A one-way ANOVA: F (df) = 36.664 (6, 21); p <0.001], and ascospores [F (df) = 79.712 (6, 21); p <0.001]. Conidia survival decreased to 34.0 % at 55°C whereas ascospores had a higher survival rate of 89.7%. Isolate 2-232 also illustrated a similar

survival pattern (**Figure 4-8**). A one-way ANOVA revealed that there was a significant effect of temperature on survival of both conidia [F (df) = 81.450 (6, 21); p <0.001], and ascospores [F (df) = 58.757 (6, 21); p <0.001]. There was a decrease to 29.0% of conidia surviving after heating at 55°C, whereas 85.0% of ascospores remained viable at the same temperature. For the final wild-type isolate 2-250, a one-way ANOVA revealed that there was a significant effect of temperature on survival of both conidia [F (df) = 349.635 (6, 21); p <0.001], and ascospores [F (df) = 283.936 (6, 21); p <0.001]. Ascospores showed 89.9% survival at 55°C, whereas only 33.0% of conidia survived at this temperature. Interestingly, both conidia and ascospores had the similar survival values (8.5%) at 60°C, but whereas some ascospores survived above this temperature, at temperatures of 65°C or higher no viable conidia were found (**Figure 4-9**).

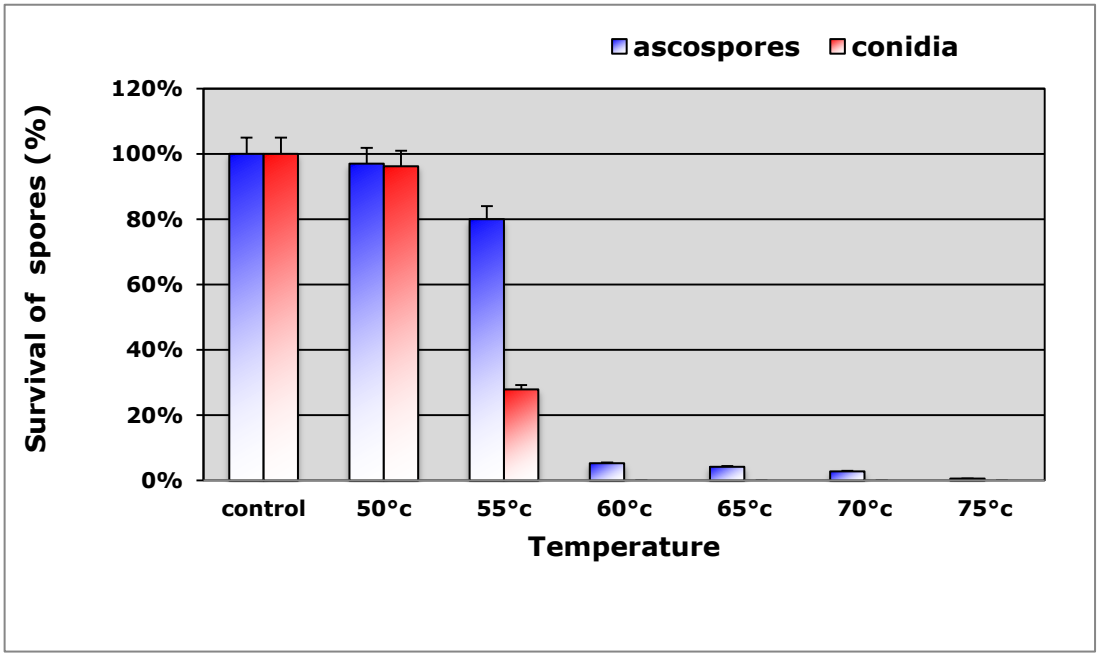

Figure 4-6 Effect of heat shock on spore viability of *A. nidulans* isolate 2-224 (wild-type). Spore survival is expressed relative to room temperature controls. Error bars indicate ± SEM.

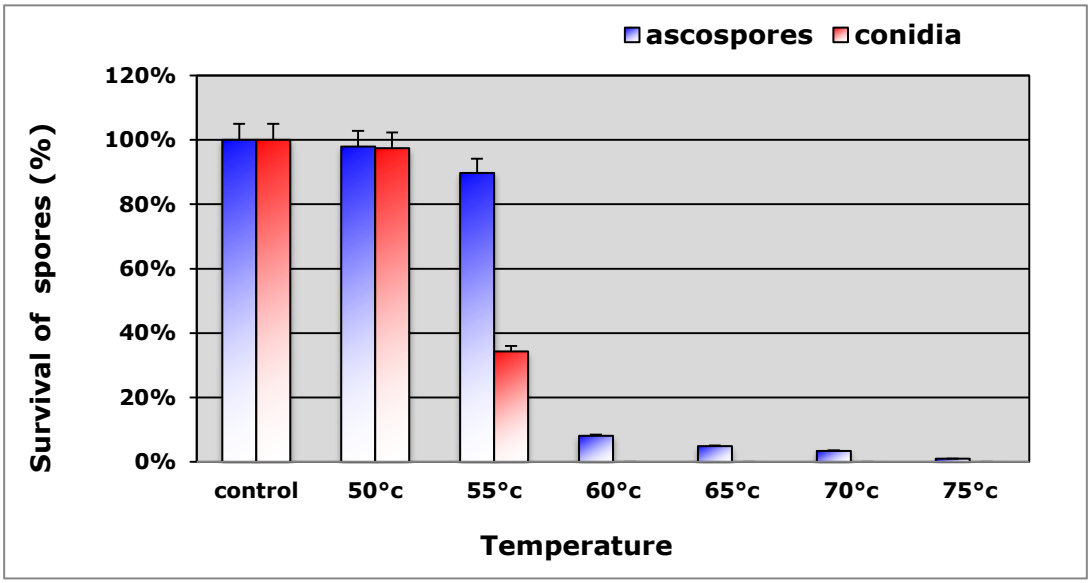

Figure 4-7 Effect of heat shock on spore viability of *A. nidulans* isolate 2-227 (wild-type). Spore survival is expressed relative to room temperature controls. Error bars indicate ± SEM.

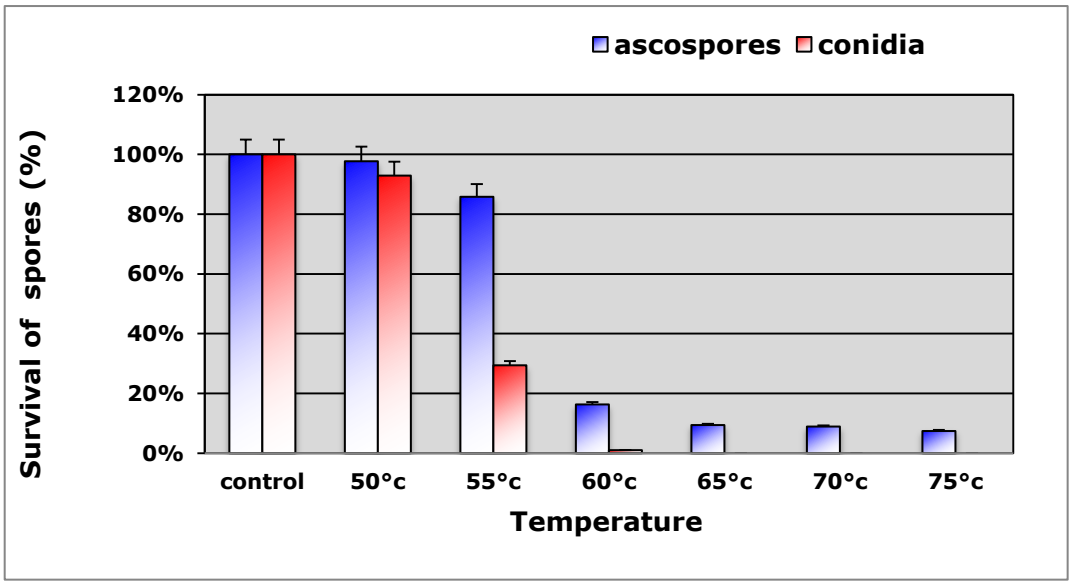

Figure 4-8 Effect of heat shock on spore viability of *A. nidulans* isolate 2-232 (wild-type). Spore survival is expressed relative to room temperature controls. Error bars indicate ± SEM.

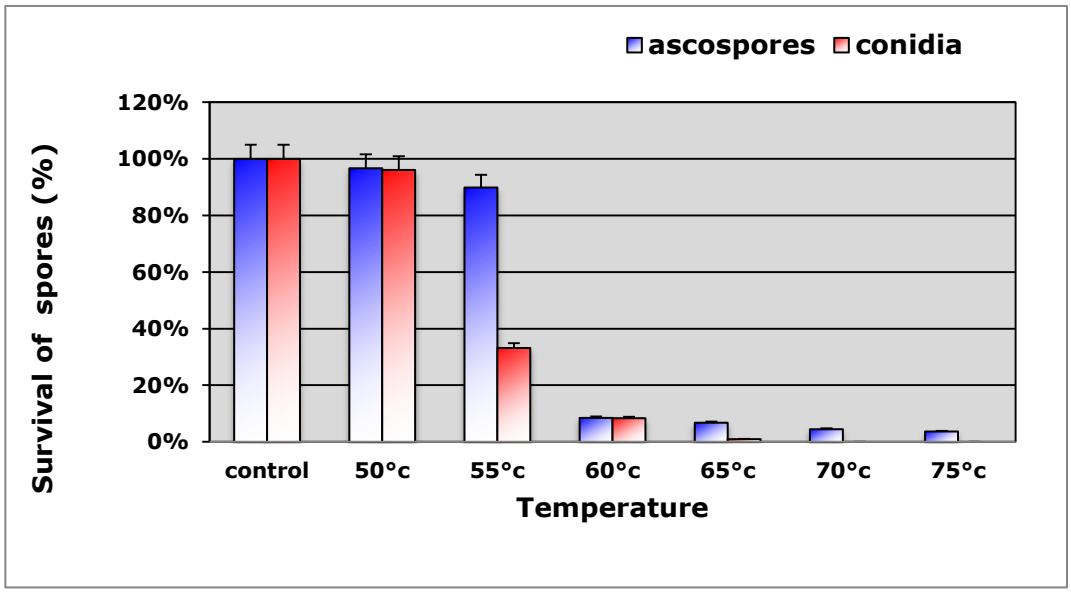

Figure 4-9 Effect of heat shock on spore viability of *A. nidulans* isolate 2-250 (wild-type). Spore survival is expressed relative to room temperature controls. Error bars indicate ± SEM.

Laboratory strains: Regarding individual isolates/strains, the laboratory strains showed less clear differences in survival between ascospores and conidia. There was a significant effect of temperature on spore survival in strain 2-154 for both conidia [A one-way ANOVA: F (df) = 98.368 (5, 18); p <0.001], and ascospores [F (df) = 172.139 (5, 18); p <0.001] (**Figure 4-10**). Similarly, a significant effect of temperature was evident of spore survival of strain 2-155 for both conidia [A one-way ANOVA: F (df) = 47.467 (5, 18); p <0.001], and ascospores [F (df) = 44.548 (5, 72); p <0.001] (**Figure 4-11**). Conidia of both strains 2-154 and 2-155 showed higher heat tolerance than the wild-type strains, a small percentage showing survival at both 60 and 65 ºC, although survival of conidia was constantly higher e.g. in the case of 2-154 conidia exhibited 57.4% survival at 55°C, while ascospores showed 90.4% at the same temperature. Both conidia and ascospores were almost entirely inactivated later at higher temperatures 70°C-75°C. However, there was unexpected decrease in the number of viable ascospores in strain 2-149 surviving at 55°C compared with conidia, only 19.40% surviving compared to 35.40% of conidia (**Figure 4-12**). Similar numbers of conidia and ascospores survived at 60°C, but at 65°C and 70°C the ascospores exhibited slightly greater levels of survival compared with the

conidia. Both conidia and ascospores were destroyed at 75°C. A one-way ANOVA revealed that there was a significant effect of temperature on survival of spores in 2-149 for both conidia [A one-way ANOVA: F (df) = 33.496 (5, 18); p <0.001], and ascospores [F (df) = 74.118 (5, 18); p <0.001].

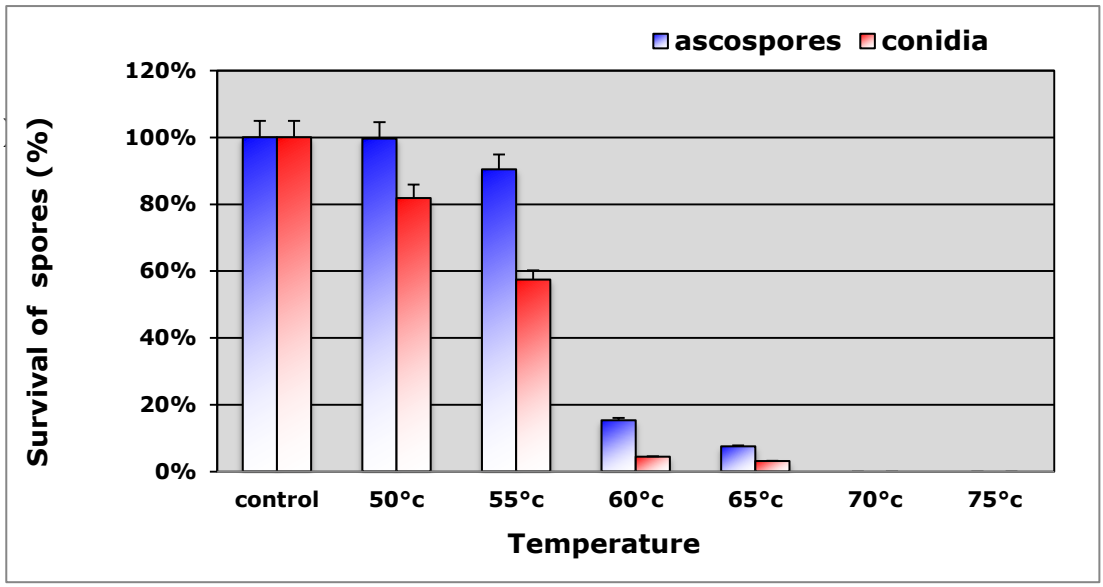

Figure 4-10 Effect of heat shock on spore viability of *A. nidulans* strain 2-154 (*ve*A1*Ku* mutant). Spore survival is expressed relative to room temperature controls. Error bars indicate ± SEM.

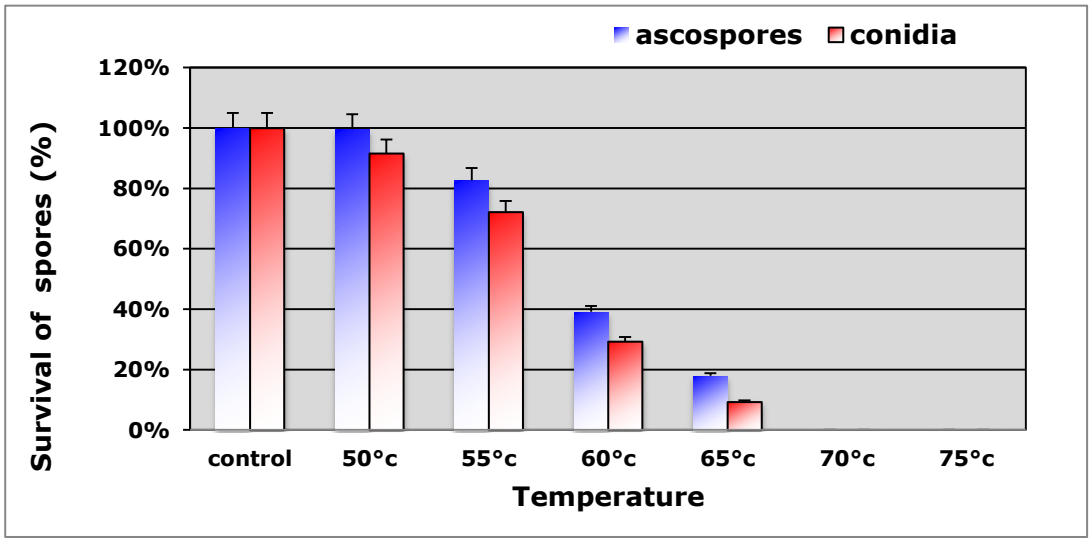

Figure 4-11 Effect of heat shock on spore viability of *A. nidulans* strain 2-155 (*ve*A1*Ku* mutant). Spore survival is expressed relative to room temperature controls. Error bars indicate ± SEM.

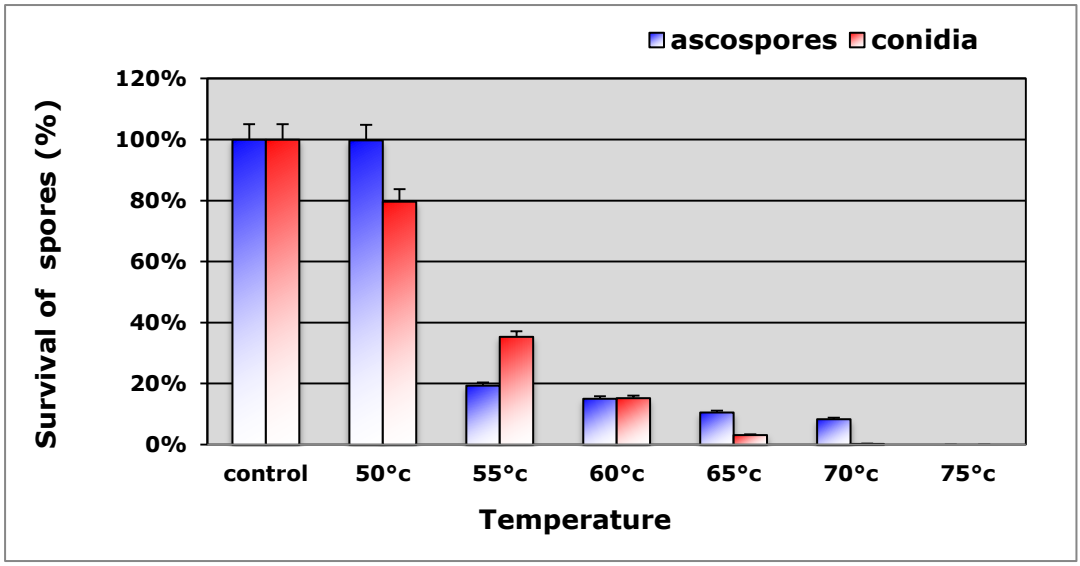

Figure 4-12 Effect of heat shock on spore viability of *A. nidulans* strain 2-149 (*ve*A1*Ku* mutant). Spore survival is expressed relative to room temperature controls. Error bars indicate ± SEM.

4.1.1.2 *D*-values for effect of heat shock on viability of conidia and ascospores of *A. nidulans*

Data from the heat shock experiments were converted to *D*-values to see whether ascospores exhibited higher *D*-values compared with conidia in the isolates/strains tested. The heat treatments for both conidia and ascospores of *A. nidulans* had shown a noticeable decrease in the number of surviving spores above 55°C. Thus, this temperature was selected to be a suitable temperature to determine the *D*-values of both conidia and ascospores. Therefore the spores of all isolates/strains were subjected to thermal exposure at 55°C for different lengths of time of 40 min, 55 min, 70 min, 85 min and 105 min to determine the D_{55}-values (section **4.3.2**). Results are shown **Table 4-1**, (also see **Appendix 2** and **Appendix 3** for graphical derivation).

The most striking result was that the D_{55}-values of conidia for all isolates/strains were consistently lower than the comparable D_{55}-value for ascospores of the same strain. This provided compelling evidence that

for isolates/strains tested that ascospores exhibit greater resistance to heat shock than conidia, at least at 55ºC. Indeed, there was a significant difference between the average D_{55}-values of conidia and ascospores when all strains were averaged [P= 0.023; T-test = 1.25 (df, 16)] (**Table 4-1**). However, there were notable differences between isolates/strains with the D_{55}-values of conidia for some *A. nidulans* actually higher than the ascospore values of others e.g. 2-155 had an ascospore D_{55}-value of only 39.5 compared to 2-154 which had a conidia D_{55}-value of only 88.5 (**Table 4-1**). It is also noted that differences in D_{55}-values were more pronounced in the wild-type than the laboratory strains.

Table 4-1 *D-values* of conidia and ascospores of nine isolates/strains of *A. nidulans* that were subjected to heat shock at 55°C for different times (40 min, 55 min, 70 min, 85 min and 105 min).

Isolate/Strain	**D_{55}-value (minutes)**	
	Conidia	**Ascospores**
2-137 wild-type (VeA)	45.5	49.0
2-139 wild-type (VeA)	67.5	78.4
2-149 mutant (VeA1)	88.2	89.8
2-154 mutant (VeA1)	88.5	90.9
2-155 mutant (VeA1)	36.6	39.5
2-224 wild-type (VeA)	46.0	58.1
2-227 wild-type (VeA)	39.4	95.5
2-232 wild-type (VeA)	85.5	101.5
2-250 wild-type (VeA)	70.9	81.3
Mean for all data	63.0 ± 8.8	76.0 ± 9.0

4.1.1.3 Effect of heat shock on viability of conidia and ascospores of *E. repens*

Figure 4-13 to **Figure 4-16** illustrate that the heat shock at different temperatures 50°C, 55°C, 60°C, 65°C, 70°C and 75°C for 30 min had a significant influence on spore viability (conidia and ascospores) for all isolates of *E. repens* assessed in the study (51-3, 51-4, 51-5 and 51-6). A two-way ANOVA of data for either conidia or ascospore survival of all isolates revealed that there was an overall effect of temperature on survival of both conidia [F (df) = 94.049 (6, 84); p <0.001], and ascospores [F (df) = 124.044 (6, 84); p <0.001], a significant effect of strain on conidia [F (df) = 33.919 (3, 84); p <0.001] and ascospores [F (df) = 20.267 (3, 84); p <0.001], and that there was a significant interaction between temperature and strain on both conidia [F (df) = 3.816 (18, 84); p <0.001] and ascospores [F (df) = 7.290 (18, 84); p <0.001]. Increasing the heat shock temperature resulted in a gradual decrease of spore viability of both conidia and ascospores. For most isolates the conidia and ascospores had similar rates of survival up to 65ºC. It was only at the higher temperatures 70^{o}C and 75^{o}C that ascospore survival was notably greater than that of conidia, which were almost entirely killed at those temperatures. A subsequent two-way ANOVA incorporating data for all isolates and survival of ascospore versus conidia in the same analysis revealed that there was a significant difference in survival of ascospores versus conidia [F (df) = 88.4 (55,1); p <0.001], a significant effect of temperature on spore survival [F (df) = 22.8 (55,6); p <0.001], and a significant interaction between spore type and temperature [F (df) = 3.5 (55,6); p =0.007].

Concerning individual isolates, **Figure 4-13** shows that there was a significant effect of temperature on isolate 51-3 on survival of both conidia [A one-way ANOVA: F (df) = 28.832 (6, 21); p <0.001], and ascospores [F (df) = 24.222 (6, 21); p <0.001]. Ascospores showed higher survival at 70^{o}C (24.90 %) than conidia (0.38 %). Similarly, a one-way ANOVA revealed a significant effect of temperature on strain 51-4 for survival of both conidia [F (df) = 18.739 (6, 21); p <0.001], and

ascospores [F (df) = 24.880 (6, 21); p <0.001] (**Figure 4-14**). The ascospores were more heat tolerant than conidia at 70°C, showing 22.7% survival compared to 6.3% for conidia. Strain 51-5 exhibited a similar set of results, with ANOVA showing a significant effect of temperature on strain 51-5 on survival of both conidia [A one-way ANOVA: F (df) = 31.751 (6, 21); p <0.001], and ascospores [F (df) = 18.838 (6, 21); p <0.001] (**Figure 4-15**). Only 5.0% of conidia survived at 70°C compared to 45.30% of ascospores at this temperature.

The one isolates to show a different pattern was 51-6 (**Figure 4-16**). A one-way ANOVA: revealed that there was a significant effect of temperature on strain 51-6 on survival of both conidia [F (df) = 35.939 (6, 21); p <0.001], and ascospores [F (df) = 320.626 (6, 21); p <0.001]. However, remarkably both the conidia and ascospores of 51-6 were both markedly less heat resistant than the other three isolates of *E. repens* investigated. For example, conidia and ascospores did not survive heat treatment above 60°C and 65°C respectively. But unlike the other isolates, in this case ascospores were significantly more heat resistant than conidia at 50, 55 and 60ºC (**Figure 4-16**).

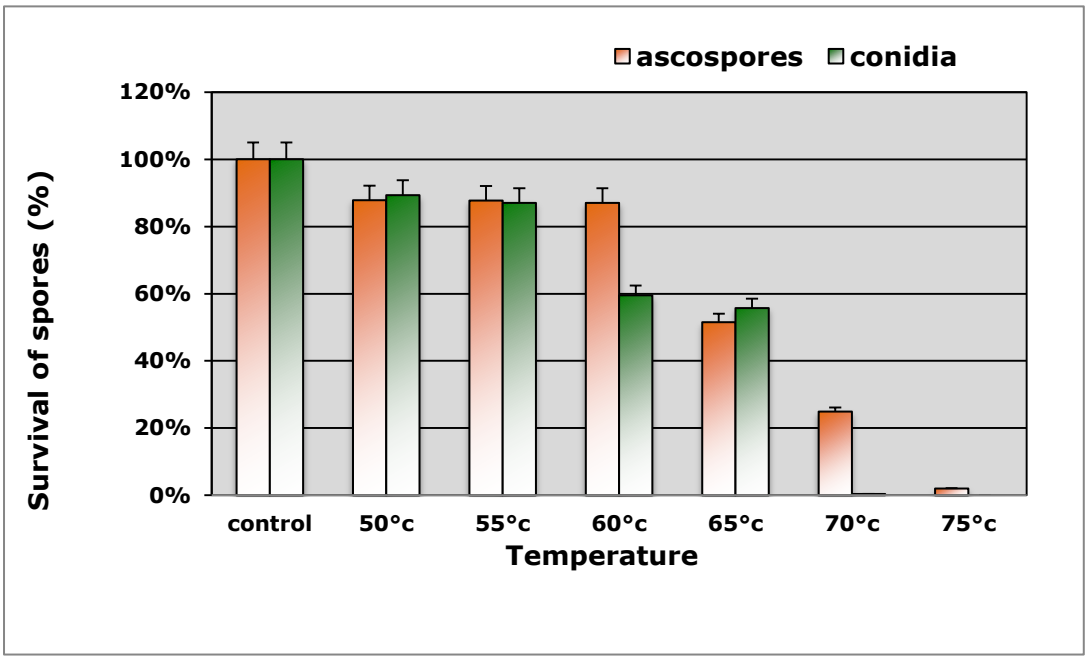

Figure 4-13 Effect of heat shock on spore viability of *E. repens* isolate 51-3. Spore survival is expressed relative to room temperature controls. Error bars indicate ± SEM.

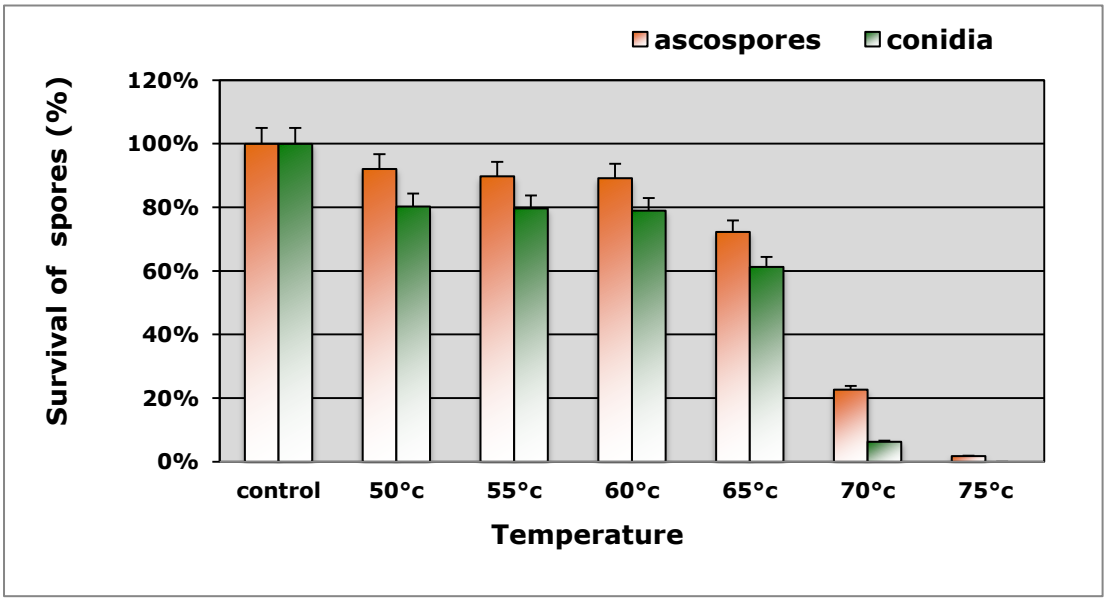

Figure 4-14 Effect of heat shock on spore viability of *E. repens* isolate 51-4. Spore survival is expressed relative to room temperature controls. Error bars indicate ± SEM.

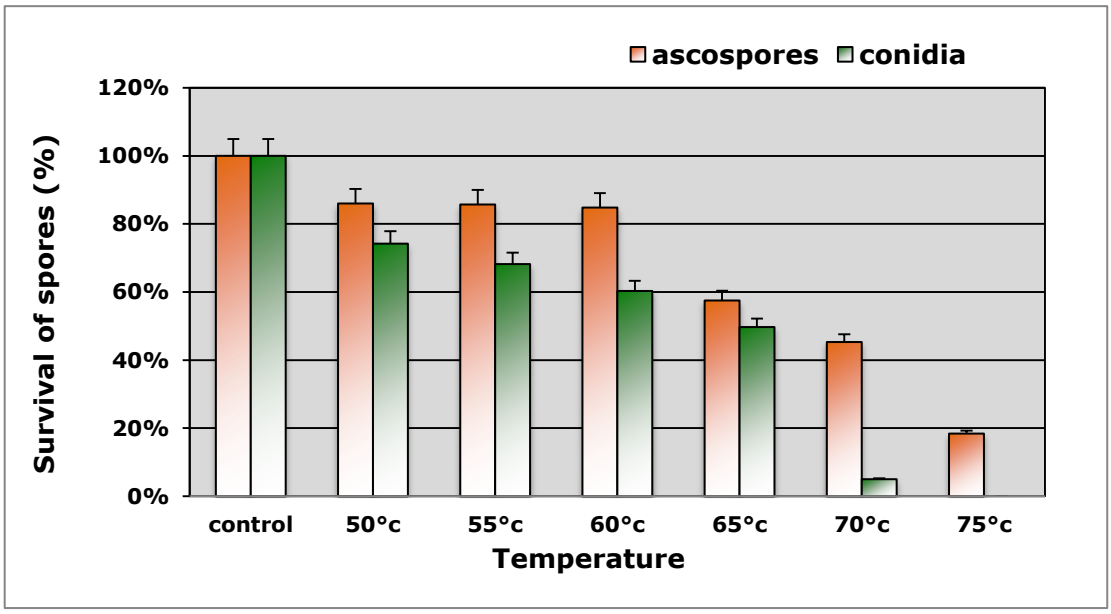

Figure 4-15 Effect of heat shock on spore viability of *E. repens* isolate 51-5. Spore survival is expressed relative to room temperature controls. Error bars indicate ± SEM.

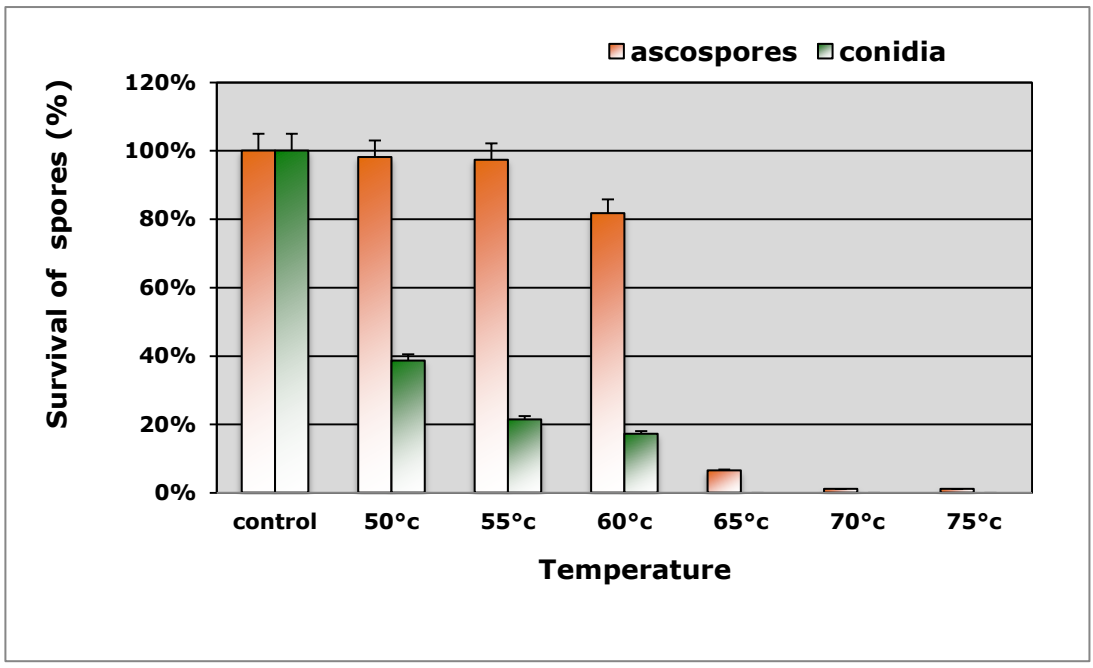

Figure 4-16 Effect of heat shock on spore viability of *E. repens* isolate 51-6. Spore survival is expressed relative to room temperature controls. Error bars indicate ± SEM.

4.1.2 Effect of UV light on viability of asexual and sexual spores of *A. nidulans*

To determine the influence of UV light on spore viability of *A. nidulans* conidia and ascospores of six wild-type isolates (2-137, 2-139, 2-224, 2-227, 2-232 and 2-250) and two *Ku* mutants (2-149 and 2-154) were exposed to UV radiation at a wavelength of 254 nm over a time course from 10 to 60 min and percentage survival calculated relative to non-exposed controls (section **4.3.3**). A two-way ANOVA between all isolates/strains illustrated that there was a significant effect of UV exposure on survival of both conidia [F (df) = 302.004 (6, 168); p <0.001], and ascospores [F (df) = 29.129 (6, 168); p <0.001], a significant effect of isolate/strain on conidia [F (df) = 23.160 (7, 168); p <0.001] and ascospore survival [F (df) = 7.415 (7, 168); p <0.001], and also a significant interaction between temperature and strain on conidia [F (df) = 11.832 (42, 168); p <0.001], but no significant effect on ascospores [F (df) = 0.902 (42, 168) ; p = 0.643].

Overall, ascospores exhibited notably elevated levels of UV resistance compared to conidia (**Figure 4-17** to **Figure 4-24**), as was apparent from a subsequent *D*-value analysis (see next section **4.1.2.1**). This was also confirmed by a subsequent two-way ANOVA incorporating data for all isolates and survival of ascospore versus conidia in the same analysis, which revealed that there was a significant difference in survival of ascospores versus conidia [F (df) = 154.7 (111,1); p <0.001], a significant effect of UV exposure on spore survival [F (df) = 34.0 (111,6); p <0.001], but there was no significant interaction between spore type and temperature [F (df) = 1.4 (111,6); p =0.225].

Regarding individual isolates, the wild-type isolates 2-137, 2-139, 2-227, 2-232 and 2-250 all showed a broadly similar pattern of results (**Figure 4-17** to **Figure 4-21**). In all cases conidia and ascospores showed steadily decreasing survival with increased UV exposure time. However, ascospores showed consistently greater rates of survival at all exposure times than conidia, and showed viability of between 5-40% even after 60

min exposure, whereas conidia were totally killed at this time of exposure (except for minimal survival by conidia of 2-227 and 2-232). A one-way ANOVA revealed a significant effect of UV exposure on the wild-type 2-137 on survival of both conidia (F (df) = 28.988 (6, 21); p <0.001], and ascospores [F (df) = 6.739 (6, 21); p <0.001]. There was also a significant effect of UV exposure on 2-139 on survival of both conidia [F (df) = 44.173 (6, 21); p <0.001], and ascospores [F (df) = 17.915 (6, 21); p <0.001]. Similarly, a one-way ANOVA showed a significant effect of UV exposure on 2-250 for both conidia [F (df) = 260.074 (6, 21); p <0.001], and ascospores [F (df) = 3.106 (6, 21); p =0.024], and also a significant effect of UV exposure on isolate 2-227 for both conidia [F (df) = 41.704 (6, 21); p <0.001] and ascospore survival [F (df) = 20.187 (6, 21); p <0.001]. Correspondingly, the one-way ANOVA analysis of isolate 2-232 also revealed a significant effect of UV exposure on survival of both conidia [F (df) = 175.001 (6, 21); p <0.001] and ascospores [F (df) = 4.492 (6, 21); p <0.004].

By contrast, the wild–type isolate 2-224 had a markedly different pattern of sensitivity to UV exposure. Conidia were inactivated by UV exposure at a similar rate as the other wild-type isolates, but in this case ascospores also showed greatly increased sensitivity to UV relative to the other wild-type isolates with only 20% ascospore survival after 20 min exposure, and no survival after 60 min (**Figure 4-22**). Thus ascospores were more delicate than those of the other wild-type isolates. As expected, a one-way ANOVA showed that there was a significant effect of UV exposure on this strain for survival of both conidia [F (df) = 38.984 (6, 21); p <0.001], and ascospores [F (df) = 72.950 (6, 21); p <0.001].

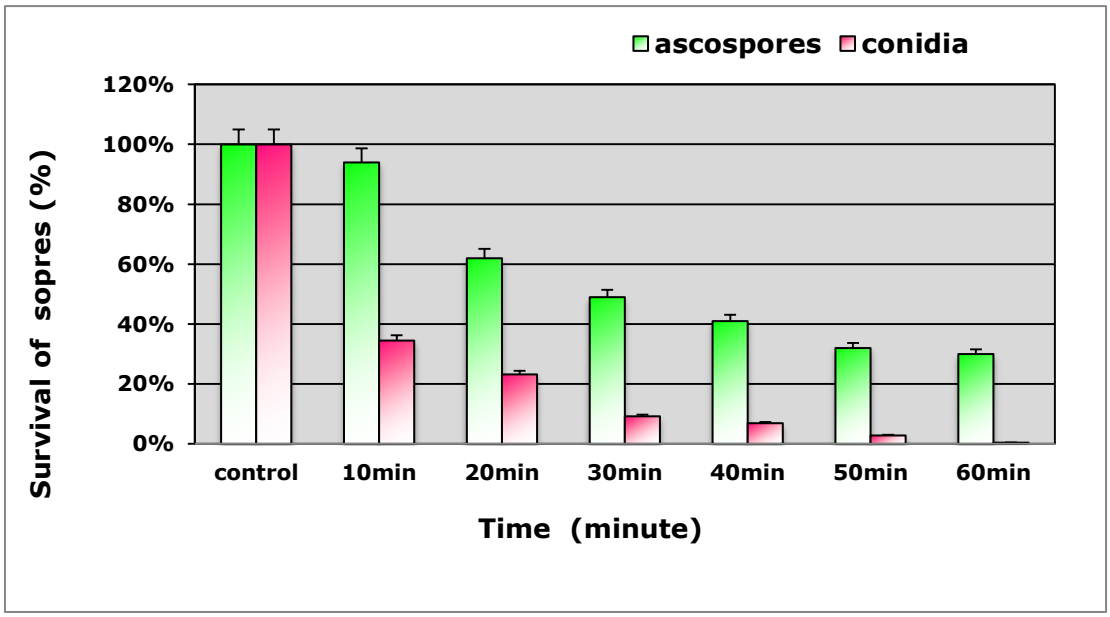

Figure 4-17 Effect of UV light at wavelength of 254 nm on spore viability of *A. nidulans* isolate 2-137. Spore survival is expressed relative to non-exposed controls. Error bars indicate ± SEM.

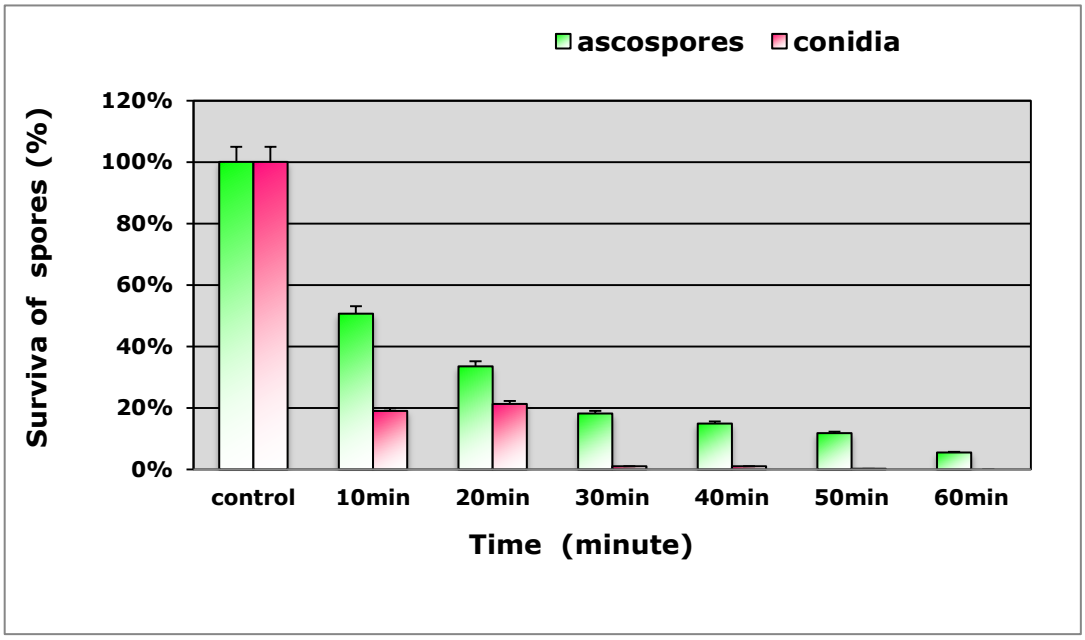

Figure 4-18 Effect of UV light at wavelength of 254 nm on spore viability of *A. nidulans* isolate 2-139. Spore survival is expressed relative to non-exposed controls. Error bars indicate ± SEM.

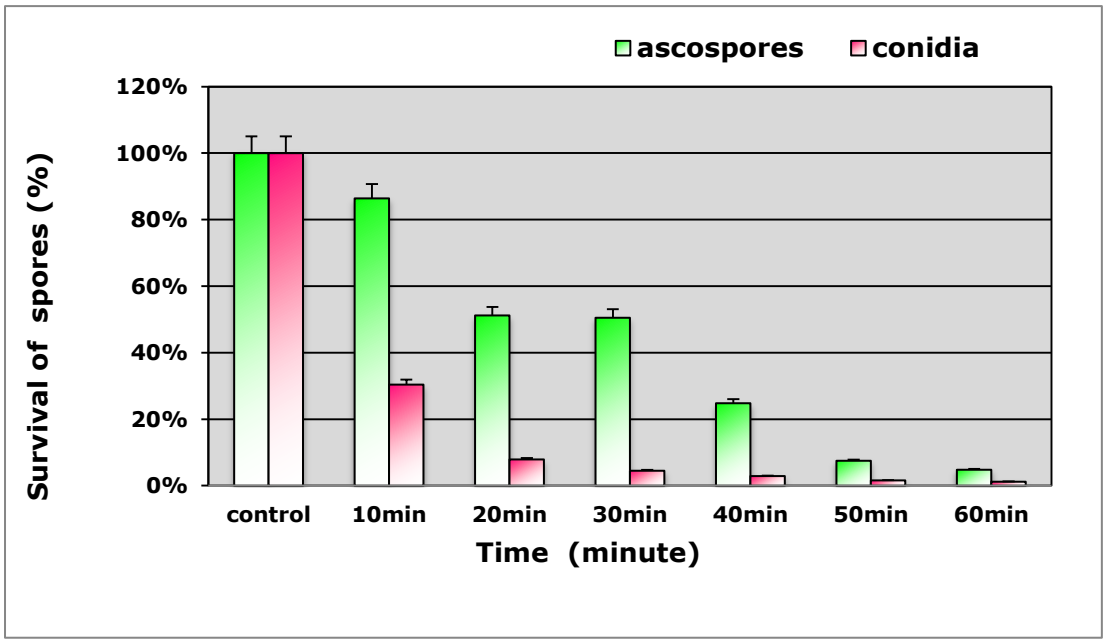

Figure 4-19 Effect of UV light at wavelength of 254 nm on spore viability of *A. nidulans* isolate 2-227. Spore survival is expressed relative to non-exposed controls. Error bars indicate ± SEM.

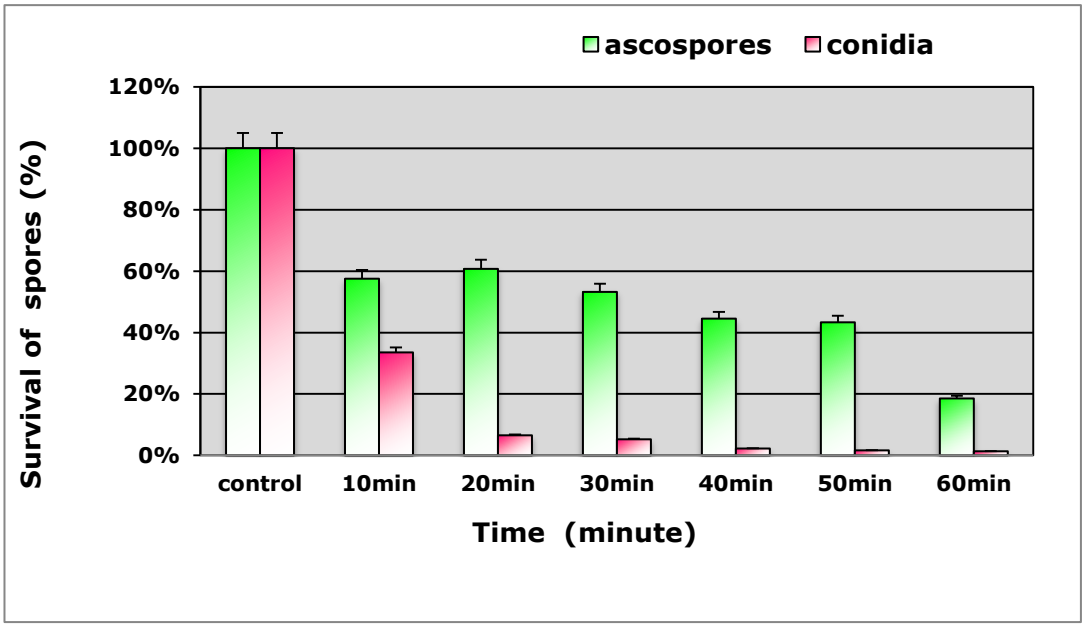

Figure 4-20 Effect of UV light at wavelength of 254 nm on spore viability of *A. nidulans* isolate 2-232. Spore survival is expressed relative to non-exposed controls. Error bars indicate ± SEM.

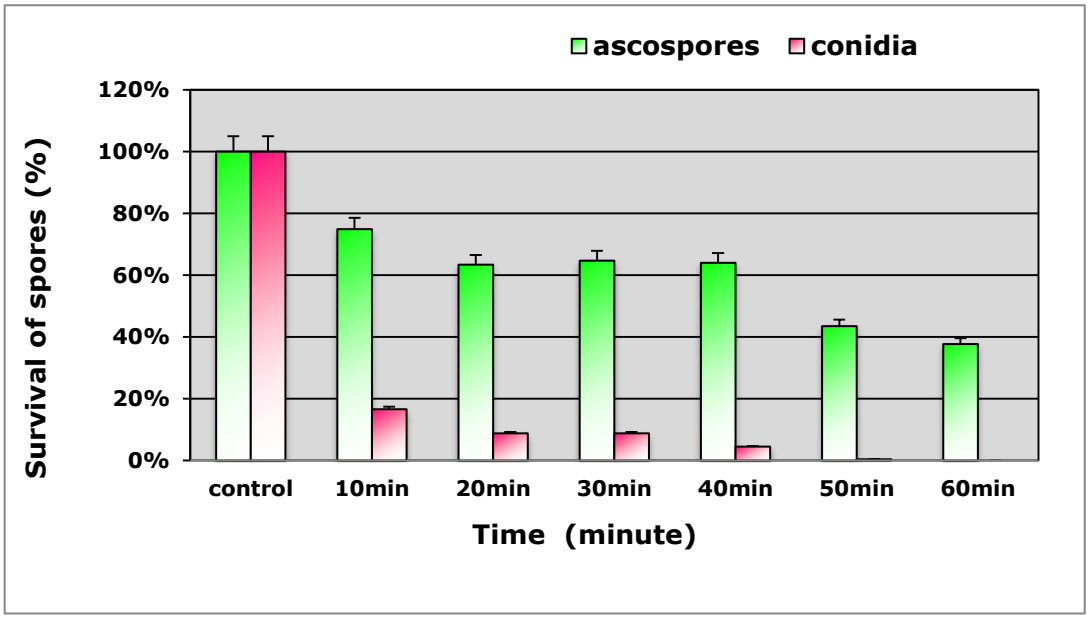

Figure 4-21 Effect of UV light at wavelength of 254 nm on spore viability of *A. nidulans* isolate 2-250. Spore survival is expressed relative to non-exposed controls. Error bars indicate ± SEM.

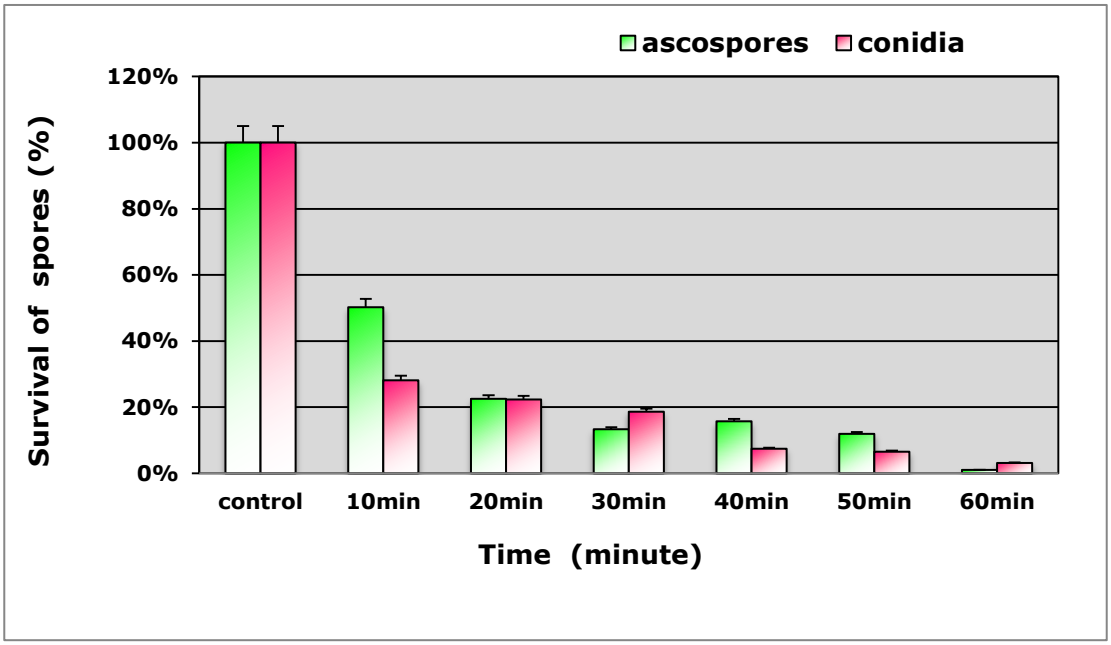

Figure 4-22 Effect of UV light at wavelength of 254 nm on spore viability of *A. nidulans* isolate 2-224. Spore survival is expressed relative to non-exposed controls. Error bars indicate ± SEM.

Regarding the laboratory *veA1ku* mutant strains of 2-149 and 2-154 gave notably different patterns. ANOVA for both isolates showed a statistically significant effect of UV exposure on survival of both conidia and ascospores: for 2-149 an effect on conidia [F (df) = 25.169 (6, 21); p <0.001], but not ascospores [F (df) = 1.676 (6, 21); p = 0.176]; for 2-154 a significant effect on conidia [F (df)=484.308 (6, 21); p <0.001] but not on ascospores [F (df) = 0.380 (6, 21); p = 0.883]. For 2-149, both conidia and ascospores showed survival up to 60 min, albeit with ascospores showing consistently higher survival rates, similar to wild-type isolate 2-224 (**Figure 4-23**). By contrast, conidia of 2-154 were killed very rapidly, with almost total kill after only 20 min exposure, whilst ascospores showed *ca.* 30% viability even at 60 min exposure (**Figure 4-24**).

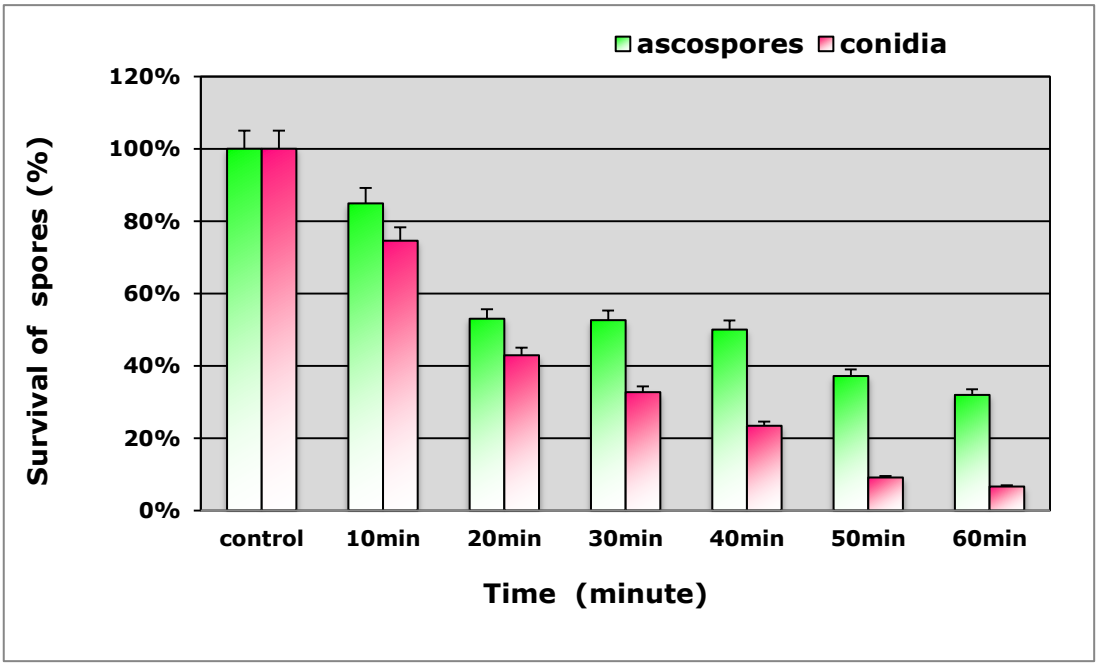

Figure 4-23 Effect of UV light at wavelength of 254 nm on spore viability of *A. nidulans* isolate 2-149. Spore survival is expressed relative to non-exposed controls. Error bars indicate ± SEM.

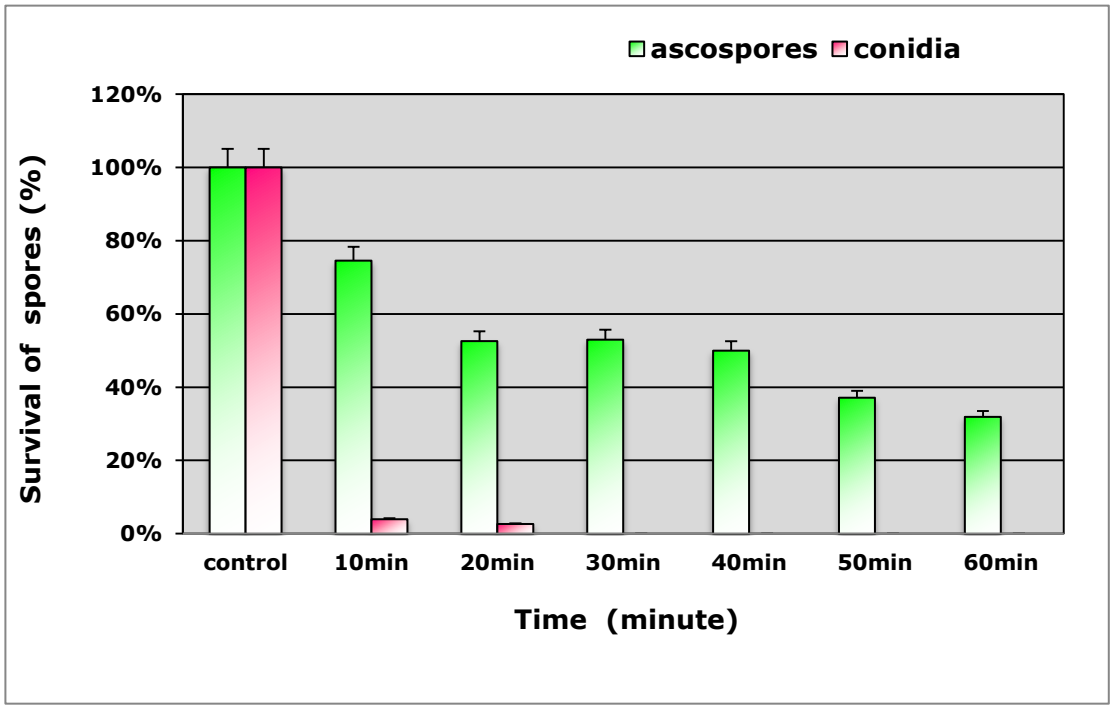

Figure 4-24 Effect of UV light at wavelength of 254 nm on spore viability of *A. nidulans* strain 2-154. Spore survival is expressed relative to non-exposed controls. Error bars indicate ± SEM.

4.1.2.1 *D*-values for effect of UV exposure on viability of conidia and ascospores of *A. nidulans*

Given that a trend was apparent showing differential survival of conidia and ascospores, it was decided to determine *D*-values for UV exposure at 254 nm to assess whether conidia and ascospores have different resistance against the UV radiation. Linear relationships for calculation were obtained for all strains (**Appendix 4** and **Appendix 5**). Ascospores were found to have consistently greater *D*-values when compared to conidia of the same isolate/strain. Of course, there was a highly significant difference between the average *D*-values of conidia and ascospores when all strains/isolates were averaged [P= 0.001;T-test = 4.85 (df, 14)] (**Table 4-2**). As with the heat shock work, there were some instances where conidia of some *A. nidulans* exhibited greater *D*-values than some conidia (e.g. 2-154 versus 2-137), but this was quite rare and the highest *D*-values were mostly for ascospores.

Table 4-2 *D*-values of conidia and ascospores of eight isolates/strains of *A. nidulans* subjected to UV at 254 nm over a range of times (0 min, 10 min, 20 min, 30 min, 40 min, 50 min and 60 min).

Isolate/Strain	***D*-value (minutes)**	
	Conidia	**Ascospores**
2-137 wild-type (VeA)	28.7	80.6
2-139 wild-type (VeA)	26.0	52.0
2-149 mutant (VeA1)	48.9	84.7
2-154 mutant (VeA1)	8.6	100
2-224 wild-type (VeA)	21.4	37.3
2-227 wild-type (VeA)	16.2	43.9
2-232 wild-type (VeA)	16.1	56.0
2-250 wild-type (VeA)	19.1	80.6
Mean for all data	23.1 ± 4.7	66.9 ± 8.0

4.1.3 Influence of heating entire cleistothecia on isolation of ascospores

Given the discovery that ascospores exhibited slightly greater heat resistance than conidia, experiments were conducted to determine whether preheating of cleistothecia, prior to ascospore isolation, might provide a useful way to heat inactivate (i.e. kill) contaminating vegetative tissues and conidia of *A. nidulans* and thereby provide a method to increase the purity of ascospore suspensions. Either conidia or ascospores from two wild-type strains of *A. nidulans* (2-137, 2-139) or two *veA1ku* mutants (2-149, 2-154) were subjected to various heat shocks (65^{o}C, 68^{o}C, 70^{o}C or 72^{o}C for 30 min). In the case of ascospores, this heat shock was applied whilst they were still in cleistothecia (which had previously been rolled carefully over 4% TWA to remove most attached conidia and fungal debris), whereas conidia were heat shocked directly in solution. Ascospores were then released by breaking down the cleistothecial wall. Spore survival was then expressed as a percentage relative to non-heat shocked room temperature solutions.

A two-way ANOVA analysis between all four isolates/strains revealed that there was a significant effect of heat shock on survival of both conidia [F (df) = 347.405 (4, 60); p <0.001] and ascospores [F (df) = 364.188 (4, 60); p <0.001], a significant effect of strain on survival of conidia [F (df) = 7.548 (3, 60); p <0.001] and ascospores [F (df) = 94.043 (3, 60); p <0.001], and also a significant interaction between heating temperature and isolate/strain on survival of conidia [F (df) = 9.247 (12, 60); p <0.001] and ascospores [F (df) = 29.905 (12, 60); p <0.001] (**Figure 4-25** to **Figure 4-28**).

Considering individual isolates, both conidia and ascospores of 2-137 were almost entirely killed over 70°C, whereas 5.4% of ascospores and 2.0% of conidia survived the heat shock at 68°C (**Figure 4-25**). A one-way ANOVA of this strain revealed a significant effect of heat shock temperature on survival of both conidia [F (df) = 485.046 (4, 15); p <0.001] and ascospores [F (df) = 784.466 (4, 15); p <0.001].

A more marked difference between spore types was evident in isolate 2-139. Both conidia and ascospores were entirely killed over 70°C. By contrast, 37.5% of ascospores survived heat shock at 68°C, whereas all conidia were completely killed at this temperature (**Figure 4-26**). A one-way ANOVA revealed a significant effect of heat shock temperature on survival of both conidia [F (df) = 21.578 (4, 15); p <0.001] and ascospores [F (df) = 23.415 (4, 15); p <0.001].

Similarly, the two VeA1 strains showed clear differences in survival of conidia or ascospores when exposed to the heat shock. For 2-149, over 92.0% of ascospores survived the heat shock at 65°C and 68°C for 30 min, whereas only approximately 10% and 3.3% of conidia survived at 65°C and 68°C, respectively (**Figure 4-27**). A one-way ANOVA revealed that there was a significant difference between heat shock temperature and survival of both conidia [F (df) = 1116.333 (4, 15); p <0.001] and ascospores [F (df) = 229.525 (4, 5); p <0.001]. Meanwhile, differences in spore survival were most evident in 2-154, for which approximately 27.0% and 10.0% of ascospores survived heat shock at 65°C and 68°C respectively, whereas no survival of conidia was observed at either temperature (**Figure 4-28**). Statistically, a one-way ANOVA revealed a significant effect of heat shock temperature on survival of both conidia [F (df) = 3072.432 (4, 15); p <0.001] and ascospores [F (df) = 337.283 (4, 15); p <0.001].

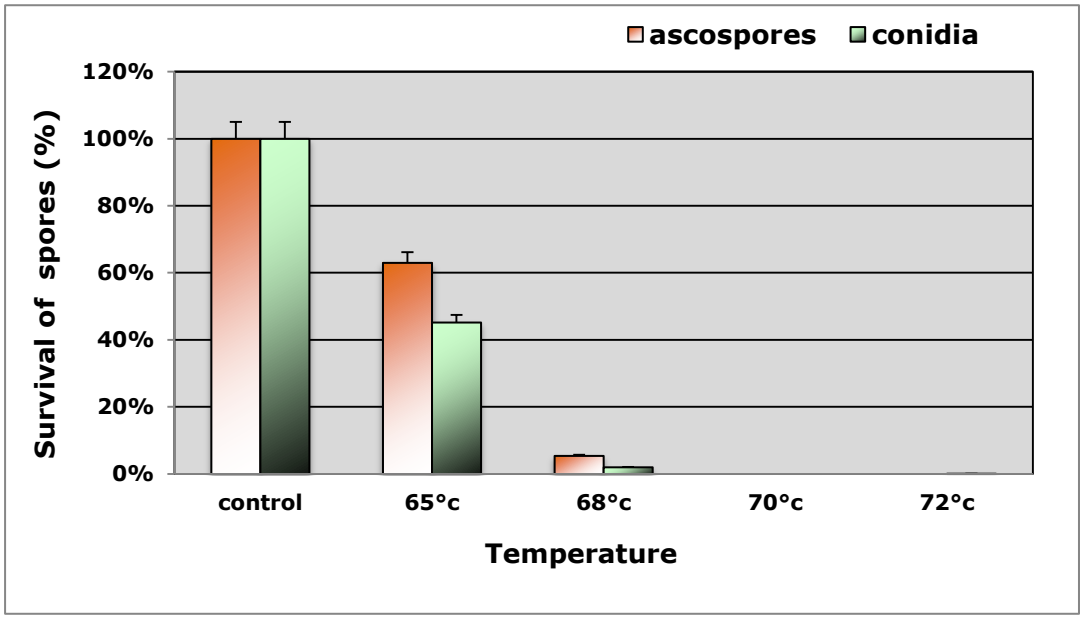

Figure 4-25 Influence of heat shock for 30 min at four different temperatures on spore viability of *A. nidulans* isolate 2-137 (*ve*A). Error bars indicate ± SEM.

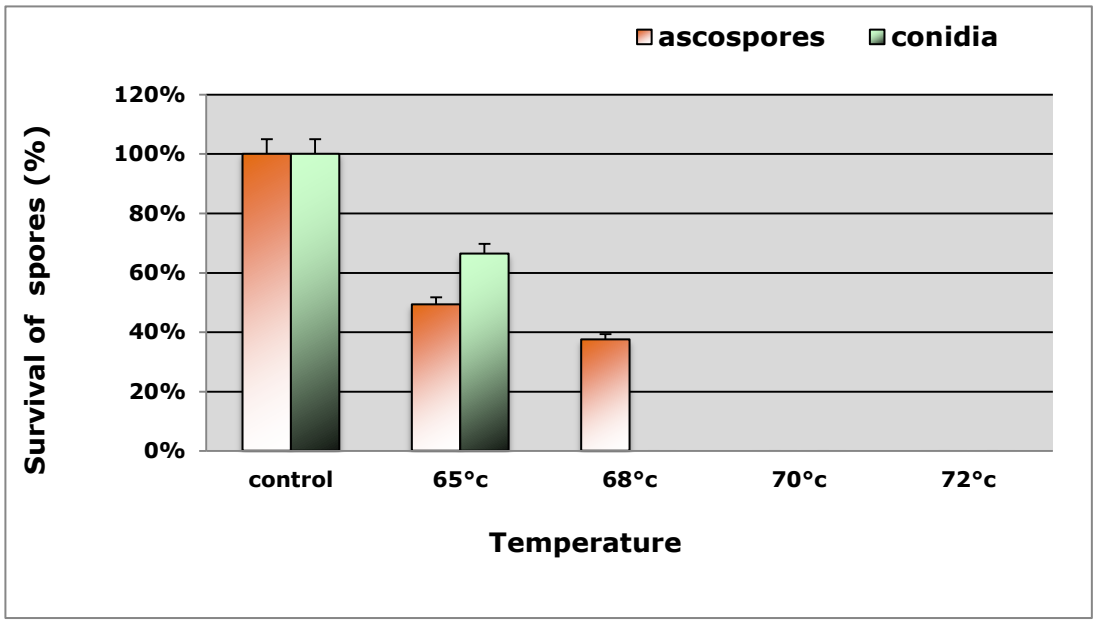

Figure 4-26 Influence of heat shock for 30 min at four different temperatures on spore viability of *A. nidulans* isolate 2-139 (*ve*A). Error bars indicate ± SEM.

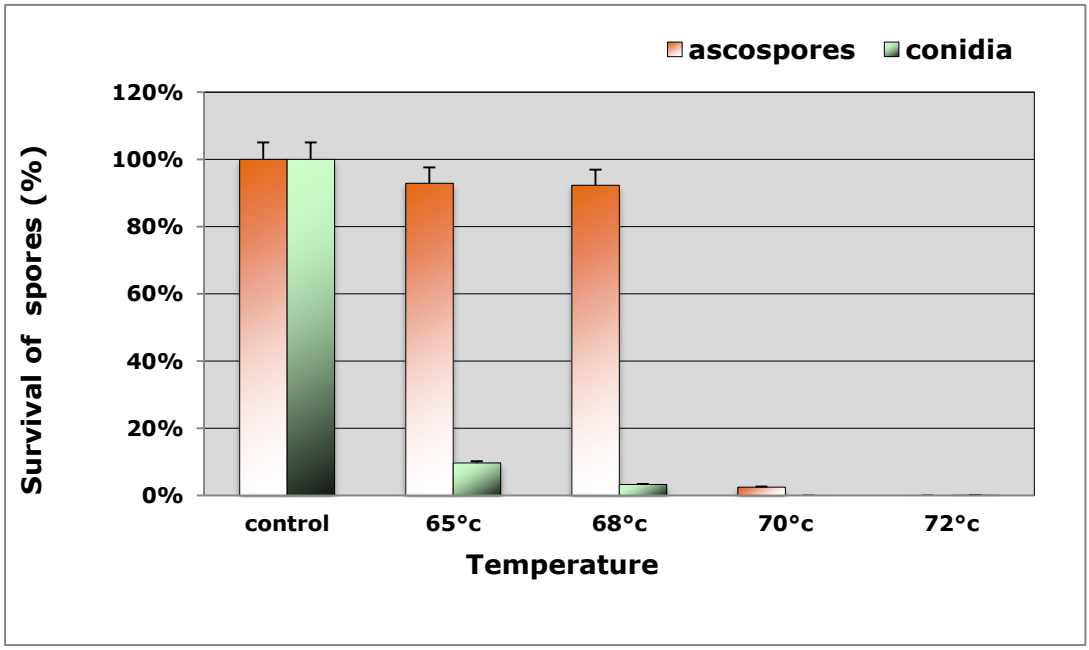

Figure 4-27 Influence of heat shock for 30 min at four different temperatures on spore viability of *A. nidulans* strain 2-149 (*veA1ku* mutant). Error bars indicate ± SEM.

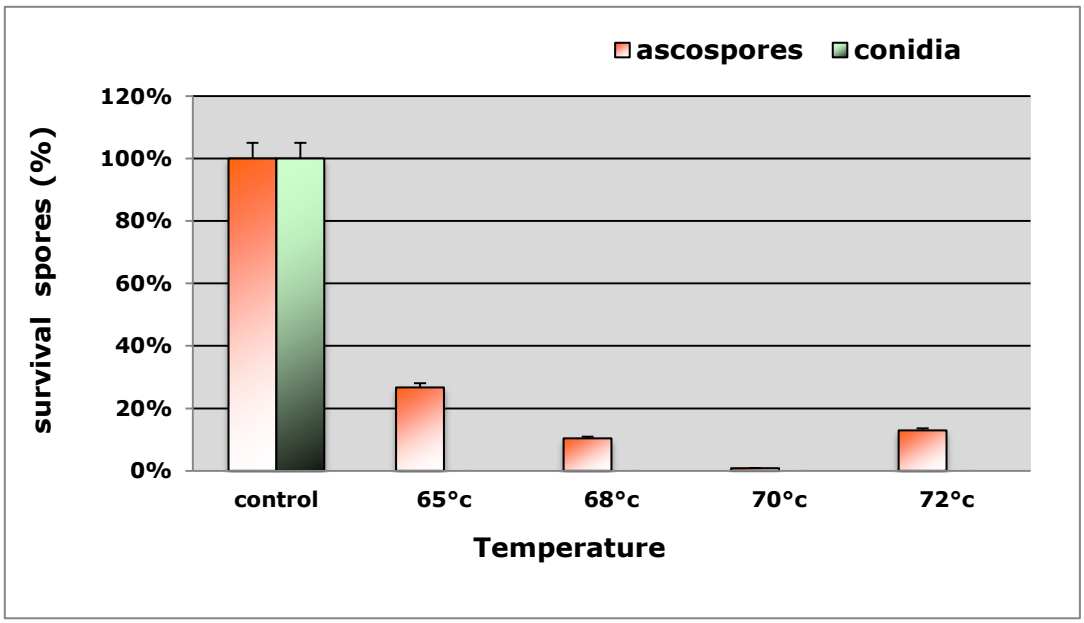

Figure 4-28 Influence of heat shock for 30 min at four different temperatures on spore viability of *A. nidulans* strain 2-154 (*veA1ku* mutant). Error bars indicate ± SEM.

4.2 Discussion

Aspergillus nidulans and *Eurotium repens* are both homothallic species which undergo sexual reproduction, normally by self fertilization, in addition to asexual reproduction. Sexual development has a higher metabolic cost than asexual reproduction and can only occur under relatively narrow environmental conditions, whereas asexual reproduction allows the production of a greater number of conidia in a shorter period of time than sexual reproduction (sections **1.3.1.1** and **1.3.4.1**). In homothallic breeding systems there is also little chance of increased genetic variation in the ascospore progeny unless a rare outcrossing event occurs. Therefore the main questions raised here are why does homothallism evolve in the first place from heterothallic ancestors and why is this breeding system then retained in *Aspergillus* species which are also able to reproduce asexually?

The overall aim of this chapter was to investigate whether sexual and asexual spores of *A. nidulans* and *E. repens* show differential rates of survival when challenged with environmental stresses such as heating and UV exposure. The reason for the evolution of sexual reproduction has long been a topic of debate both in fungi and eukaryotes in general, with arguments such as the production of offspring with improved fitness due to novel gene combinations, and genome repair mechanisms being proposed (Stearn, 1987; Otto and Lenormand, 2002; Kück and Pöggeler, 2009). However, a consensus has not yet been reached. The research conducted here suggests yet another evolutionary possibility in the especial context of fungal biology because sexual ascospores were shown to have an increased survival rate relative to asexual conidia. This might be due to factors such as the increased structural rigidity of cell walls, cell internal physiology, and/or a binuclear state as will now be discussed.

4.2.1 The effects of temperature on asexual and sexual spores

For both asexual and sexual spores of *A. nidulans* and *E. repens* a relationship was found between spore viability and temperature, with decreased survival of spores as they were subjected to increased heat shock at higher temperatures. This indicates that temperature has a major effect on spore viability. This is to be expected as elevated temperatures affect the chemical structures of fungal cells and molecules start to break down (Deacon, 2006). For instance, proteins begin to unfold and denature causing alterations in their secondary and tertiary structures and loss of viability as essential proteins become non functional, and at the same time changes in membrane fluidity occur with leakage and possible damage to cell components (Riezman, 2004).

4.2.1.1 The influence of heat shock on spore viability (conidia vs. ascospores) of *A. nidulans* and *E. repens*

Although some *Aspergillus* species, such as members of the genus *Neosartorya* and related *Talaromyces* species, are known to produce heat-resistant ascospores (Dijksterhuis and Samson, 2006) less is known about heat resistance in other teleomorphic genera such as *Emericella* and *Eurotium.* Indeed, in the case of *A. nidulans* (teleomorph *Emericella nidulans*) it has been assumed that conidia and ascospores exhibit similar resistance against environmentally harsh conditions (Braus *et al.,* 2003). Thus, species such as *A. nidulans* and *E. repens* might be expected to evolve to asexuality using conidia to disperse to new habitats rather than undergo complicated sexual development (Harrub and Thompson, 2004). Therefore, the purpose of this experiment was to examine in detail whether conidia and ascospores might exhibit as yet undetected differences in spore viability when subjected to heat shock at different temperatures for certain amounts of time.

Spores from nine strains of *A. nidulans* (comprising wild-type and *veA1ku* mutants) and also four isolates of *E. repens* were exposed to various levels of thermal stress from 50-75°C for 30 min (sections **4.3.1.1**).

Results obtained revealed that at certain temperatures ascospores were significantly more heat resistant than conidia in all isolates/strains investigated. Sometimes these differences were slight, but ascospores consistently showed better rates of spore survival. In the case of *A. nidulans,* further experimentation was performed involving the calculation of *D*-values (decimal reduction time) to determine whether ascospores and conidia exhibited different resistance levels when subjected to heat shock at 55°C over a time course of 40 min, 55 min, 70 min, 85 min and 105 min (section **4.3.2**). A linear relationship was obtained and the time (in minutes) to inactivate 90% of conidia and ascospores of all strains investigated was specified. Results again showed that ascospores of a given isolate/strain had consistently greater *D*-values than conidia (**Table 4-1**), showing that ascospores had greater resistance to heat exposure at 55°C than conidia. It is noted that some of the data from the *D*-value analysis was inconsistent with results from the earlier heat shock work. For example ascospores of 2-149 were found to have relatively high heat resistance in the *D*-value experimentation, whereas initial heat treatment work suggested low resistance (**Figure 4-12**). Reasons for this inconsistency were unclear but might arise from the use of different spore batches and/or occasional spurious data. The D_{55}-values obtained of between 36-102 min compare with *D*-values obtained for conidia of *A. flavus, P. expansum, P. roqueforti* and *P. citrinum* of between 3.5 and 230 min at 54-56°C (Bröker *et al.*, 1987). *D*-values between 44.9 and 81 min have been reported for conidia of *Wallemia sebi*, *Eurotium rubrum*, *Aspergillus niger*, *Penicillium chrysogenum* and *P. glabrum* when exposed to thermal shock between 56°C and 62°C in buffered solutions (Baggerman and Samson, 1988).

Although the sexual ascospores of a given isolate/strain were consistently more heat resistant than conidia from the same strain, there were nevertheless some interesting differences between spore survivals in certain isolates/strains. Ascospores from all wild-type isolates aforementioned (**Figure 4-4 to Figure 4-9**) and both *veA1ku* mutants 2-154 and 2-155 (**Figure 4-10 to Figure 4-11**) exhibited similar levels of heat resistance with between 60-80% survival at 55°C. By contrast,

conidia of the wild-type strains were almost entirely destroyed at 55°C, whereas the conidia of the *veA1ku* laboratory mutants showed up to 60% survival at this temperature, and were only inactivated at 60°C-65°C i.e. they had increased heat resistance. Thus, there were clear differences in spore survival rates between isolates/strains. This reflects the often underestimated genetic diversity of fungal species, and the need to ensure that multiple isolates/strains of a species are included in laboratory studies before broad-ranging conclusions can be drawn. For example, Mcnelly-Ingle and Frost (1965) found that the optimum temperature for sexual reproduction varied for different strains of *N. crassa.*

It is noted that all of the laboratory strains 2-149, 2-154 and 2-155 are thought to be derived from a common ancestor first used in laboratory work by Pontecorvo (1953). Thus, they might have been expected to show a similar response to heat shock. Although 2-149 and 2-154 had similar *D*-values, strain 2-155 had markedly lower *D*-values for both conidia and ascospores (**Table 4-1**). The reason for this is unclear but it might be that the particular nutritional mutations present in 2-155 rendered it particularly sensitive (**Table 3-1**) or that other mutations had occurred in the lab to change the characteristics and behaviours of this strain leading to less heat resistance. The *veA1* and *ku* mutations had no apparent effect on spore viability given the relatively high *D*-values for both 2-149 and 2-154.

In a similar fashion, ascospores of *E. repens* were found to exhibit slightly greater levels of heat resistance compared to conidia, although for most isolates this was only marked at 70ºC and above. However, as observed for *A. nidulans* there was again noticeable variation between isolates, with three of the four isolates showing similar behaviour but one (51-6) showing especial sensitivity to heat shock. Both conidia and ascospores of the former three isolates of *E. repens* (51-3, 51-4 and 51-5) were markedly more heat resistant than the equivalent spores of *A. nidulans*. Even conidia of these *E. repens* isolates showed 40-60% viability at 65ºC compared to the ascospores of *A. nidulans* which only

showed between 5-20% viability when exposed to the same heat shock. In parallel work heat resistance of ascospores of the xerophilic species *E. herbariorum* and *E. chevalieri* has been evaluated (Kocakaya Yildiz and Coksöyler, 2002). It was found that ascospores of *E. chevalieri* exhibited relatively high heat resistance, with between 18-25% of ascospores surviving a heat shock at 70°C for 10 min in a medium of 98 a_w and pH 3.8. Up to 0.5% of these ascospores also survived in similar conditions at 80°C (Kocakaya Yildiz and Coksöyler, 2002). On the other hand, the conidia of *E. chevalieri* were much less heat resistant, with only 0.1% surviving a heat shock at 60°C for 10 min. Thus, elevated heat resistance of ascospores might be a general feature of *Eurotium* species, of possible ecological importance of survival in high temperature and low-water availability conditions.

Many other investigations have been conducted in the past to study fungal spore survival at elevated temperatures. But these have tended to focus on species producing ascospores with exceptionally high temperature resistance. Thus, the present study is one of the few to assess spore survival in more 'standard' fungal species. Ascospores, conidia, sclerotia and chlamydospores are more heat resistant compared to vegetative fungal cells (Dijksterhuis, 2007). With regard to the species with elevated heat resistance, ascospores of both *T. flavus* and *N. fischeri* could survive in fruit products after pasteurisation (Van Der Spuy *et al.*, 1975), whilst ascospores of *A. fischeri* var. *glaber* were found to survive in hot water at 100°C for 5 min, and also in canned fruits despite the canning process. Moreover, they were found to survive under anaerobic conditions more than two months (Mcevoy and Stuart, 1970). In addition, Mcevoy and Stuart (1970) figured out that ascospores of *Byssochlamys fulva* and *A. fischeri* var. *glaber*, both contaminants of canned strawberries, are highly heat resistant. Ascospores of *B. fulva* required exposure to 90.5°C to be killed, whilst ascospores of *A. fischeri* var. *glaber* were able to survive at both higher temperatures and longer exposure times than those used in the canning process. By contrast, conidia of *A. fischeri* var. *glaber* did not survive above 70°C. However, all cells were killed in 10 min of extreme temperature between 95°C and

100°C. This is the so-called "thermal death point" (Mcevoy and Stuart, 1970). The high heat resistance of ascospores of these species is apparent when they are compared to even moderately heat resistant species. For example, the D_{83} value of *E. chevalieri* was 3.77 min whereas that of *N. fischeri* was 63.51 and 69.27 min in mango and grape juices at pH 3.5, respectively (Rajashekhara *et al.*, 1998). Beuchat (1986) reported *D*-values for ascospores of *T. flavus* as being 2.9–5.4 min at 91°C in several fruit products, whilst Scott and Bernard (1987) found a *D*-value of 2.2 min at 90.6°C for *T. flavus* in apple juice, and a *D*-value of 1.4 min for *N. fischeri* was at 87.8°C. Conversely, *E. chevalieri* has lower sensitivity to temperature increases at lower temperatures in terms of a *z*-value of 12.8°C, which is high compared to other heat resistant fungi (Kocakaya Yildiz and Coksöyler, 2002). In one detailed investigation, the effect of pasteurisation at 84°C for 20 sec in red currant juice at pH 3.2 was studied. Juice was subsequently bottled at 80-82°C and post-pasteurised in a rain-pasteuriser at 72°C for 20 min and finally stored for a few weeks at 15-20°C. It was consequently found that 1% of 100,000 bottles were infected with *N. fischeri* var. *glaber*. It was hypothesised that this red currant juice might have contained approximately 10 ascospores per bottle after pasteurisation which caused spoilage of 1 in 10,000 bottles which was considered an acceptable loss (Baggerman and Samson, 1988).

There are several possible reasons why ascospores might have elevated heat resistance compared to conidia. These include the structure of spores, presence of sugar such as trehalose and mannitol, water activity (a_w) effect, age of culture, and number of nuclei. Firstly, the structure of spores has a fundamental role in providing resistance against adverse conditions such as heat, toxic chemicals and other environmental stressors and it is vital that DNA remains intact within dormant spores (Sussman and Douthit, 1973). It was found that conidia of the thermotolerant species *B. fulva* were less heat resistant than ascospores of the species. Further research identified that the ascospore cell wall was thicker than that of the conidium, leading to the hypothesis that this might be a key factor increasing the heat tolerance (Partsch *et al.*, 1969).

Similarly, the structure of ascospores of some yeasts is thought to lead to an increase in resistance against various chemical and physical stresses, particularly the heat shock (Coluccio *et al.*, 2008). It was also observed that the ascospores of *S. cerevisiae* and *Schizosaccharomyces pombe* were more resistance than vegetative cells, being able to survive passage through the gut of *Drosophila melanogaster* and a variety of treatments such as incubation at 42^{o}C, exposure to very high salt concentrations and high or low pH mimicking natural stressors. It was suggested that the structure of the ascospore walls was essential for this resistance as they contain chitosan and dityrosine layers not found in vegetative walls (Coluccio *et al.*, 2008). Similarly Smits *et al.* (2001) suggested that the ascospore cell wall of *S. cerevisiae* is thicker and more extensive than the cell wall of vegetative cells, which might provide essential resistance to environmental assaults, with ascospore walls thought to be composed of four layers of different polymers. Despite the ascospores of various yeasts including *S. cerevisiae* being highly resistance to laboratory treatments such as exposure to ether vapur or temperature shock at 55^{o}C, the relevance of these treatments to stresses in natural environment is still unclear (Dawes and Hardie, 1974; Briza *et al.*, 1990). Similarly, the walls of vegetative cells of various fungal species are usually thinner and less complex than the cell walls of sexual spores for several species of terrestrial phycomycetes, basidiomycetes, slime molds and ascomycetes (Sussman and Douthit, 1973). The thickness and chemical structure of the cell wall of spores is thought to play a fundamental role in reducing protein denaturation and damage to cells due to heat shock via increasing the heat-resistance of spores against harsh conditions (Partsch *et al.*, 1969). Thick-walled hyphal fragments and chlamydospores of *Paecilomyces variotii, Fusarium* spp. and sclerotia of *Eupenicillium* are also said to exhibit high thermo-resistance (Splittstoesser and King, 1984).

Of relevance to the development of thick cell walls in ascospores is the observation that heat resistance of ascospores can be strongly affected by the age of spores at which spores are harvested. This is presumably because the ascospore cell wall requires a longer time to mature than the

less complex conidial wall. For example, the heat resistance of ascospores of *N. fischeri* increased with age and was dependent on the temperature at which ascospores were produced (Conner and Beuchat, 1987). It was observed that young cells of *Hansenula anomala* were less resistant than old cells (Su *et al.*, 1985). Conner *et al.* (1987) also reported that the heat resistance of *N. fischeri* increased very significantly in mature ascospores versus young ascospores. Similar observations were made for *Aspergillus* species found as contaminants of canned strawberries, and it was argued that mature ascospores might be changed physiologically over time and that would be positively reflected in their heat resistance (Kavanagh *et al.*, 1963). Further investigations using transmission electron microscopy (TEM) and chemical analyses illustrated that the ultrastructural and chemical changes occurred in the ascospores of *N. fischeri* when they reached an age of 11-25 d. This was correlated with an elevated heat resistance, indicating a possible relationship between these changes and increase in ability to resist thermal shock (Conner *et al.*, 1987).

A second factor contributing to high temperature resistance is the so-called "glassy state" of ascospores, referring to the presence of a highly dense cytoplasm inside the ascospores which increases heat resistance by preventing proteins from denaturation, maintaining a low moisture content and protecting other organelles (Dijksterhuis, 2007). Linked to this, many heat-resistant ascospores have been shown to accumulate organic materials such as trehalose, mannitol and glycerol which can protect cellular membranes and the proteins (Crowe *et al.*, 1984; Prestrelski *et al.*, 1993). A relatively high amount of mannitol and trehalose has been detected in the ascospores of many heat resistant fungi and is thought to have essential roles in protecting proteins from denaturation (Conner *et al.*, 1987; Dijksterhuis, 2007) and preventing oxidative reactions that could cause damage to DNA (Ruijter *et al.,* 2003). Of particular relevance to the present study is that mannitol and trehalose are found in the ascospores of *A. nidulans* (Fillinger *et al.,* 2001). Both trehalase and invertase were found to be compartmentalised in ascospores of *Neurospora tetrasperma*, which could contribute to heat

resistance (Sussman, 1966; Thevelein, 1984). It was suggested that the trehalose found in heat-resistant ascospores, which was lacking from heat-sensitive ascospores, may stabilise membranes against dehydration due to heat shock or freezing stress (Rudolph and Crowe, 1985; Rudolph *et al.*, 1986). Additionally, intracellular trehalose can reduce the heat inactivation of enzymes of the slime mould *Dictyostelium discoideum* resulting in more heat resistant spores (Emyanitoff and Wright, 1979). More recently, the *treB* gene that encodes a protein for intracellular trehalase in *A. niger* has been deleted and the intracellular levels of trehalose during conidial outgrowth of this fungus was then analysed. As result of the gene deletion the number of conidia formed on conidiophores was found to decrease, indicating a role for trehalose in sporogenesis (Svanstro and Melin, 2013). In order to investigate the function of trehalose as a protectant of fungal structures against extreme stresses, the conidia of the same *ΔtreB* mutant were subjected to acid and salt stresses. They were shown to be somewhat more sensitive than the wild type conidia, suggesting the role of trehalose as a stress protectant in *A. niger* (Svanstro and Melin, 2013). Further investigations exhibited similar results when spores of *ΔtreB* of *A. nidulans* and *A. niger* were heat shocked at 55^{o}C showed to be highly sensitive. This may explain the importance of *treB* gene for surviving and the role of trehalose for protecting spores against the heat stress (Svanstro and Melin, 2013). Conner *et al.* (1987) stated that the amount of mannitol accumulating in ascospores of *N. fischeri* is also a key factor increasing the heat resistance. Sugars, such as mannitol and trehalose, have been correlated with the presence of a glassy state (Dijksterhuis *et al.,* 2002) as they substitute water in hydrogen binding leaving the spore dehydrated. Mannitol on the other hand has the ability to quench reactive oxygen species, which can cause damage to the DNA and proteins of the ascospore, and may have a role in prevention of oxidative damage (Ruijter *et al.,* 2003).

In terms of the water activity effect, it was observed that a lowered water activity elevated the heat resistance of conidia of *A. niger* (Lubieniecki and Heiss, 1975). Another study showed that decreasing the

internal water activity, via the accumulation of high amounts of mannitol, trehalose and glycerol, in ascospores of *N. fischeri* aged from 11-25 d appeared to be an important factor in the development of heat resistance and maintenance of enzyme viability in this species (Conner *et al.*, 1987).

A third factor linked to increased heat resistance of ascospores might be the number of nuclei present per spore, It has been reported that each ascospore of *A. nidulans* contains two nuclei (i.e. a binuclear state) compared with only one nucleus (i.e. mononuclear state) in each conidium (Elliot, 1969). As well as double the number of nuclei, it would also be expected that ascospores would have increased amounts of cytoplasm with higher numbers of other organelles, proteins, and sugars etc. The overall effect would be to provide more 'buffering capacity' against thermal shock and hence increased heat resistance of ascospores over conidia. However, much more work remains to be investigated in this context. Preliminary investigations using TEM were made to attempt to confirm the binuclear state of ascospores of *A. nidulans*, but it was not possible to gain convincing data due to technical difficulties (data not shown).

4.2.2 The influence of UV radiation on spore viability of *A. nidulans*.

This experimental work aimed to analyse whether conidia and ascospores of *Aspergillus nidulans* have different levels of resistance when exposed to UV light for various lengths of times. The underlying hypothesis was that ascospores might exhibit greater survival than conidia, which would confer evolutionary advantages to ascospore production given exposure to UV radiation in the atmosphere. Both ionising and non-ionising radiation are known to negatively affect spore viability, due to damage to the DNA of fungal cells (Moore-Landecker, 1996). Although many studies have been caried out in the past to assess the effect of UV on growth and reproduction on various fungi, to our knowledge no investigations have yet been reported examining either the overall effect of UV on spore viability (asexual and sexual) of *A. nidulans* nor the possible differential

resistance between spore types in *Aspergillus* species in general. Therefore, spores from 8 isolates/strains of *A. nidulans* were exposed to UV shock at a wavelength of 254 nm for a time course of 10 to 60 min and survival of conidia and ascospores determined over this period.

ANOVA revealed that there was an overall significant effect of UV exposure on spore viability for all isolates/strains with decreased survival of spores as the period of UV exposure was increased. This was to be expected because ultraviolet light (UV) is a strong mutagen in the wavelength range 225-300 nm, corresponding to the part of the spectrum at which DNA absorbs radiation. The mutagenic effect arises primarily due to the generation of dimers between adjacent pyrimidine bases on the same DNA strand. These dimers cause the strand to buckle, disrupting normal base pairing. This prevents proper replication and can result in point mutations, deletions, and chromosomal rearrangements, although the effects of mutagenesis can be reversed by photoreactivation, a light-dependent DNA repair mechanism (Davis and De Serres, 1970). Related to these effects, gamma radiation dosage from 0.25 to 6.0 kGy have been used for decontamination of fungal species including *Aspergillus, Penicillium, Rhizopus, Mucor, Alternaria* and *Fusarium* present on *Nigella sativa* seeds in Egypt (Zeinab *et al.*, 2001).

Although both conidia and ascospores of *A. nidulans* were inactivated by exposure to UV light, my results as a whole showed that ascospores exhibited significant better resistance to UV exposure than conidia. This was apparent from the greater *D*-values of ascospores compared to conidia (**Table 4-2**). Indeed, these differences were more marked than differences in *D*-values of ascospores compared to conidia when exposed to heat shock (**Table 4-1**). The ascospores generally were more heat resistant than conidia with around 20% of increase (**Table 4-1**) when heat shocked. Whereas, they showed roughly 65% after UV exposure (**Table 4-2**). This suggested that in the case of *A. nidulans* that ascospores might have a more important role in resistance to UV irradiation than heat shock.

Looking at specific data, most isolates/strains showed a similar pattern of spore survival, with 50-60% of ascospores surviving at the first three timepoints of UV shock (10-30 min), but survival then gradually decreased after this with the increased exposure time. By contrast only 0-20% of conidia survived after the first three timepoints of UV shock (10-30 min) for most strains (**Figure 4-17** to **Figure 4-24**). There were some exceptions, such as 2-224 which exhibited low survival of both ascospores and conidia, and 2-149 which had quite large survival of *ca.* 30% of conidia at 30 min, but these were unusual. The latter strain was of *veA1ku* genotype, suggesting that these mutations did not result in increased UV sensitivity at least at the spore stage (although this might conceivably occur at the vegetative growth stage). Of particular interest was the observation that conidia of strain 2-154 were particularly sensitive to UV exposure, with minimal survival of less than 5% of conidia even after only 10 min exposure (**Figure 4-24**). This was highly significant because 2-154 is an orange spore colour mutant strain (**Table 3-1**). It has been suggested that melanin pigments contributing to the wild-type green colour of conidia of *A. nidulans* protect the fungus against the lethal effects of UV radiation, visible light and natural sunlight inactivation, with a yellow pigment mutant not exhibiting this protection (Al-Rubeai and El-Hassi, 1986). Results with 2-154 are consistent with this assertion. On the other hand the yellow mutant and wild type of *A. nidulans* showed an equal sensitivity to visible light in the presence of toluidine blue (Thomas *et al.*, 1981). Pigment mutations are found in some strains of *A. nidulans* (Ayling, 1969). The green and yellow pigments are thought to be situated near or in the conidium cell wall (Rowley and Pirt, 1972). Elsewhere, the role of conidial pigmentation has been studied in the entomopathogenic fungus *Metarhizium anisopliae.* This produces wild-type dark green conidia but in addition yellow, purple and white conidia colour mutants. The effect of pigmentation on survival against solar UV radiation was investigated. The results obtained showed a significant variation in tolerance, with green conidia exhibiting better resistance than the other three-color mutants. Indeed, the white conidia

were the least resistant and were more sensitive than the mutants with yellow conidia (Brags *et al.*, 2006).

Elsewhere, Rotem *et al.* (1985) figured out that solar radiation in general and its UV portion specifically are the main factors that cause mortality of *Peronospora tabacina*, *Uromyces phaseoli* and *Alternaria solani*. Caesar and Pearson (1982) observed that survival of ascospores of *Sclerotinia sclerotiorum* decreased when subjected to sunlight, and that the survival rate of ascospores was 49.0 and 13.4% under plastic films that transmitted 10 and 50-60% of UV radiation in the 300-400 nm range, respectively. Similarly, a few hours of direct solar radiation was sufficient to inactivate conidia of *Metarhizium anisopliae* in the field, with exposure to both solar UVA (320–400 nm) and UVB radiation (290–320 nm) effectively inactivating conidia (Roberts and Campbell, 1977; Braga *et al.*, 2001b). More recent investigations showed that the exposure to UVB light at 302 nm for 5 to 20 min reduced the ability of mutants of *A. flavus* and *A. parasiticus* to accumulate aflatoxin precursor metabolite by five-fold compared to common aflatoxin-producing strains or pigmented mutants that normally accumulate aflatoxin precursors (Ehrlich *et al.*, 2010).

The reasons why ascospores of *A. nidulans* have greater resistance to UV exposure than conidia are at present unknown. However, some speculation can be made. One factor might be the presence of pigments in the ascospores that might absorb the ionizing radiation and thus confer protection to the ascospores. The characteristic red colour of *A. nidulans* ascospores is due to the presence of the pigment asperthecin (Szewczyk *et al.*, 2008), which might absorb UV radiation. Indeed, a colourless ascospore mutant of *A. nidulans* was obtained during the course of studies, but unfortunately time limitation prevented analysis of the UV resistance of its ascospores.

Elsewhere, melanins have been widely considered to protect cells of several fungal species against environmental stresses including UV radiation, oxidation, and heat shock (Bartley *et al.*, 1990; Jacobson,

2000; Langfelder *et al.*, 2003). This suggests that melanins play a fundamental role as important structural components of the cell wall of conidia and hyphae (Butler and Day, 1998; Butler *et al.*, 2001). Adams *et al.* (1998) have described the biosynthesis of melanins and melanin-like compounds during the conidiation of *A. nidulans*, whilst Tsai *et al.* (1999) described their production during conidiation of *A. fumigatus*. Al-Rubeai and El-Hassi (1986) asserted that melanin is the most important group of pigments protecting conidia and other fungal structures from radiation, describing the role of green pigments (a melanin protein complex) of *Penicillium cyclopium* in protecting conidia from Far-UV radiation (FUV) (200-122 nm). Consistent with this assertion was the finding that conidia of wild-type *P. cyclopium* were around 100 times more resistance to FUV than unpigmented conidia. In addition, carotenoids were found to increase the resistance of a melaninless mutant of *Wangiella dermatitidis* against the lethal effects of UV irradiation. However, these pigments were less effective in the wild-type fungus due to the superior shielding effect of melanin pigments (Geis and Szaniszlo, 1984). Pigments in some lichens are also known to provide protection against UV radiation (Gauslaa and Solhaug, 2001; Sundin *et al.*, 2002). Taking all those aforementioned observations in account, spore pigmentation can clearly play an essential role in protecting spores against UV radiation (Braga *et al.*, 2001a; 2001b; Gómez and Nosanchuk, 2003).

In addition to pigmentation, there might be other factors enhancing the resistance of ascospores of *A. nidulans* as described before in relation to elevated heat resistance e.g. the unique structure of ascospores versus conidia comprising the thickness of cell wall, elevated amount of trehalose and mannitol, water activity effect. In particular the presence of two nuclei (versus one in conidia) might be a critical factor, allowing some DNA damage to occur in one nucleus whilst leaving a functional nucleus intact. Moreover, the ascospore walls of some fungi, including the model species *Neurospora crassa*, contain sporopollenin which is a highly stable polymeric molecule that may play a fundamental protective role in their ascospore walls (Gooday *et al.*, 1974). Sporopollenin has also found to be a constituent of the outer wall of pollen grains and has

been detected in the wall layers of *Daldinia concenfrica* ascospores, where it has been linked with their environmental resistance (Beckett, 1976; Read and Beckett, 1996). However, much remains to be learnt about this correlation and the precise mechanism by which sporopollenin might confer environmental resistance.

4.2.2.1 Assessment of heat shock of cleistothecia to yield contaminant free ascospore suspensions

As an adjunct to work in the present chapter, studies were also undertaken to determine whether it might be possible to use heat shock of ascospores within cleistothecia to produce contaminant free ascospore suspensions. This would be in contrast to the traditional 'rolling of cleistothecia on TWA' (Todd *et al.*, 2007), which is subject to occasional contamination.

It has been assumed that ascospores and conidia of *A. nidulans* have very similar sensitivity to heat shock (Braus *et al.,* 2002). However, results from the present chapter illustrated that ascospores exhibited marginally increased heat resistance than conidia, although for many isolates/strains both conidia and ascospores survived heat shock at the same temperature. This contrasts with, for example, *Neosartorya* species where conidia can be heat inactivated by heat shock at 70-80ºC whilst leaving ascospores intact (O'Gorman *et al.,* 2009). Thus, it was considered that ascospores of *A. nidulans* within the asci within clesitothecia might gain some protection against heat shock compared to contaminating 'naked' vegetative mycelia and conidia on the exterior of the cleistothecial wall, which together with their inherent increased heat resistance might allow purification of ascospores. Although results of the heat shock varied considerably between isolates and strains, nevertheless there was a general pattern that ascospores within cleistothecia showed better survival against heat shock at 65-72^{o}C for 30 min than conidia. Thus, heat shock at 68^{o}C appeared to provide a useful method to reduce the contamination of ascospore suspensions for many isolates/strains of *A. nidulans*.

4.3 Conclusion

Data obtained in this chapter has demonstrated that both heat shock and UV exposure influenced significantly the spore viability of *A. nidulans*. Ascospores had greater levels of resistance than conidia to both heat shock and UV exposure. This was also shown by *D*-value comparisons between spore types (**Table 4-1** and **Table 4-2**). Similar results were obtained with four isolates of *E. repens* showing that the ascospores were also more resistant than conidia against temperature shock.

These results are of significance for various reasons. Firstly, they address the key enigma of why does homothallism occur in species that can also reproduce asexually? It was found that the formation of ascospores in homothallic species such as *A. nidulans* and *E. repens* could be of ecological advantage in allowing the survival of spores in harsh environmental conditions under which conidia might lose viability. For example, the enhanced irradiation resistance could be of importance as infrared, visible light, and near-UV (NUV) region reach the earth due to solar radiation. Although ozone in the atmosphere absorbs most Far-UV (FUV) light at wavelength from 220 to 300 nm, nevertheless some irradiation will reach the surface of earth, which can be absorbed by nucleic acid with possible mutagenic effects (Al-Rubeai and El-Hassi, 1986). Meanwhile, the genus *Aspergillus* might have tropical origins, so increased heat resistance might allow improved environmental survival (Raper and Fennel, 1965). The increased heat and UV resistance complements the fitness advantage contributed by the 'selection arena' occurring during sex in homothallic fungi (Bruggeman *et al.*, 2004).

The enhanced environmental resistance of ascospores can also explain the evolution of homothallism from heterothallic ancestors since a major advantage of homothallism is to populate an area from just one isolate without the need for outcrossing, so called 'reproductive assurance' (Murtagh *et al.,* (2000). It is also noted that the ascospores of *A. nidulans* and *E. repens* germinated readily at room temperature, unlike

the ascospores of some highly heat resistant species which require either a heat shock or high pressure to germinate (see section **4.1.3**). Thus, there might be a trade off between increased ascospore resistance and ability to germinate readily. Results from this chapter are also of industrial relevance for the food and medical sectors. Data suggests that sexual ascospores of many aspergilli might have higher than expected resistance to common sterilisation procedures. Therefore there is an increased risk of industrial spoilage problems (such as contamination of high sugar foods by *E. repens*) and also some infection by medical diseases such as aspergillosis (principally caused by *A. fumigatus*, but with some reports attributed to *A. nidulans*). More investigations are now needed to further examine this novel discovery, such as examining the precise physical and chemical reasons behind the differential resistance between ascospores and conidia in these species.

Chapter 5 Loss of Sexual Fertility and The Evolution of Asexuality

5.1 Introduction

5.1.1 Background to sexual and asexual development of ascomycete fungi

In general, filamentous ascomycete fungi propagate either by asexual and/or sexual means through mitotic or meiotic processes. As a consequence conidia (conidiospores) and ascospores are produced, respectively (Dyer and Paoletti, 2005). Fungi reproduce either sexually or asexually primarily based on the prevalent conditions. They commonly generate asexual spores where there are plentiful nutrient and suitable environmental conditions (e.g. pH, moisture, oxygen and other elements). However, they generate sexual spores under more restrictive conditions such as at the end of growing seasons when insufficient nutrient are available (Dyer *et al.*, 1992; Duncan, 1998).

5.1.2 Why have some fungal species lost their sexual reproductive ability?

Despite the many supposed benefits of sexual reproduction, approximately 20% of fungal species are only known to reproduce by asexual means (Dyer and Paoletti, 2005). Many asexual species are closely related to sexual species phylogenetically, indicating that they are descended from sexual ancestors. For example, *Penicillium* allied with *Eupenicillium* and *Talaromyces* (Lobuglio and Taylor, 1993; Berbee *et al.,* 1995), *Cercospora* with *Mycosphaerella* (Groenewald *et al.,* 2006), *Bipolaris* with *Cochliobolus*, and *Phaeoacremonium* with *Togninia* (Mostert *et al.,* 2006). There are various genetic and physiological reasons that might explain the lack of a sexual cycle in fungi as follows:

A. A key possible reason for asexuality could be due to mutation or loss of a key gene(s) required for sex (Dyer and Paoletti, 2005). For example, *Candida parapsilopsis* has a mutation in the *MAT**a**1* gene, which leads to formation of a nonfunctional MAT protein, hence providing a functional reason for asexuality (Logue *et al.*, 2005). However, other than these examples, genome-wide surveys of known sex-related genes of putative asexual species including *A. niger* (Pel *et al.*, 2007), *A. oryzae* (Galagan *et al.*, 2005; Machida *et al.*, 2005), *P. marneffei* (Woo *et al.*, 2006), *C. albicans* (Tzung *et al.*, 2001) and *C. glabrata* (Muller *et al.*, 2008) have failed to detect any evidence of mutation of genes required for sex. Moreover, intact non-mutated *MAT* genes, together with expression of *MAT* genes and the presence of pheromone precursor genes, has instead been shown in some asexual species including *Fusarium avenaceum*, *F. culmorum*, *F. poae*, *F. semitectum* (Kerenyi *et al.*, 2004)*, B. sacchari* (Sharon *et al.*, 1996), *Alternaria brassicae*, *A. brassicicola* and *A. tenuissima* (Berbee *et al.*, 2003). An initial report suggested that there was a mutation of a *pro1* gene needed for sex in *A. niger*, resulting in a premature stop codon (Pel *et al.*, 2007), However, this was later proven incorrect due to the presence of a previously non-annotated intron (PS Dyer, pers. comm.). It is noted that in addition to the approximately 200 genes known to be required for sex in ascomycete fungi (which have been used for genome screening purposes), e.g.(Galagan *et al.*, 2005) that up to 400 genes in total may have a role in sexuality (Dyer *et al.*, 1992), and a mutation in any of these major genes could lead to asexuality. Therefore, a mutation in an as yet unidentified gene(s) may be a reason for asexuality.

B. A gradual decline in sexual reproductive ability may also occur via a series of gene mutations and/or altered levels of gene expression. This might lead eventually to lack of function and loss of sex within populations as a whole, the so-called 'slow decline hypothesis' of Dyer and Paoletti (2005). This is illustrated by the

classic experimental work of Mather and Jinks (1958) who found that the production of cleistothecia (then termed perithecia) and ascospores was gradually lost in *Aspergillus glaucus* when the fungus was propagated for several generations by conidia, which they suggested might be due to various changes in the cytoplasm. However, sexual fertility could then be restored by propagation by ascospore transfer (**Figure 5-1**). A similar pattern was observed in prior experimental work by Jinks (1954) with *A. nidulans*, which was also reported to exhibit a gradual decline in sexual fertility when propagated by conidia, but this paper lacked many experimental details. *Neurospora crassa* was also said to exhibit a decline in sexual fertility when propagated by hyphal tips [(Sheng, 1951), cited in Mather and Jinks (1958)]. Interestingly though, in the case of the aspergilli, production of conidia was never lost as a result of sexual propagation (Jinks, 1954; Mather and Jinks 1958).

C. For many fungal species, the early stages of sexual morphogenesis can occur independently of the *MAT* genes. Some fungi are hermaphrodites, which are able to form both antheridia and ascogonia (Bistis, 1996; Bobrowicz *et al.*, 2002; Coppin *et al.*, 2005). However, some heterothallic fungal species include isolates which may be able to produce only antheridia (termed 'males') or ascogonia (termed 'females'), thus superimposing a second layer of sexual compatibility in addition to that mediated by *MAT* genes. Thus, for some heterothallic *Fusarium* species (Leslie and Summerell, 2006) there can be *MAT1-1* female, *MAT1-1* male, *MAT1-2* female and *MAT1-2* male, as well as *MAT1-1* and *MAT1-2* hermaphrodites. It is necessary to have males and females of opposite mating type present for viable sexual reproduction (Nauta and Hoekstra, 1992; Leslie and Summerell, 2006). The gradual accumulation of isolates of only one sex would limit a species ability to undergo sexual reproduction thereby rendering a species asexual in most circumstances.

D. A physical absence of partners of opposite mating type in a certain geographical region can also render species asexual. For example,

MAT1-1 isolates of *Ascochyta rabiei* were limited to asexual reproduction in Tunisia until the last decade, when *MAT1-2* isolates were inadvertently introduced into Tunisia allowing development of the *Didymella rabiei* teleomorph. Similarly, Snyder *et al.* (1975) found that isolates of *Hypomyces solani* f. sp. *cucurbitae* (anamorph: *Fusarium solani* f. sp. *cucurbitae*) were restricted to asexual reproduction for most of its worldwide distribution due to lack of a suitable mating partner, but retained the ability to mate when artificially introduced to isolates of the opposite mating type.

E. The failure to discover sexual states in many asexual fungi might simply be due to oversight or lack of observation. For example, it had been thought that *Pseudocercospora herpotrichoides* was a purely asexual anamorphic species but a sexual state (teleomorph) named '*Tapesia yallundae'* was discovered in the late 1980s by careful observation of field material. Subsequently apothecia were found in field surveys on straw stubble that had been left throughout the winter throughout much of its range in the UK (Lucas *et al.*, 2000; Dyer *et al.*, 2001). Similarly, a sexual cycle was recently discovered for *Septoria passerinia* in its natural environment, which belonged to the genus *Mycosphaerella* (Ware *et al.*, 2007). Furthermore, Armstrong-Cho and Banniza (2006) and Guerber and Correll (2001) discovered teleomorphs (sexual states) of the plant pathogens *Glomerella tuncata* (anamorph: *Colletotrichum tuncatum* and *Glomerella acutata* (anamorph: *Colletotrichum acutatum*), respectively. In addition, the sexual states of supposedly 'asexual' species have been successfully discovered in some other species of economical and biological importance since the millennium, including sexual cycles of *Togninia minima* (anamorph: *Phaeoacremonium aleophilum*) that causes Petri disease in grapevines, *Cordyceps bassiana* (anamorph: *Beauveria bassiana*), which is used as a biological control agent against insects, and *Fusarium tucumaniae* that causes soybean sudden death syndrome (Huang *et al.*, 2002; Mostert *et al.*, 2006; Covert *et al.*, 2007).

F. Another reason for supposed asexuality of species is that the anamorph (asexual state) and teleomorph (sexual state) have been described as separate entities and accidentally not been connected by taxonomists due to oversight. For example, it was only in the 1970s that the phytopathogen *Mycosphaerella graminicola* was linked with its anamorph *Septoria tritici* (Sanderson, 1972). Parallel investigations allowed the joining of the industrial species *Trichoderma reesei*, long known as a purely asexual propagating species, to its long established teleomorph *Hypocrea jecorina* (Kuhls *et al.*, 1996; Druzhinina *et al.*, 2006). This latter work involved the identification of a *MAT1-2* gene from the genome sequence of *T. reesei* QM6a and then crossing to a MAT1 partner that had already been defined in *H. jecorina* (Seidl *et al.*, 2009). As a further example, Houbraken *et al.* (2008) have recently reported that the anamorphic and teleomorphic state of the foodstuff, soil and plant contaminant *Byssochlamys spectabilis* (anamorph: *Paecilomyces variotii*) were connected and the species has been given a new name of *Talaromyces spectabilis.*

G. A final reason for supposed asexuality of species might be that conditions required for sex are very infrequent in nature. This might possibly lead to decline of sexuality in these fungi. For instance *C. albicans* has intact *MAT* and pheromone genes and mating has been induced in laboratory manipulated conditions, indicating that the failure to discover sex in the wild is not due to lack of functional genes, but may be as a result of lack of correct environmental conditions (Hull *et al.*, 2000; Magee and Magee, 2000; Tzung *et al.*, 2001). Magee and Magee (2000) found mating of *C. albicans* occurred when isolates were injected into a mice after deleting the MTL **a** or MTLα regions and incubation at 30°C. This suggested that skin might provide appropriate niches to induce mating in *C. albicans*, but mating in this fungus has not yet been observed in nature (Hull *et al.*, 2000; Magee and Magee, 2000; Magee and Magee, 2004). Related to this, many fungi may

apparently be asexual because appropriate conditions have not yet been detected to induce the sexual state *in vitro*.

Many of the above explanations for asexuality indicate that many supposedly 'asexual' species might have the potential for sexual reproduction. And indeed there is accumulating evidence that sexual reproduction might be possible in some supposedly 'asexual' species. For example, population genetic studies of various 'asexual' fungi have provided evidence of recombination in field populations, such as in the food spoilage agent *A. flavus,* which was later discovered to have a sexual stage (Taylor *et al.*, 1999b). Meanwhile, genome-sequencing studies revealed complements of genes required for sexual reproduction in the considered 'asexual' pathogenic species *C. albicans* and *A. fumigatus* (Tzung *et al.*, 2001). Later investigations with both of these species revealed the potential for sexual mating, and in the case of *A. fumigatus* a complete sexual cycle (Miller and Johnson, 2002; Tavanti *et al.*, 2004; O'Gorman *et al.*, 2009). Kück and Pöggeler (2009) argued that solving the 'mystery' of a lack of a sexual cycle in some putatively asexual species would provide a very important tool for understanding aspects of the evolution in fungi.

5.1.3 Aims of this chapter

One of the principal aims of the present study is to study the possible evolution of asexuality in *Aspergillus* species. As mentioned above, there are classical studies by Jinks (1954) and Mather and Jinks (1958) (both published in the journal *Nature*) suggesting that sexual fertility and ultimately sexual reproduction can be lost when sexual species are sub-cultured repeatedly in the laboratory solely by asexual transfer, as shown in **Figure 5-1.**

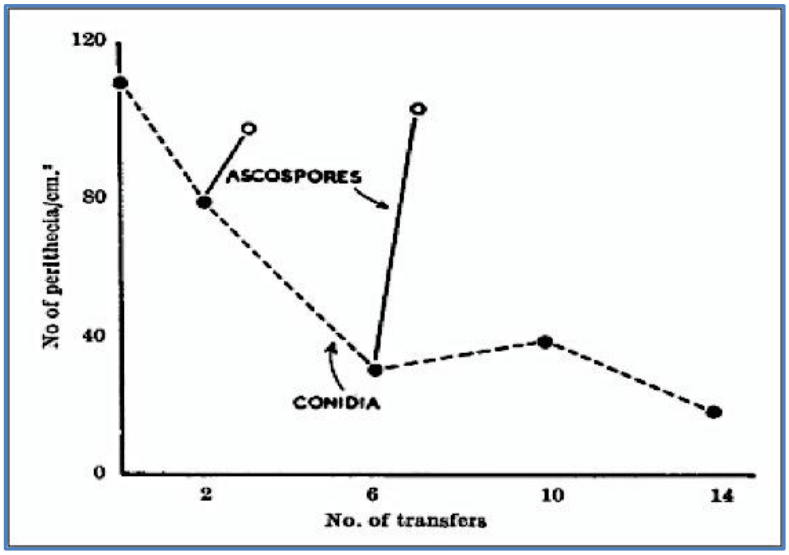

Figure 5-1 The effects of propagation by conidia and by ascospores on the frequency of perithecial production in *Aspergillus glaucus*. Continued propagation by conidia (dotted line) result in a gradual decline of perithecial production. This is, however, immediately restored to its original level by a single propagation through an ascospore. [Adapted from (Mather and Jinks, 1958)].

This offers a key insight into how asexual species might evolve. However, these papers provided very few experimental details (e.g. precise definition of a 'transfer') and no statistical validation or replication of these results, possibly reflecting scientific practice at the time. Also the stages between assessing sexual fertility between 'transfers' appear quite large. Given the possible significance of these observations work was therefore undertaken in the present study to assess whether these results could be repeated, and whether new insights could be gained by applying modern microscopic and molecular techniques. For these studies the species *A. nidulans* and *E. repens* (anamorph *A. glaucus*) were selected, as originally used by Jinks (1954) and Mather and Jinks (1958). In addition work was conducted with *N. fischeri* as a further example of an independent *Aspergillus* species. All species selected were homothallic to allow relatively rapid induction of the sexual cycle.

Specific aims were:

1. To repeat the work of Jinks (1954) and Mather and Jinks (1958) to assess whether a loss of sexual fertility can be shown to arise due to sub-culturing by conidial transfer.
2. To correlate the number of vegetative transfers with number of mitotic divisions to provide a quantifiable guide as to how rapidly loss of sexual fertility might occur.
3. To investigate whether sexual fertility levels can be restored by passage via ascospores, as suggested by Jinks (1954) and Mather and Jinks (1958), to thereby providing insights into the possible genetic basis of such changes e.g. whether it is a mutational and/or epigenetic effect.

5.2 Materials and Methods

5.2.1 Fungal isolates and asexual and sexual growth

A total of twelve isolates from the three homothallic species *A. nidulans*, *N. fischeri* and *E. repens* (i.e. four isolates per species) were investigated to assess whether loss of sexual fertility can be shown to occur due to sub-culturing by conidial transfer. The four representatives of *A. nidulans* were 2-137, 2-139 (both wild-type), 2-149 (*pyrG89, veA1, pyroA4, nkuA*::*argB*) and 2-154 (*yA1, pabaA1, niiA4, nkuA*::*bar*), grown on ACM [supplemented with 10 mM Uracil (1.12 g/L) and 5.0 mM Uridine (1.2 g/L) in the case of 2-149 using a pyrimidine stock of 4.48 g Uracil + 4.88 g Uridine in 200 ml dH_2O]. The four isolates of *N. fischeri* were 53-2, 53-14, 53-15 and 53-16 (**Table 3-2**) and those of *E. repens* were 51-3, 51-4, 51-5 and 51-6 (**Table 3-3**). All isolates were known to be sexually fertile from previous work in the present study. Work conducted in chapter 3 had already identified suitable conditions to favour either asexual or sexual growth of *A. nidulans*, *N. fischeri* and *E. repens* (section **3.5**), and these conditions were therefore adopted for the present experimental work as described below.

5.2.1.1 Asexual growth conditions and harvest of conidia

All species were incubated at 28ºC under white light (2.5 µmol m^{-2} s^{-1}) in unsealed 9.0 cm diameter Petri dishes. *Aspergillus nidulans* and *N. fischeri* were grown on ACM, whilst *E. repens* was grown on M40Y to favour asexual reproduction. Asexual spores of all species were harvested using 0.01% Tween 20 phosphate buffer solution, which were then strained through a Miracloth filter as previously described (section **4.3.1.1**). The spore concentration was then measured using a haemocytometer (sections **4.3.1.1** and **4.3.4**).

5.2.1.2 Sexual growth conditions, fertility assay and harvest of ascospores

To assess the sexual fertility of cultures, sexual reproduction was induced in *A. nidulans* and *E. repens* as previously described (sections **3.3.3.1** and **4.3.1.2**). This involved spreading 1 x 10^5 spores over the surface of a 5.0 cm Petri plate containing either ACM or M40Y, respectively. Six replicate plates were set up per isolate/strain. In the case of *A. nidulans*, Petri dishes were incubated individually (i.e. not in stacks!) in the dark at 32^{o}C for 15 hr and then plates were sealed with two layers of Nescofilm before being returned back to the incubator and left for a further 30 d to allow maturation of cleistothecia (section **3.3.4**). For *E. repens*, Petri dishes were incubated unsealed at 28ºC for 7 d under white light (2.5 µmol m^{-2} s^{-1}). Meanwhile for *N. fischeri* sexual reproduction was induced by inoculating ACM plates with 1 x 10^5 spores. These plates were sealed straight away with two layers of Nescofilm, and placed in the dark at 28 ºC.

Finally the number of cleistothecia formed after 4 weeks was scored visually by microscopy, counting numbers of cleistothecia formed in replicate viewing areas of known size (75.36 mm^2), with arising data converted to numbers of cleistothecia formed per 100 mm^2 as described in section **3.3.3.1**. Six pseudo-replicate counts were made per individual plate, which was averaged to give a datapoint for each replicate, with six replicate plates scored per assay. Particular care had to be taken when

counting cleistothecia of *E. repens*, as these were often very abundant and could be hidden underneath mycelium and were present at different depths of the culture. Therefore, for the latter species the surface mycelium was gently removed with a sterile cotton bud to reveal all cleistothecia, and various depths of focus used to identify and score all cleistothecia.

To harvest ascospores, 50-100 mature cleistothecia of a given species/isolate were collected using a sterilised needle and cleaned on 4% TWA before release of ascospores as previously described (section **4.3.1.2**) (Todd *et al.*, 2007). In the particular case of *A. nidulans* cleaned cleistothecia were preheated at 68ºC for 30 min prior to rupture to obtain pure ascospores according to previous results of the present study (section **4.3.4**). The ascospore concentration was then measured using the haemocytometer method and diluted to the desired 625 spores per ml before being spread over suitable growth media to allow isolation of individual ascospore-derived cultures (sections **4.3.1.1** and **4.3.4**).

5.2.2 Procedure for assessing the possible loss of sexual fertility (slow decline) arising from conidial sub-culturing

At the start of the experiment all cultures of *A. nidulans*, *E. repens* and *N. fischeri* were restored to a 'default' state by passage through the sexual cycle (section **5.2.1.2**). Resulting sexual ascospores were spread over plates of suitable growth medium (ACM for *A. nidulans* and *N. fischeri,* M40Y for *E. repens*) using a sterile spreader and incubated 2-3 d at 28ºC under light to identify individual germinating colonies. Six morphologically typical colonies were then transferred to new 9.0 cm diameter Petri dishes containing the same growth medium, which were all incubated (unsealed) at 28ºC in light for 5-7 d. A typical culture, as judged by morphological appearance (e.g. growth rate, evidence of asexual sporulation, pigmentation, lack of sectoring), was then chosen and labelled as 'culture transfer 0' at the start of the major transfer

experiment for each isolate/strain of each species under study (i.e. a total of 12 cultures).

The 'transfer 0' cultures were then used for two purposes. Firstly the sexual fertility at this starting point was determined. This involved the harvesting of conidia from an exterior part of the plate (ca. 40 mm from the centre), the setting up of sexual cultures by spreading the conidial spore suspension (1 x 10^5 conidia) on suitable growth medium for each species, incubation under appropriate conditions to induce sexual reproduction for each species, and the final scoring of plates for production of cleistothecia as described above (section **5.2.1.2**). This provided the important starting 'transfer 0' value for sexual fertility. Secondly, conidia from this 'transfer 0' culture were used to establish the first of the planned series of asexual transfers by conidial transfer. To achieve this, a small amount of conidia were collected using a sterile loop from the edge of the plate (ca. 40 mm from the centre; **Figure 5-2**) and then spores were streak spread out on growth plates (ACM for *A. nidulans* and *N. fischeri,* M40Y for *E. repens*). Plates were incubated at 28ºC for 1-2 d then six newly emerging single spore colonies were picked off and grown on new 9.0 cm diameter growth medium plates at 28ºC in the light for 5-7 d. A morphologically 'average' looking culture was then chosen and labelled as culture 'transfer 1' (**Figure 5-2**). The original 'transfer 0' plate was sealed with Nescofilm, stored safely in the cold room, dated and labelled.

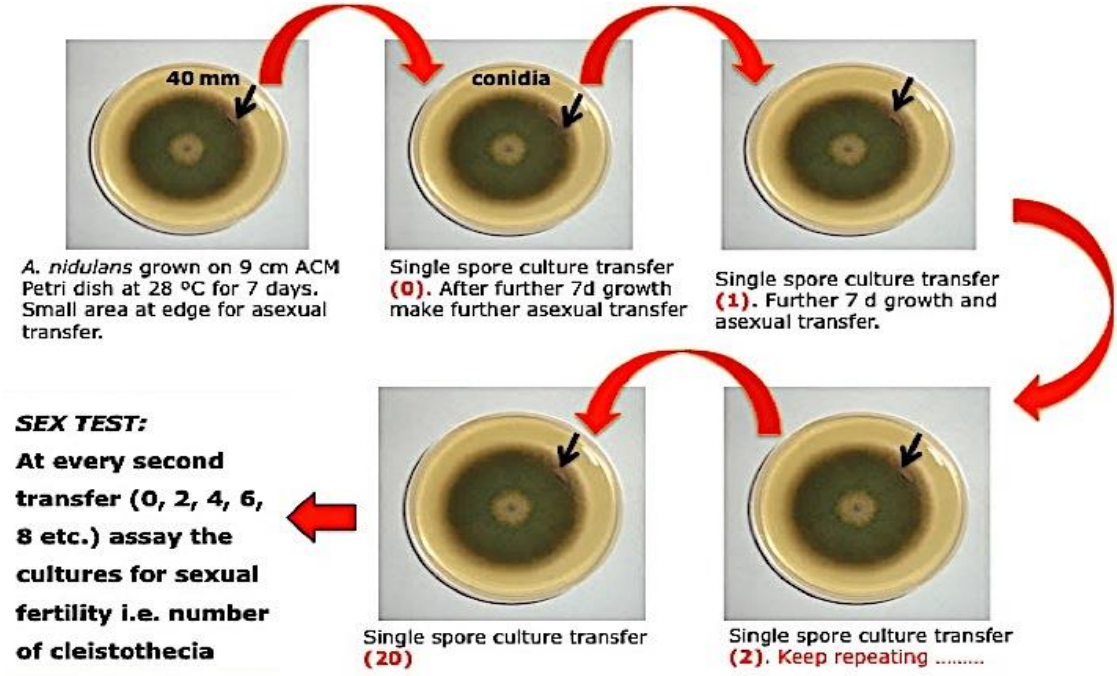

Figure 5-2 Asexual culture transfer protocol used to assess possible decline in sexual fertility resulting from conidial subculture (0-20 rounds). Sexual fertility tests were made at even transfer numbers (0,2,4, 6 etc.).

Some conidia were then collected from the edge (ca. 40 mm from the centre; **Figure 5-2**) of the new 'culture transfer 1' plate using a loop and then conidia were streak spread out on a new 9.0 cm diameter growth plate, which was incubated at 28ºC for 1-2 d. Six newly emerging single spore colonies were then picked off and grown on new 9.0 cm diameter plates at 28ºC in the light for 7 d. A morphologically 'average' looking culture was chosen and labelled as culture 'transfer 2'. The 'transfer 1' plate was stored safely in the cold room, dated and labelled. Conidia from the new 'transfer 2' plate were used in two ways. Firstly, conidia were collected from the exterior part of the plate (ca. 40 mm from the centre), and used to set up sexual fertility test cultures (**Figure 5-2**) as per instructions above (section **5.2.1.2**). These six replicate plates of cleistothecia were stored safely at 4ºC. Secondly, some conidia from edge of the plate were collected and then streak spread out on growth plates. Plates were incubated at 28ºC for 1-2 d then six newly emerging single-spore colonies were picked off and grown on new 9.0 cm diameter

growth plates at 28ºC in the light for 5-7 d. An 'average' looking culture was again chosen and labelled as culture 'transfer 3'. The original 'transfer 2' plate was kept and stored safely in the cold room, sealed with Nescofilm, dated and labelled. The above protocols were then sequentially repeated until a total of 20 culture transfers had been made. Thus, the odd number transfers (1, 3, 5, 7, 9, 11, 13, 15, 17 and 19) were used only for asexual transfer purposes, whereas the even number transfers (0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20) were also used to assess levels of sexual fertility. Sexual fertility data was analysed by ANOVA and plotted as graphs showing the number of cleistothecia formed per 100 mm^2 against the number of cell cycles (mitotic division) (see below) that correlated to sub culturing transfers from 0, 1, 2, 3, 4, and 5 up to 20.

5.2.3 Procedure for assessing possible restoration of sexual fertility by ascospore transfer

During the progress of experimentation it became apparent that a gradual loss in sexual fertility was evident in many isolates as a result of the serial culture by conidial transfer. Therefore another experiment was set up to determine whether cultures could be restored to initial levels of sexual fertility (the 'default' state) by establishing new single spore culture from sexual ascospores rather than asexual conidia, as described by Mather and Jinks (1958) (**Figure 5-1**). This sex 'restoration' experiment was carried out by collecting ascospores from cleistothecia produced from three different serial culture transfer stages (transfers 12, 18 and 20) of all 12 species/isolates under study. Single ascospore-derived colonies were derived as described above (sections **4.3.1.2** and **5.2.1.2**) following Todd *et al.* (2007), and six independent single ascospore cultures established on 9.0 cm diameter Petri dishes. A typical plate was chosen based on colony morphology. A sexual fertility assay was then made as described above (section **5.2.1.1**) and six replicate plates were scored for the formation of cleistothecia (section **5.2.1.2**).

5.2.4 Quantification of rate of decline in sexual fertility relative to number of mitotic nuclear divisions

The previous work of Jinks (1954) and Mather and Jinks (1958) used a number of rather undefined 'transfers' to trace the loss of sexual fertility. However, for the purposes of the current project a more defined, quantifiable measure was sought for the period over which sexual fertility might decline. Attempts were therefore made to correlate the decrease in fertility with number of mitotic divisions occurring during serial transfer. This was achieved by staining cell walls and nuclei and observing nuclei and apical tips according to rationale proposed by Kaminskyj and Hamer (1998) and Schoustra *et al.* (2007); (2010) (section **5.2.4.1** and **Figure 5-3**). They argued that if the average length of hyphae (measured in μm) occupied per nuclei (***x***) can be determined, therefore one can calculate how many nuclei (***y***) are present in a straight line from the centre to the edge of a fungal colony (e.g. ***y*** = 40,000 μm/***x*** for a colony in a 9 cm diameter Petri dish extending to a radius of 40 mm). Given that nuclear division only occurs at the growing tip in the aspergilli [based on observations in *A. nidulans* (Kaminskyj and Hamer, 1998)], then the average number of nuclei per apical cell (***z***) will be equivalent to how many nuclei are added per mitotic cycle. This is based on the logic that at each mitotic division all nuclei in the apical tip divide synchronously to give rise to double the number of nuclei, with half of the resulting nuclei partitioned off in new sub apical cells, each with typically 3-4 nuclei (**Figure 5-3**). The next round of mitotic division will then occur when sufficient growth has occurred to allow the next round of nuclear division and so forth. Therefore, one can calculate how many mitotic divisions (***n***) have occurred in a colony by dividing ***y***/***z*** (Schoustra *et al.*, 2007; 2010).

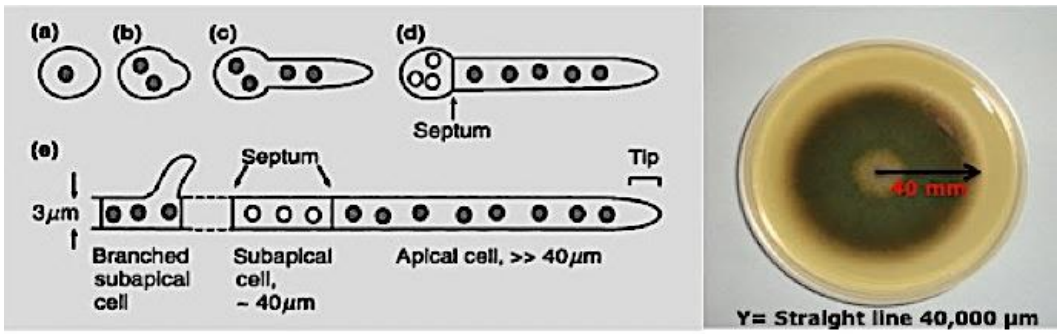

Figure 5-3 Cartoon summarising the morphology of cells in wild-type *A. nidulans* hyphae grown at 28 °C. A conidium (a) germinates and, (b) nuclei migrate into the germ tube as it grows, (c) the germling undergoes nuclear division giving rise to, (d) hyphae which are tubular, 3 μm in diameter, and have (e) apical cells that are variable in length and usually much longer than 40 μm. Apical cells contain many nuclei, which undergo synchronous division and partition to form subapical cells which are delineated by septa on average about 40 μm apart. Subapical cells contain three to four evenly spaced nuclei. [Adapted from (Kaminskyj and Hamer, 1998)]. A straight line from the centre to the edge of a growing colony in a 9 cm diameter Petri dish represents approximately 40,000 μm mycelial growth.

5.2.4.1 Staining nuclei, cell walls and septa to determine number of mitotic divisions correlated with colony growth

To implement the rationale of Schoustra *et al.* (2007); (2010) cultures were stained as described below and 10 observations made of independent hyphae and apical cells of representative isolates/strains of *A. nidulans, E. repens* and *N. fischeri* to calculate the average of length of hyphae per nucleus and the average of number of nuclei per apical cell. Various solutions were used in this work as follows, according to Momany (2001) and Momany and Kaminskyj (pers. comms).

Fixative solution 50 ml of 37% formaldehyde solution, 50 ml of 500 mM potassium phosphate buffer pH7 (sections **2.1.2.8** and **2.1.2.9**) and

2 ml of Triton X-100 were mixed thoroughly. This solution was used to fix germlings and had to be made shortly before use.

Antifade mounting solution 50 ml of 500 mM potassium phosphate buffer pH7 (sections **2.1.2.8** and **2.1.2.9**), 10 ml of 50% glycerol and 5.0 ml of 0.1% n-propyl gallate solution were mixed thoroughly and stored at 4ºC. This solution was almost colourless when freshly made, and was replaced when it turned yellow.

Calcofluor solution 1.0 mg of Calcofluor dye was dissolved into 1.0 ml distilled water (dH_2O). To ensure that the chemical was fully dissolved, 1-2 drops of 25 mM NaOH (5 µl in 50 ml dH_2O) were added to provide alkaline conditions. This Calcofluor stock was stored at 4°C in the dark (covered with foil). Calcofluor solution was used to stain cell walls.

Hoechst 33258 solution (Sigma bis-benzmide B2883) 1.0 mg of Hoeschts 33258 was dissolved into 1.0 ml distilled water (dH_2O). Hoeschts stock was stored at -20°C in the dark (covered with foil). This solution was used to stain nuclei.

Staining solution A typical working solution was made by mixing 2.0 µl of Calcofluor solution (as described above) and 1.0 µl of Hoechst 33258 solution (as described above) in 5.0 ml dH_2O. The diluted staining solution was made shortly before use (at least 1 min) and was covered with foil to protect from light.

Nuclei, cell walls and septa of hyphae of the different *Aspergillus* species were stained aseptically following the protocol of Momany (2001) using Hoechst 33258 and Calcofluor solution as follows.

A coverslip was placed in the bottom of a small, clean sterile 5.0 cm diameter Petri dish using flame-sterilised forceps.

10 ml of appropriate sterilised liquid medium for each strain (ACM or M40Y broth) were placed in a sterile disposable 15 ml centrifuge tube. Then 1 x 10^6 conidia were added to the medium and a tube was thoroughly inverted several times to ensure mixing of contents.

The medium with conidia was then poured over the coverslip. To prevent any growth of hyphae on underside of the coverslip, it was pressed down with the sterile end of the tube (the screw cap end). This removed any air bubbles trapped under the coverslip and resulted in the coverslip being sealed to the bottom of the plate.

The plate with coverslip was incubated at 28ºC for between 15-20 hr.

A small Coplin jar was filled up with fixation solution above. The coverslip was carefully lifted from the liquid medium with forceps and placed in the jar and incubated for 30 min.

The coverslip was rinsed briefly by immersion in a small beaker of sterilised water.

A piece of Parafilm (of size approximately 9.0 x 2.5 cm) was rubbed with a straight edge on plexiglass or on a 9.0 cm diameter Petri dish to make a smooth working surface, then 450 µl of the Calcofluor/Hoechst staining solution (as described above) was pipetted on this piece and the coverslip was placed on this drop, with hyphae facing down. All materials were covered with the lid of a box or piece of aluminium foil to protect from light, and were incubated at room temperature for a further 5 min.

After 5 min of incubation, the coverslip was rinsed briefly by immersion in a small beaker of sterilised water.

7 µl of antifade mounting solution (as described above) was pipetted onto a clean slide, then the coverslip was carefully placed on this mounting medium and pressed it down gently to prevent introducing any bubbles.

The slide with coverslip was covered with a box or aluminium foil to protect from light and left to air-dry for 1 hr. The edges of the coverslip were then sealed with nail polish.

Finally the slide was viewed with a fluorescence microscope with a camera (ZEISS-Axioskop - Carl Zeiss Jena GmbH) attached and equipped with Zeiss Filter Set 02 (DAPI, Hoechst) and lengths of hyphae/apical tips

determined using annotation software provided with the microscope (Axio Vision).

The slide was covered with aluminium foil to protect from light and was stored at 4°C. Nuclei and septa remained visible for several months.

5.2.5 Statistical analysis

Statistics software (SPSS, version 21) for Macintosh (Mac OS X version 10.6.8) was used. ANOVA (analysis of variance) was used to analyse the data and to determine whether the results were significant at a 5% confidence level according to Barnard *et al.* (2007).

5.3 Results

5.3.1 Colony growth and number of mitotic divisions in *Aspergillus nidulans*

In order to determine whether a 'slow decline' of sexual fertility in *A. nidulans* was observed as result of propagation by asexual reproduction, 20 serial conidial subculture transfers were carried out as described above (section **5.2.2**). To determine how many mitotic divisions were correlated with these transfers, fresh hyphae (16 hr old) were stained using Hoechst 33258 and Calcofluor solution (section **5.2.4.1**). Based on 10 independent observations of septa and apical cells of hyphae of isolates 2-139 and strain 2-154 (i.e. a total of 20 readings, 10 from each isolate/strain), the average length of hyphae per nucleus (***x***) was 5.6 ±3.02 µm while the average of number of nuclei per apical cell **(*z*)** was 12±1.44 (**Figure 5-4**). There was no statistical difference between these independent isolates [P= 0.92; T-test = 0.100 (df, 18)]; [P= 0.57; T-test = 0.585 (df, 18)]. Therefore, following the logic of Schoustra *et al.* (2007); (2010), the number of nuclei **(*y*)** per 40 mm radius in a 9.0 cm diameter Petri dish = 40,000 µm /5.6 = 7,100 nuclei approximately, and the total number of mitotic divisions (*n*) in this colony = 7,100/12 = 590

approximately mitoses per transfer. This data was used to calibrate all of the following data regarding possible decline in sexual fertility.

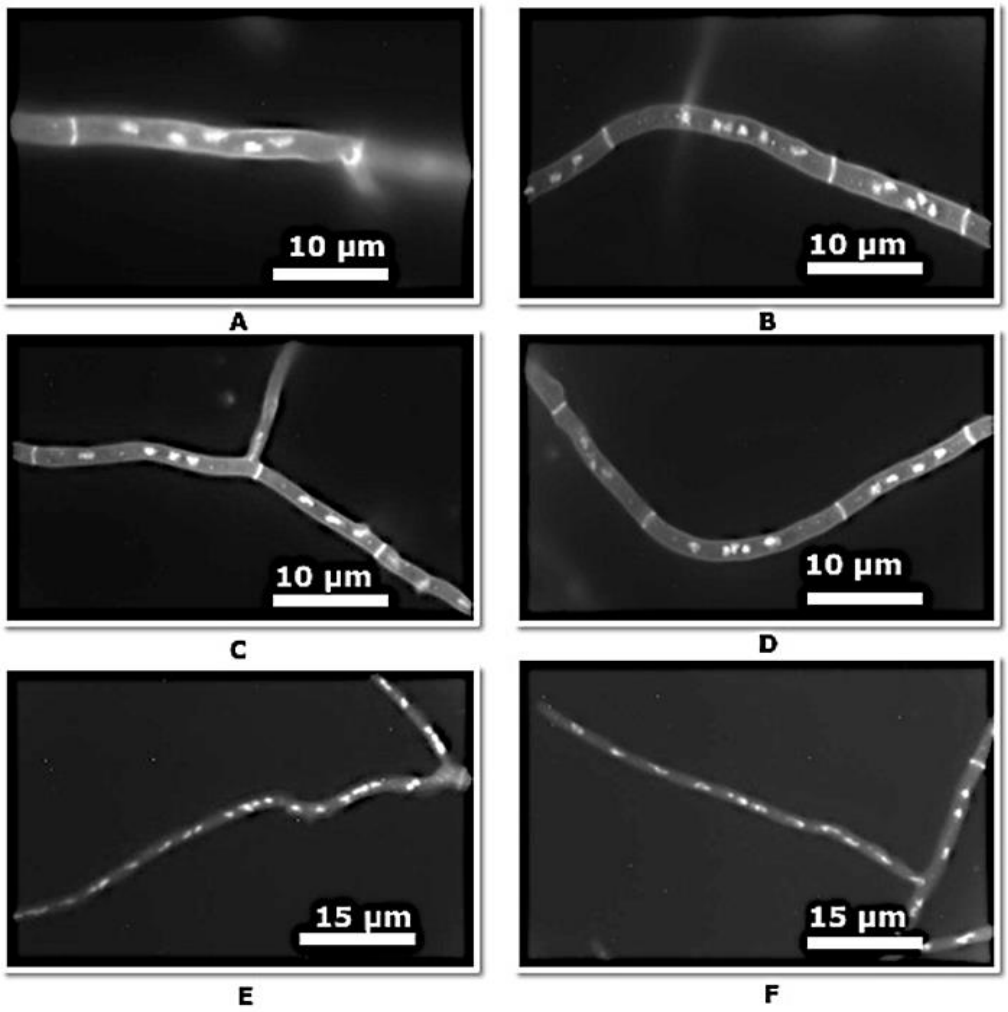

Figure 5-4 Representative staining of mycelia (hyphae, septa) and nuclei of *A. nidulans* with Hoechst 33258 and Calcofluor solution: (A) nuclei, (B) septa, (C,D) number of nuclei per hyphae (*x*), (E,F) number of nuclei per apical cell (*z*). A, B, C and D scale bar= 10 µm. E and F scale bar=15 µm.

5.3.1.1 Evolution of asexuality: loss of sexual fertility in *Aspergillus nidulans*

The possible slow decline in sexuality following serial subculture by conidia was investigated in four isolates/strains of *A. nidulans*, namely 2-

137 and 2-139 (wild type) and 2-149 and 2-154 (*veA1,kuA*) (**Table 3-1**). Overall results showed that there was clearly a gradual decrease in level of sexual fertility (in terms of production of cleistothecia) for all strains to between 55-10% of original fertility levels over 20 serial transfers involving single conidial sporing from 9.0 cm diameter Petri dishes, corresponding to approximately 10,000 rounds of mitotic division (section **5.3.1**, **Figure 5-4**). A comprehensive two-way ANOVA was made, incorporating data for numbers of cleistothecia formed at all transfer assay stages (i.e. T_0, T_2, T_4, T_6 ….T_{20}) for all isolates. This revealed that there was a significant overall effect of asexual cultural transfers on formation of cleistothecia [F (df) = 6.233 (10, 220); p <0.001)], a significant effect of isolate/strain on formation of cleistothecia [F (df) = 5.919 (3, 220); p <0.001)], and a significant interaction between strain and conidial cultural transfers on production of cleistothecia [F (df) = 5.625 (30, 220); p <0.001)]. Furthermore, it was found that the sexual fertility of all strains could be restored back to between 13% to 107% of the original fertility levels when fungi were sub-cultured by a single ascospore at transfers 12, 18 or 20.

Regarding individual *A. nidulans* isolates/strains, the levels of sexual fertility of 2-137 decreased significantly from a maximum of around 130 cleistothecia per 100 mm^2 at the fourth rounds of asexual transfer (2,950 mitotic divisions) to only 20 cleistothecia per 100 mm^2 at the end (12,390 mitotic divisions) (**Figure 5-5**), equivalent to a 85% decrease. However, there was an unexpected increase in sexual fertility at the fourth transfer during initial rounds of sub-culturing (this result was verified by independently repeating the sexual fertility assays twice, as this result was unexpected) (**Figure 5-5**). Statistically, a one-way ANOVA revealed that there was a significant variation in number of cleistothecia formed in respect of 2-137 over the transfer period [F (df) = 3.279 (10, 55); p =0.002)]. Meanwhile, as shown in (**Table 5-1**) sexual fertility at the latest T_{20} stage of transfer (12,390 mitotic divisions) could be restored from approximately 25% back to 46% of the original sexual fertility when the isolate was sub-cultured by a single ascospore transfer. However,

there were no clear differences resulting from ascospore transfer at the T_{12} and T_{18} stages of transfer (**Table 5-1**).

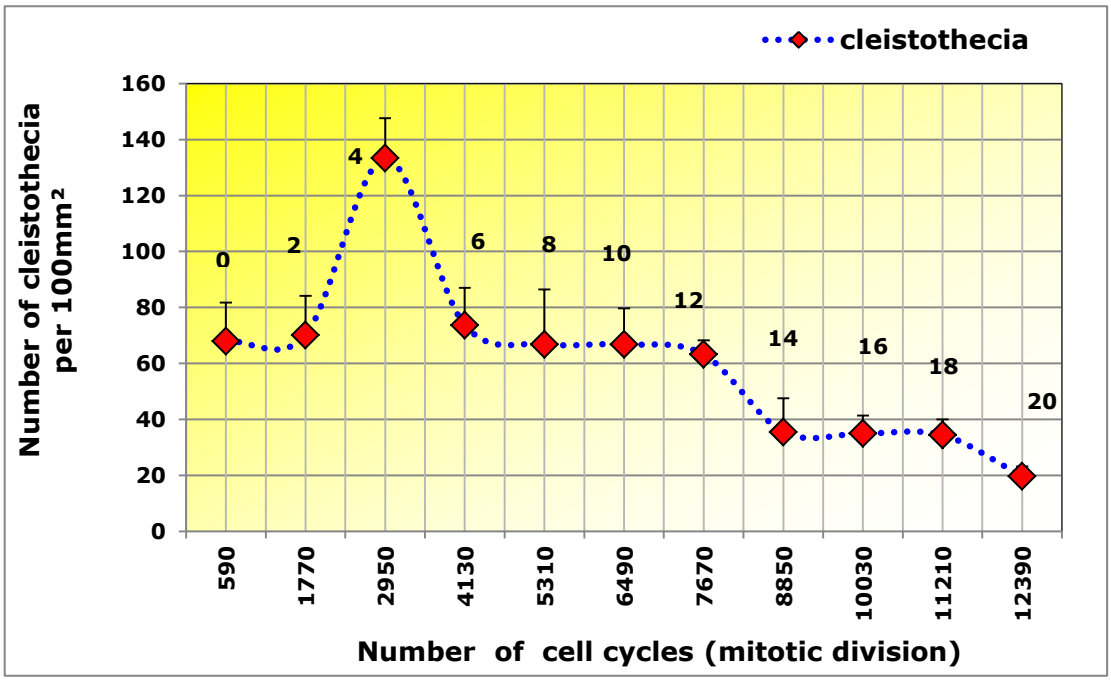

Figure 5-5 The effect of propagation by conidia (0-20 transfers) on production of cleistothecia in *A. nidulans* isolate 2.137. Error bars indicate ±SEM.

There was also a very clear trend showing that the number of cleistothecia produced by isolate 2-139 decreased significantly from approximately 80 to only 10 cleistothecia per 100 mm^2, corresponding to an almost 90% decrease, when the strain was sub-cultured over 12,000 mitotic divisions by conidial transfer (**Figure 5-6**). A one-way ANOVA showed that there was a high significant variation in number of cleistothecia formed by this strain (F (df) = 3.279 (10.55); p = 0.002). Meanwhile, as shown in (**Table 5-1**), sexual fertility at the latest T_{20} stage of transfer (12,390 mitotic divisions) could be restored from approximately 10% back to 50% of the original sexual fertility when the isolate was sub-cultured by a single ascospore transfer. However, there were no clear differences resulting from ascospore transfer at the T_{12} and T_{18} stages of transfer (**Table 5-1**).

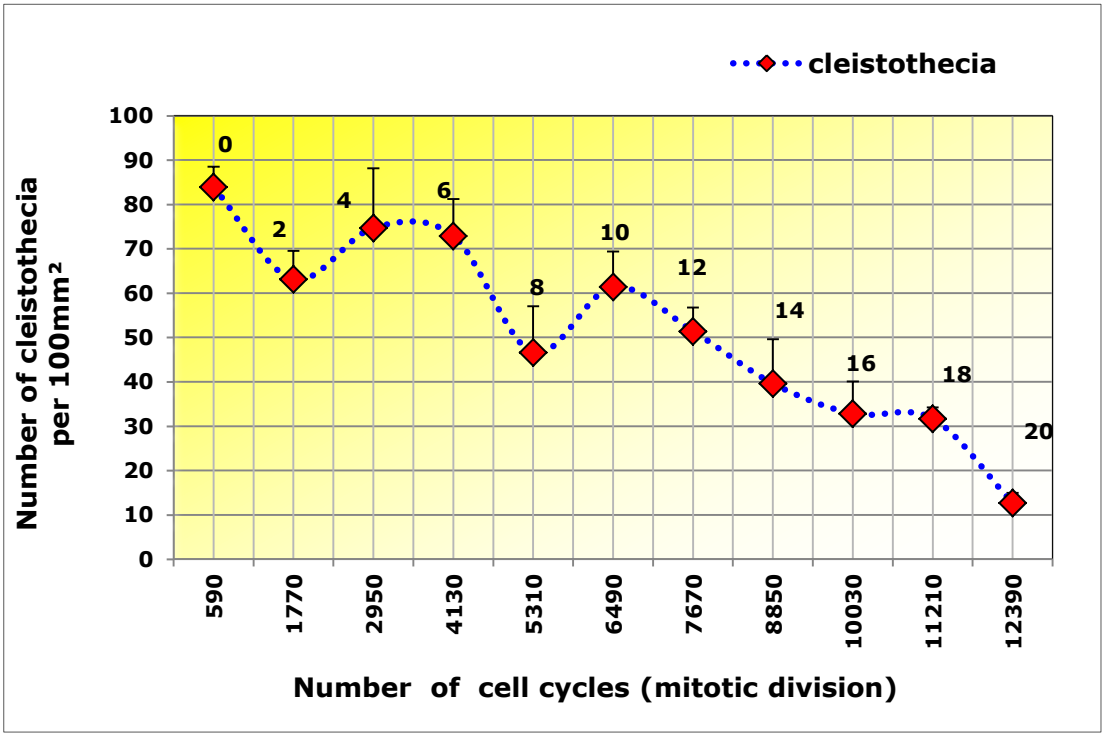

Figure 5-6 The effect of propagation by conidia (0-20 transfers) on production of cleistothecia in *A. nidulans* isolate 2.139. Error bars indicate ±SEM.

Despite a gradual drop in number of cleistothecia formed by 2-149 during the first three rounds of conidial transfer (2, 4 and 6), there was unexpectedly a fluctuating increase in this number particularly at the later transfers (12, 14, 16 and 18 = 7,670, to 11,210 mitotic divisions), with the highest amounts of cleistothecia (around 60 per 100 mm^2) formed during this period. However, this increase was followed by a sharp decrease at the final T_{20} transfer, which formed only around 30 cleistothecia per 100 mm^2, representing an approximate 45% decrease in fertility (**Figure 5-7**). A one-way ANOVA illustrated that there was a highly significant variation in number of cleistothecia formed by this strain (F (df) = 3.068 (10.55); p = 0.004). Meanwhile, as shown in (**Table 5-1**), sexual fertility at the latest T_{20} stage of transfer (12,390 mitotic divisions) could be restored from approximately 55% back to 100% of the original sexual fertility, with a near two-fold increase in cleistothecia when the isolate was sub-cultured by a single ascospore transfer. There was no clear difference resulting from ascospore transfer

at the T_{12} (7,670 mitotic divisions). However, very strangely, transfer by single ascospore at the T_{18} stage (11,210 mitotic divisions) resulted in greatly decreased fertility relative to the original conidial transfer (**Table 5-1**).

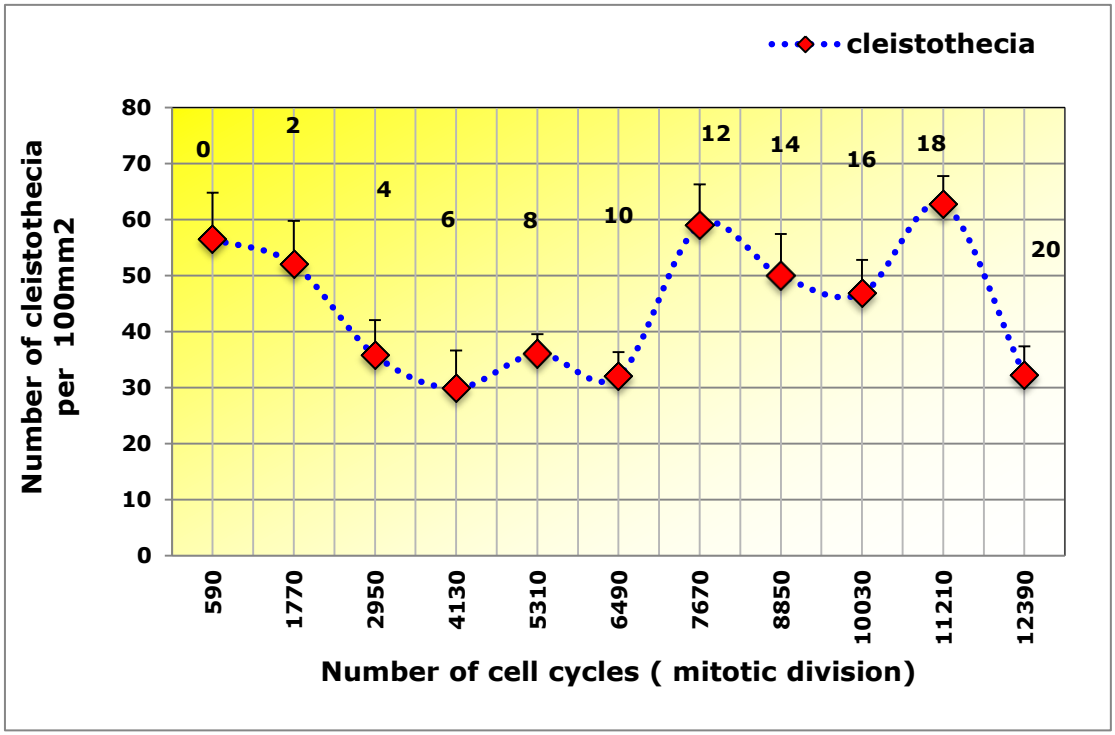

Figure 5-7 The effect of propagation by conidia (0-20 transfers) on production of cleistothecia in *A. nidulans* strain 2.149. Error bars indicate ±SEM.

Finally, (**Figure 5-8**) shows that there was a significant effect of conidial culture transfer on the number of cleistothecia generated by strain 2-154, with a highly significant variation in this number [A one-way ANOVA: F (df) = 7.561 (10, 55); p <0.001)]. The amount of cleistothecia declined sharply from around 55 cleistothecia at the beginning of transfers to only 5 cleistothecia per 100 mm^2 at the last round of asexual transfer (12,390 mitotic division) i.e. a reduction in sexual fertility of up to 90%. Meanwhile, the sexual restoration tests using transfer from a pure ascospore revealed that the sexual fertility of 2-154 was improved by transfer at the T_{12} and T_{18} stages (7,670 and 11,210 mitotic divisions

respectively) back to approximately 50% of the original levels. However, unlike transfers with the other *A. nidulans*, ascospore transfer at the final stage did not result in any notable restoration of fertility to original levels, although the ascospore transfer did result in a significant increase compared to the conidial culture at this point (**Table 5-1**).

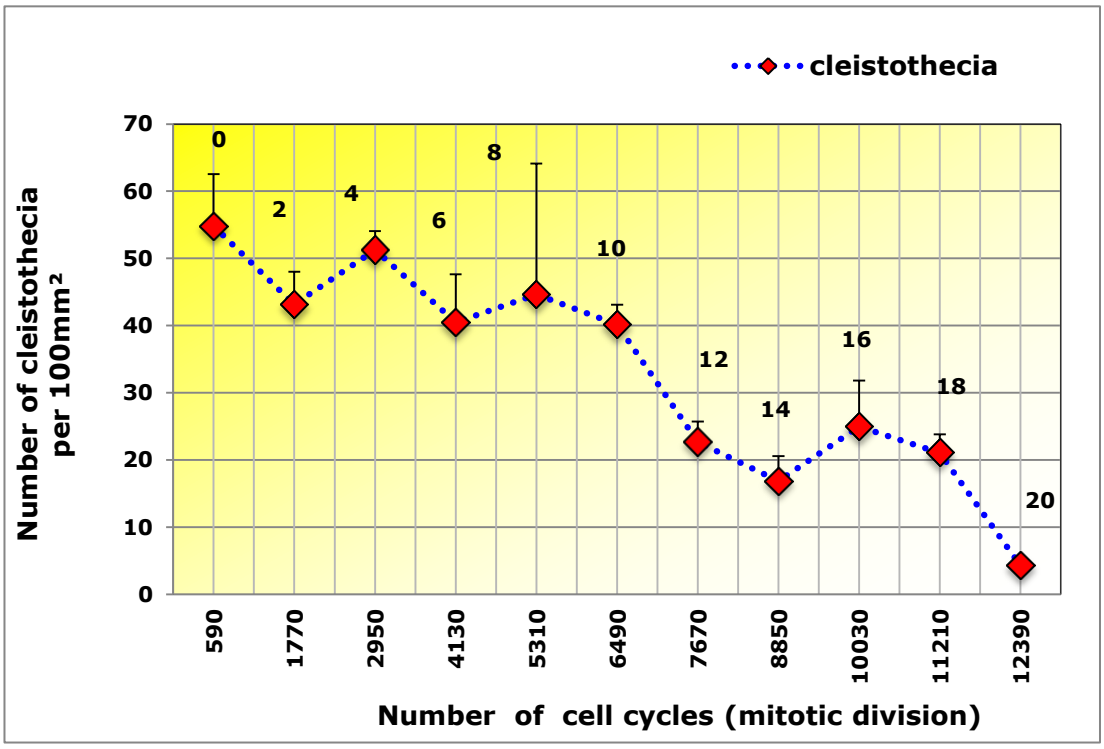

Figure 5-8 The effect of propagation by conidia (0-20 transfers) on the production of cleistothecia in *A. nidulans* strain 2.154. Error bars indicate ±SEM.

Table 5-1 Shows number of cleistothecia (per 100mm²) produced by strains/isolates of *A. nidulans* that were transferred by either conidia or ascospores. Numbers are ±SEM and figures in parenthesis are % fertility of original Transfer 0 cultures.

Strain Isolate	**Propagule Source**	**T_{12}** (7,670) *	**T_{18}** (11,210) *	**T_{20}** (12,390) *
2-137	Conidia transfer	63.0±5.0 (93.1±7.4%)	34.2±5.6 (50.2±8.2%)	20.1±3.5 (29.1±5.1%)**
	Ascospore transfer	63.2±5.0 (93.1±7.4%)	41.2±4.4 (60.1±6.4%)	31.2±4.3 (46.2±6.3%)**
** Not significance according to T-test [p=0.07; T-test = 2.01 (df, 10)]				
2-139	Conidia transfer	51.1±5.3 (61.1±6.4%)	31.2±2.5 (37.2±3.0%)	13.1±2.3 (16.1±2.7)**
	Ascospore transfer	61.2±7.6 (73.2±9.1%)	31.1±2.5 (37.1±3.0%)	47.2±5.1 (57.2±6.3%)**
** High significance according to T-test [p <0.001; T-test = 6.05 (df, 10)]				
2-149	Conidia transfer	59.1±7.4 (105±13.1%)	62.2±5.1 (110±9.0%)	32.2±3.5 (57.3±6.2%)**
	Ascospore transfer	60.2±6.1 (107±10.9%)	7.0±4.3 (13.0±7.6%)	58.0±4.3 (104±7.6%)**
** High significance according to T-test [p <0.001; T-test = 2.48 (df, 10)]				
2-154	Conidia transfer	23.2±3.0 (43.2±5.6%)	21.2±2.7 (39.2±5.0%)	4.0±0.3 (7.0±0.6%)**
	Ascospore transfer	37.1±5.5 (69.1±10.3%)	29.2±2.1 (54.2±3.9%)	14.1±1.0 (26.2±1.8%)**
** High significance according to T-test [p <0.001; T-test = 9.55 (df, 10)]				

*** Number of mitotic division in parenthesis.**

**** T-tests were only performed for data of T_{20} (last column).**

5.3.2 Colony growth and number of mitotic divisions in *Neosartorya fischeri*

The same procedures of Momany (2001) used earlier (section **5.2.4.1)** were carried out to find out the number of mitotic divisions correlated with 20 serial-conidial cultural transfers of *N. fischeri*. The number of mitotic divisions was calculated based on 10 independent observations of septa and apical cells of hyphae of *N. fischeri* isolates 53-14 and 53-16. The average length of hyphae per nucleus (***x***) was 4.4±1.42 µm, while the average of number of nuclei per apical cell (***z***) was 9±0.63 (**Figure 5-9**). There was no statistical difference between these independent isolates [P= 0.81; T-test = 0.25 (df, 18)]; [P= 0.67; T-test = 0.43 (df, 18)]. Therefore, according to Schoustra *et al.* (2007); (2010) the number of nuclei (***y***) per 40 mm Petri dish = 40,000 µm /4.4 = 9,090 nuclei approx. Therefore the total number of mitotic divisions (***n***) = 9,090/9 = 1,010 mitoses per transfer approximately. This data was used to calibrate all of the following data regarding possible decline in sexual fertility.

5.3.2.1 Evolution of asexuality: loss of sexual fertility in *Neosartorya fischeri*

The possible slow decline in sexuality following serial subculture by conidia was investigated in four isolates of *N. fischeri*, namely 53-2, 53-14, 53-15 and 53-16 (**Table 3-2**). Each of the isolates showed a different pattern in decline in fertility, but as a whole sexual fertility (in terms of cleistothecia formation) was reduced by over 90% in most isolates. This decline was correlated with over 20,000 rounds of mitotic division (corresponding to 20 serial cultural transfers and single sporing from 9.0 cm Petri dishes). A comprehensive two-way ANOVA was made, incorporating data for numbers of cleistothecia formed at all transfer assay stages (i.e. T_0, T_2, T_4, T_6 ….T_{20}) for all isolates. This revealed that there was a significant overall effect of conidial cultural transfers on formation of cleistothecia [F (df) = 24.536 (10, 220); p <0.001)], a significant effect of isolate on formation of cleistothecia [F (df) = 16.502 (3, 220); p <0.001)], and a significant interaction between isolate and

transfer on production of cleistothecia [F (df) = 4.693 (30.220); p <0.001)]. Furthermore, it was found that the sexual fertility of all strains could be restored back to between 21% to 165% of the original fertility levels when fungi were sub-cultured by a single ascospore at transfers 12, 18 or 20.

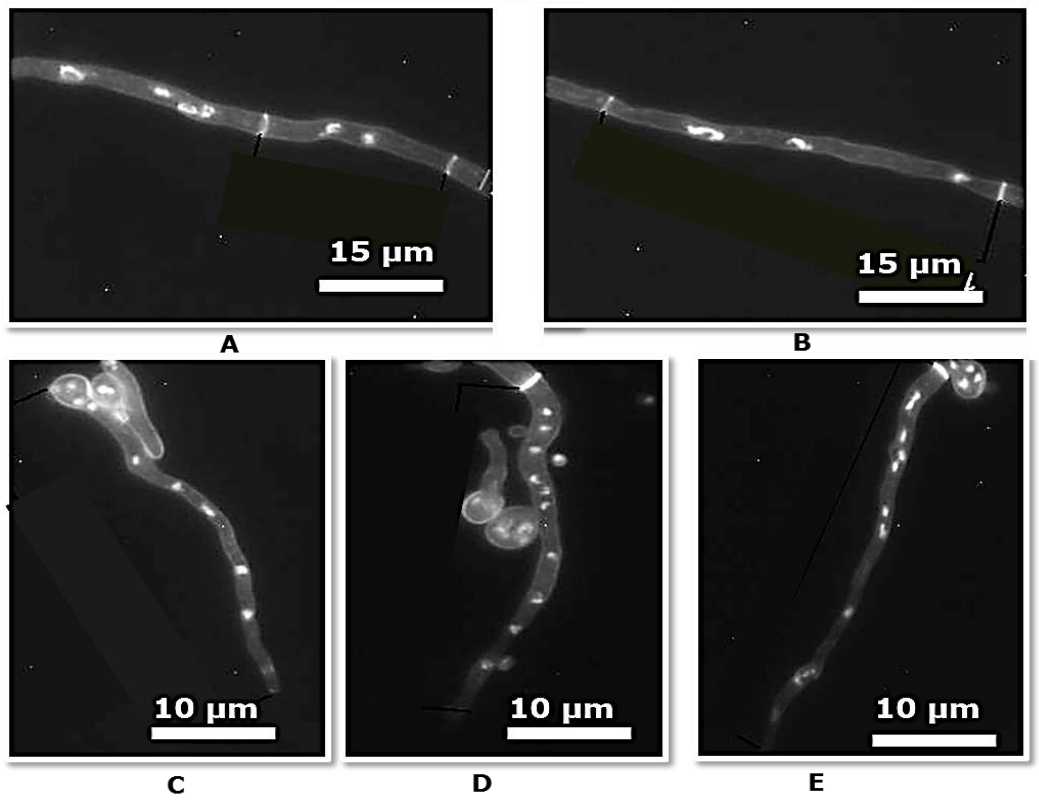

Figure 5-9 Representative staining of mycelia (hyphae, septa) and nuclei of *N. fischeri* with Hoeschts 33258 and Calcofluor solution: (A) septa, (B) number of nuclei per hyphae (*x*), (C, D, E) number of nuclei per apical cell (*z*). A and B scale bar= 15 µm. C, D and E scale bar=10 µm.

Regarding individual *N. fischeri* isolates, **Figure 5-10** illustrates that the level of sexual fertility of 53-2 was slightly higher during initiation rounds of asexual cultural transfer, forming around 15-20 cleistothecia per 100 mm², then the number decreased almost constantly following sub-culturing by conidia until the mid point of the serial asexual transfers (11,110 mitotic divisions) with less than 5.0 cleistothecia produced per 100 mm^2. However, there was then a sudden increase in amount of cleistothecia giving around 15 per 100 mm^2 at the eight rounds of

asexual transfers (14,140 mitoses) before a marked drop showing very small number of cleistothecia at the last two rounds of transfers. A one-way ANOVA revealed that there was a highly significant variation in number of cleistothecia formed by this isolate during the serial transfer [F (df) = 6.429 (10, 55); p <0.001)]. Meanwhile, the sex restoration test was successfully able to restore the fertility rate of this strain to approximately 50% of the original levels when ascospores transfers were made at transfers 12, 18 and 20 (12,130, 18,180 and 20,200 mitotic divisions, respectively) (**Table 5-2**).

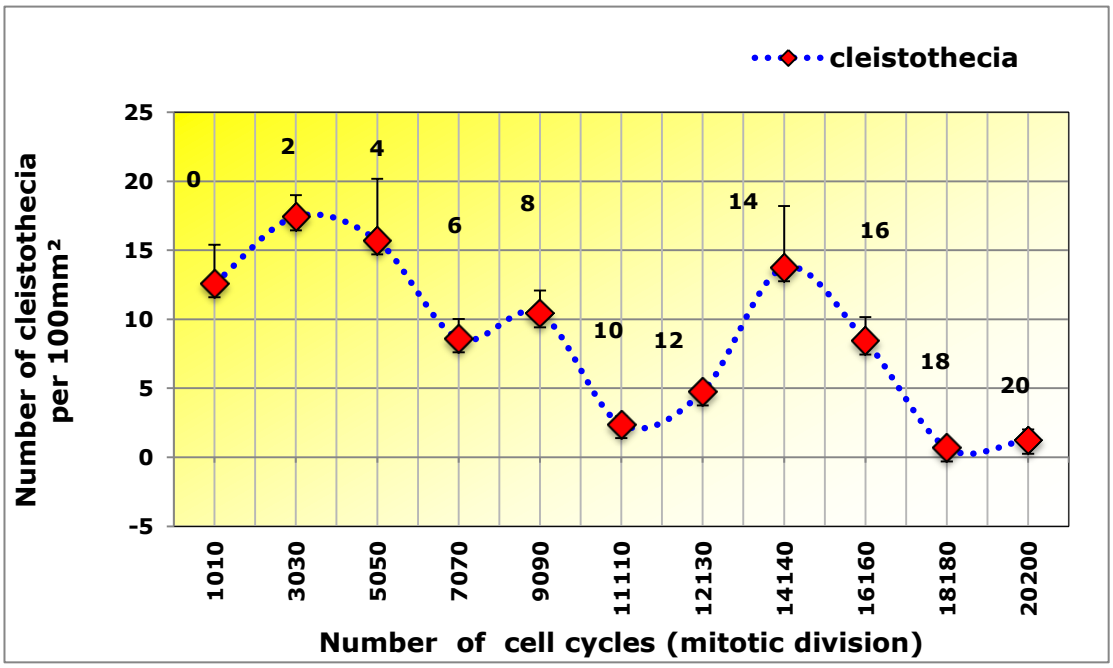

Figure 5-10 The effect of propagation by conidia (0-20 transfers) on production of cleistothecia in *N. fischeri* isolate 53-2. Error bars indicate ±SEM.

Regarding isolate 53-14, **Figure 5-11** illustrates that there was a considerable drop in amount of cleistothecia produced following 20 asexual culture transfers (20,200 mitotic division). However, there was unexpected increase at the beginning to about 15 cleistothecia per 100 mm², but later on the sexual fertility decreased sharply by around 90% at 11,110 mitoses before sex was almost lost at the end (>20,000

mitotic divisions). Statistically, there was a highly significant variation in number of cleistothecia formed via this isolate during the serial transfer [A one-way ANOVA: F (df); =7.714 (10, 55); P<0.001)]. The sexual fertility could however be effectively restored to roughly 60% of the original levels by sub-culturing by a single ascospore at all three stages of sexual test 12,18 and 20 (12,130, 18,180 and 20,200 mitotic divisions respectively (**Table 5-2**).

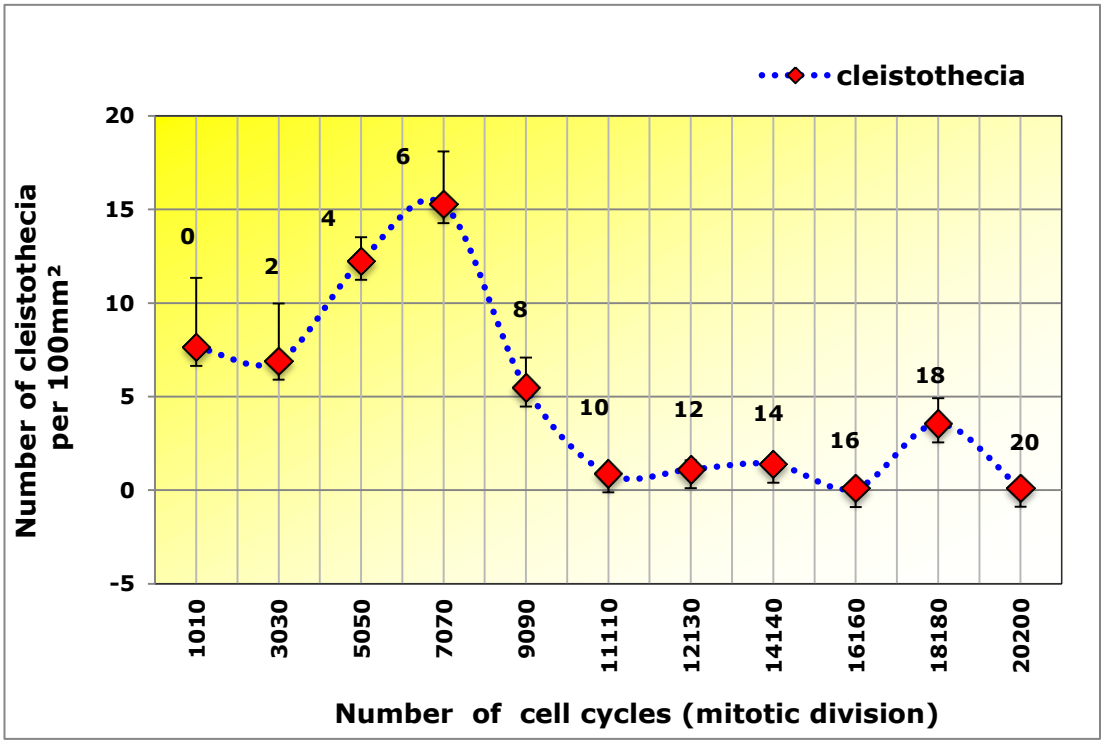

Figure 5-11 The effect of propagation by conidia (0-20 transfers) on production of cleistothecia in *N. fischeri* isolate 53-14. Error bars indicate ±SEM.

A fluctuation in fertility levels, but overall decline, was also observed after sub-culturing isolate 53-15 over 20 transfers using conidia (**Figure 5-12**). The number of cleistothecia formed was around 18 per 100 mm² at the initiation of the transfer work. Levels of sex then subsequently dipped, showing an approximate 90% reduction, and at the two final transfers only one cleistothecium was observed per 100 mm². A one-way ANOVA exhibited that there was a high significantly variation between the number of cleistothecia produced and asexual transfer period [F (df) =

5.398 (10, 55); p <0.001]. Although no marked increase in sexual fertility of 53-15 was observed after transfer from a single ascospore at transfer rounds 12 and 18 (12,130 and 18,180 mitoses, respectively), a very extreme climb in fertility was observed at the final transfer (20,200 mitotic divisions) leading to as high as 30 cleistothecia per 100 mm². This remarkably represented approximately a two-fold increase of sexual fertility rate compared with the commencement of asexual transfers (**Table 5-2**).

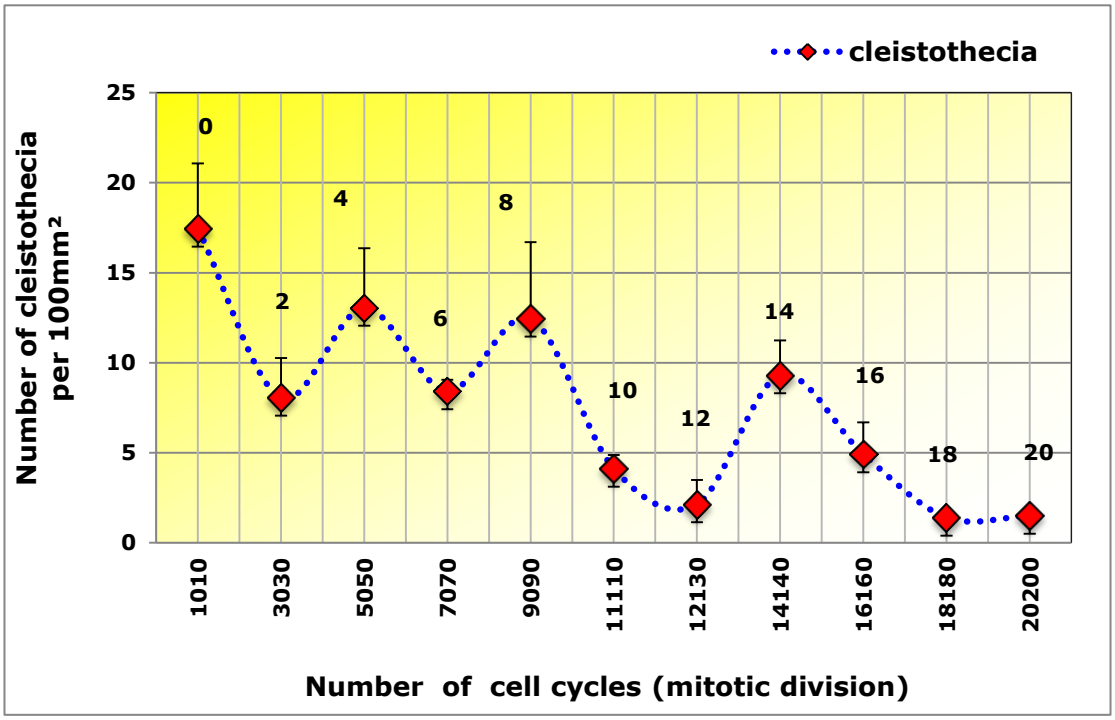

Figure 5-12 The effect of propagation by conidia (0-20 transfers) on production of cleistothecia in *N. fischeri* isolate 53-15. Error bars indicate ±SEM.

Figure 5-13 illustrates that there was a significant variation in the number of cleistothecia produced by isolate 53-16 over the period of transfer [A one-way ANOVA: F (df); =16.707 (10, 55); P<0.001)]. The sexual fertility was dramatically lost following 20 asexual transfers. At the first round of conidial propagation approximately 35 cleistothecia per 100 mm² were formed. This was followed by a substantial drop by the sixth

round (7,070 mitotic divisions) where only *ca.* 7 cleistothecia per 100 mm² were formed, before a levelling out and further reductions by the end of transfer (**Figure 5-13**). The sexual fertility of 53-16 could be restored considerably by transfer from a single ascospore at the final T20 transfer stage (20,200 mitotic divisions) back to greater than 90% of the original fertility. However, ascospore transfer at the T_{12} and T_{18} stages did not result in such an increase (**Table 5-2**).

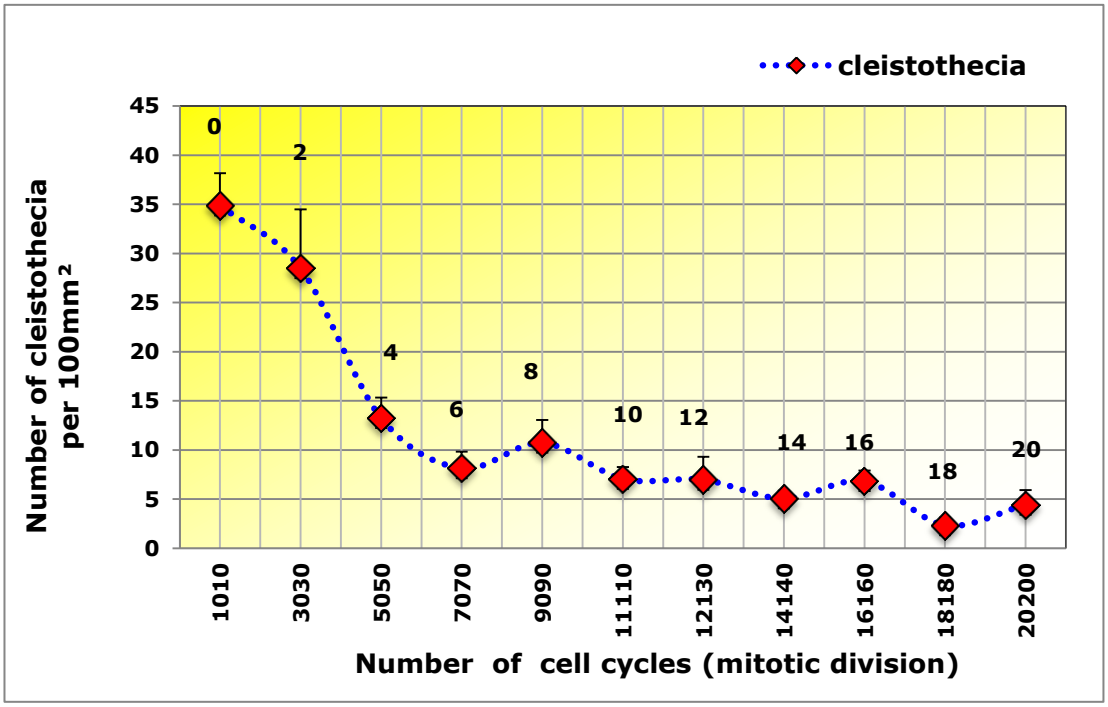

Figure 5-13 The effect of propagation by conidia (0-20 transfers) on production of cleistothecia in *N. fischeri* isolate 53-16. Error bars indicate ±SEM.

Table 5-2 shows number of cleistothecia (per 100mm²) produced by isolates of *N. fischeri* that transferred by either conidia or ascospores. Numbers are ±SEM and figures in parenthesis are % fertility of originalTransfer 0 cultures.

Isolate	**Propagule Source**	**T_{12}** (7,670) *	**T_{18}** (11,210) *	**T_{20}** (12,390) *
53-14	Conidia transfer	1.0±0.5 (13.0±6.0%)	4.0±1.4 (50.1±17.0%)	0.1±0.1 (1.3±0.6%)**
	Ascospore transfer	9.0±4.2 (113±52.4%)	7.0±1.4 (88.0±18.0%)	8.0±0.3 (100±3.5%)**
** High significance according to T-test [p <0.001; T-test = 26.7 (df, 10)]				
53-15	Conidia transfer	2.0±1.3 (12.2±7.9%)	1.4±0.3 (8.2±1.5%)	1.5±0.2 (8.8±1.4)**
	Ascospore transfer	5.0±0.8 (29.2±4.9%)	4.4±0.9 (26.3±5.6%)	28.2±2.9 (165±17.0%)**
** High significance according to T-test [p <0.001; T-test = 8.95 (df, 10)]				
53-16	Conidia transfer	7.0±2.3 (20.2±6.7%)	2.3±0.8 (6.6±2.3%)	4.4±1.5 (13.1±4.3%)**
	Ascospore transfer	7.4±1.2 (21.3±3.4%)	10.5±0.8 (30.1±2.2%)	30.4±5.4 (87.1±15.4%)**
** High significance according to T-test [p <0.001; T-test = 4.63 (df, 10)]				
53-2	Conidia transfer	4.8±0.2 (37.2±1.3%)	0.7±0.1 (5.3±0.9%)	1.3±0.3 (7.0±0.6%)**
	Ascospore transfer	9.0±4.2 (69.1±32.0%)	6.9±1.4 (53.2±0.6%)	7.7±0.3 (59.2±2.2%)**
** High significance according to T-test [p <0.001; T-test = 7.96 (df, 10)]				

*** Number of mitotic division in parenthesis.**

**** T-tests were only performed for data of T_{20} (last column).**

5.3.3 Colony growth and number of mitotic divisions in *Eurotium repens*

Calcofluor/Hoechst staining (section **5.2.4.1**) was also used to stain the cell walls, septa and nuclei of two representative isolates (51-4 and 51-6) of *E. repens* in order to correlate colony growth with number of mitotic divisions. Ten independent observations of hyphae per isolate were made to determine the average length of hyphae occupied by nuclei (***x***) and also the number of nuclei occupied in the apical cells (***z***). The number of mitotic divisions that correlated with 20 conidial cultural transfers (***n***) was successfully calculated as follows. The value of (***x***) and (***z***) were found to be 2.6±1.69 µm and 7±0.68 nuclei, respectively (**Figure 5-14**). There was no statistical difference between these independent isolates [P= 0.89; T-test = 0.144 (df, 18)]; [P= 0.60; T-test = 0.560 (df, 18)]. Thus, y = 40,000 µm /2.6 (***x***) = 15,384 nuclei approx. Therefore (***n***) = ***y***/ ***z*** **=** 15.300/07 = 2,185 mitoses per transfer approximately. This data was used to calibrate all of the following data regarding possible decline in sexual fertility.

5.3.3.1 Evolution of asexuality: loss of sexual fertility in *Eurotium repens*

The possible slow decline in sexuality following serial subculture by conidia was investigated in four isolates of *E. repens*, namely 51-3, 51-4, 51-5 and 51-6 (**Table 3-3**). Results showed that the level of sexual fertility (as judged by cleistothecial formation) showed a clear 70-90% decrease in fertility as a consequence of repeatedly asexual transfers in all isolates. This occurred over 20 serial transfers, which correlated with >40,000 mitotic divisions. However, in all strains except 51-5 an unexpected increase in sexual fertility was observed during the initiation stages of sub-culturing. A comprehensive two-way ANOVA was made, incorporating data for numbers of cleistothecia formed at all transfer assay stages (i.e. T_0, T_2, T_4, T_6 ….T_{20}) for all isolates. This revealed that there was a significant overall effect of asexual cultural transfers on

formation of cleistothecia [F (df) = 184.636 (10, 220); p <0.001)], a significant effect of strain on production of cleistothecia [F (df) = 208.650 (3, 220); p <0.001)], and a significant interaction between strain and asexual cultural transfers on formation of cleistothecia [F (df) = 58.398 (30, 220); p <0.001]. Furthermore, it was found that the sexual fertility of all strains could be restored back to between 16% to 100% of the original fertility levels when fungi were sub-cultured by a single ascospore at transfers 12, 18 or 20.

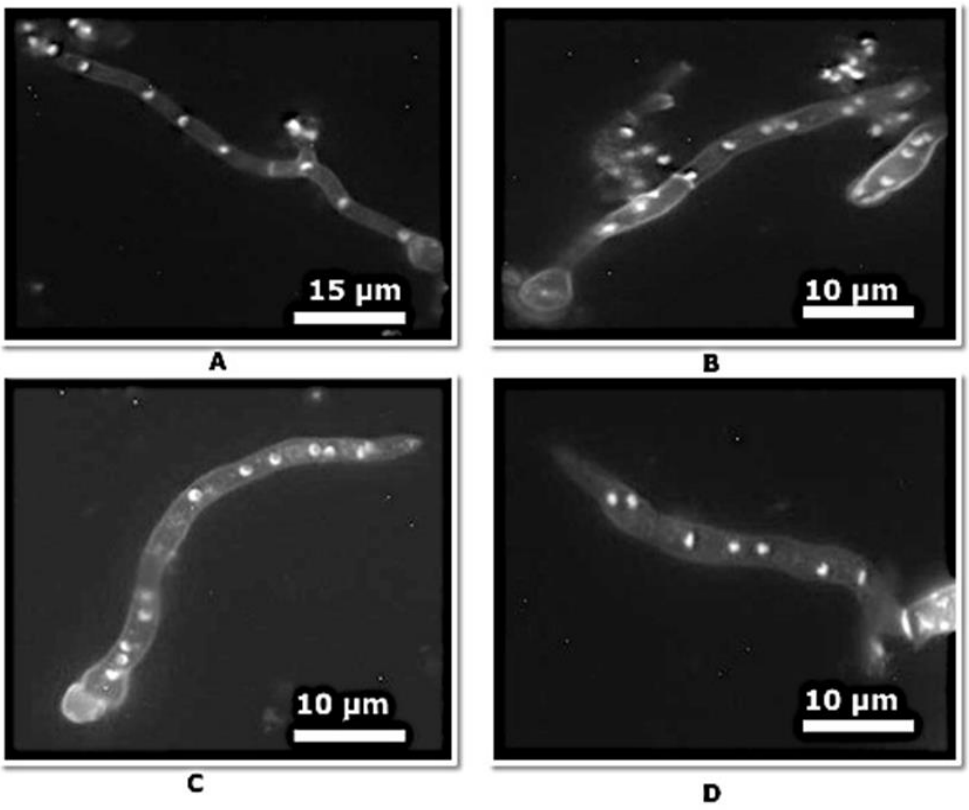

Figure 5-14 Representative staining of mycelia (hyphae, septa) and nuclei of *E. repens* with Hoeschts 33258 and Calcofluor solution: (A) nuclei and septa, (B) number of nuclei per hyphae (*x*), (C, D) number of nuclei per apical cell (*z*). A scale bar= 15 µm. B, C and D scale bar =10 µm.

Regarding individual *E. repens* isolates **Figure 5-15** shows that the level of sexual fertility of 51-3 reduced noticeably with approximately a 90% decrease in sexual fertility following prolonged asexual sub-culturing equal to over 45,000 mitotic divisions. The number of cleistothecia was around 90 per 100 mm² at the beginning of transfers (2,185 mitotic divisions), and fell down considerably later on. However, there was unexpectedly a marked increase in this number during the middle stage of transfers (24,035 mitotic divisions), reaching a peak of around 110 cleistothecia per 100 mm². A one-way ANOVA showed that there was a highly significant variation in amount of cleistothecia produced by this strain over successive transfers [F (df) = 58.809 (10, 55); p <0.001)]. Meanwhile, the sexual fertility could be successfully restored back to almost the initial levels of sex when the fungus was sub-cultured by a single ascospore at the final T_{18} and T_{20} stage (41,515 and 45,885 mitotic divisions), resulting in the formation of 70-80 cleistothecia per 100 mm². However, oddly a decrease in fertility was seemed when transferring by single ascospore at the T_{12} stage (28,405 mitotic divisions) (**Table 5-3**).

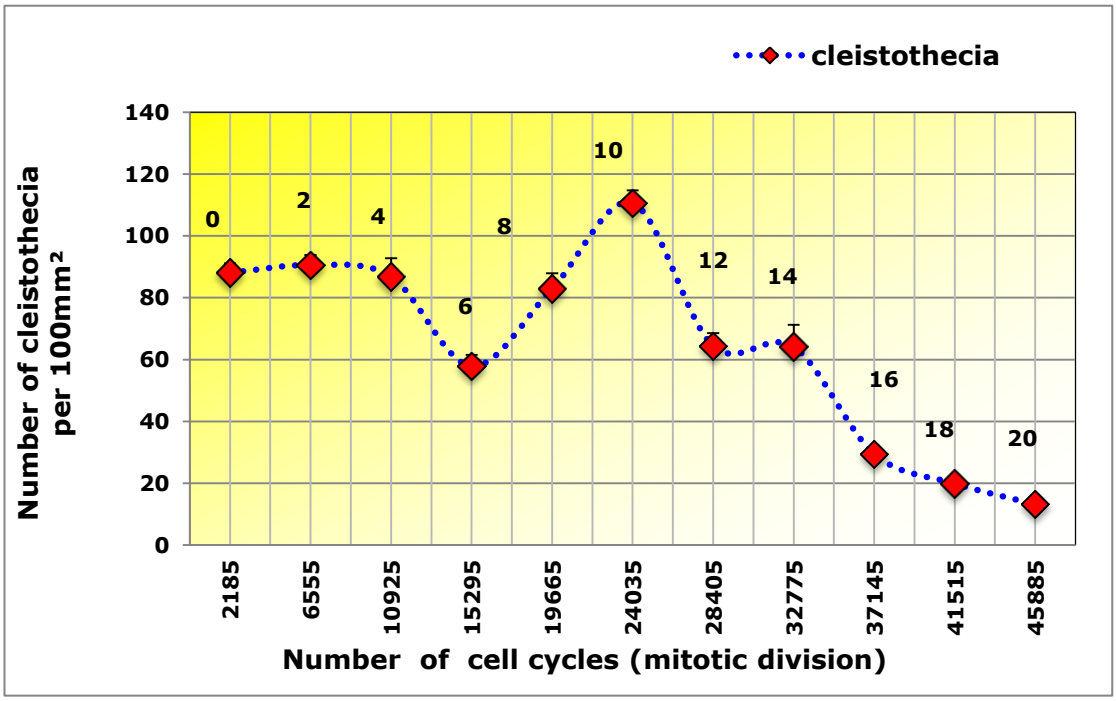

Figure 5-15 The effect of propagation by conidia (0-20 transfers) on production of cleistothecia in *E. repens* isolate 51-3. Error bars indicate ±SEM.

Similar to isolate 51-3 above, isolate 51-4 also showed a considerable reduction (approximately 70%) in the number of cleistothecia formed following 20 repeated asexual conidial transfers (roughly 45,885 mitotic divisions). However, there was again some fluctuation in the course of transfers with an unexpected peak elevation of sex of 120 cleistothecia per 100 mm² at the eighth rounds of asexual transfers (19,665 mitosis) (**Figure 5-16**). A one-way ANOVA showed that there was a significant variation in number of cleistothecia formed by this isolate over the transfer period [F (df) = 32.045 (10, 55); p <0.001]. Meanwhile, a dramatic increase in numbers of cleistothecia was observed, up to around 90% of the original values, when the fungus was sub-cultured by a single ascospore at the final T_{18} and T_{20} stages (41,515 and 45,885 mitotic divisions), resulting in the formation of about 90 cleistothecia per 100 mm². However, there was no clear increase in fertility when transferring by single ascospore at the T_{12} stage (28,405 mitotic divisions) (**Table 5-3**).

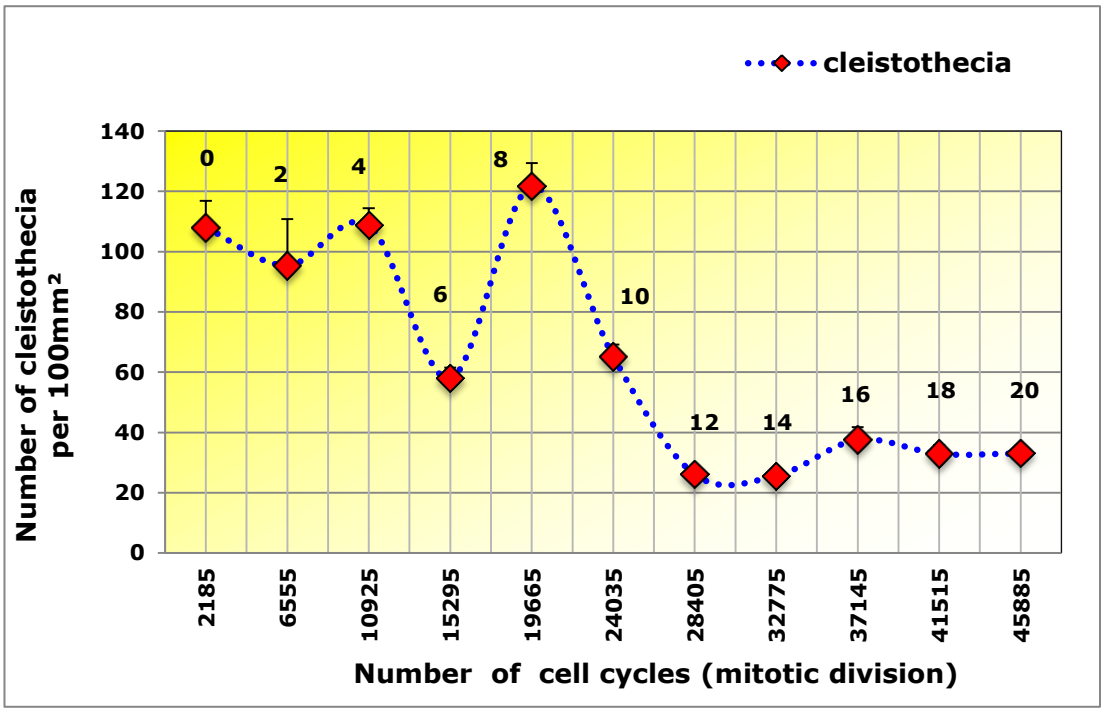

Figure 5-16 The effect of propagation by conidia (0-20 transfers) on production of cleistothecia in *E. repens* isolate 51-4. Error bars indicate ±SEM.

The sexual fertility of isolate 51-5 also showed a tremendous decrease to around 10% of the starting value following 20 conidial cultural transfers, corresponding to over 45,000 mitotic divisions (**Figure 5-17**). There was a high significant decrease in amount of cleistothecia produced from 250 at the first rounds of asexual transfers to around 50 cleistothecia per 100 mm² at the fourth rounds of transfers (corresponding to 10,925 mitoses) then the sexual levels showed a slight but steady decrease in fertility during the remaining. A one-way ANOVA showed that there was a significant variation in number of cleistothecia produced over the transfer period [F (df) = 197.171 (10, 55); p <0.001]. However, there was no obvious restoration of sexual fertility as a result of propagation using a single ascospore at the T_{18} and T_{20} stages, although a two-fold increase in formation of cleistothecia was observed at the twelfth transfer round (corresponding to 28405 mitotic divisions) when propagating by ascospore (relative to the T_{12} conidial transfer) (**Table 5-3**).

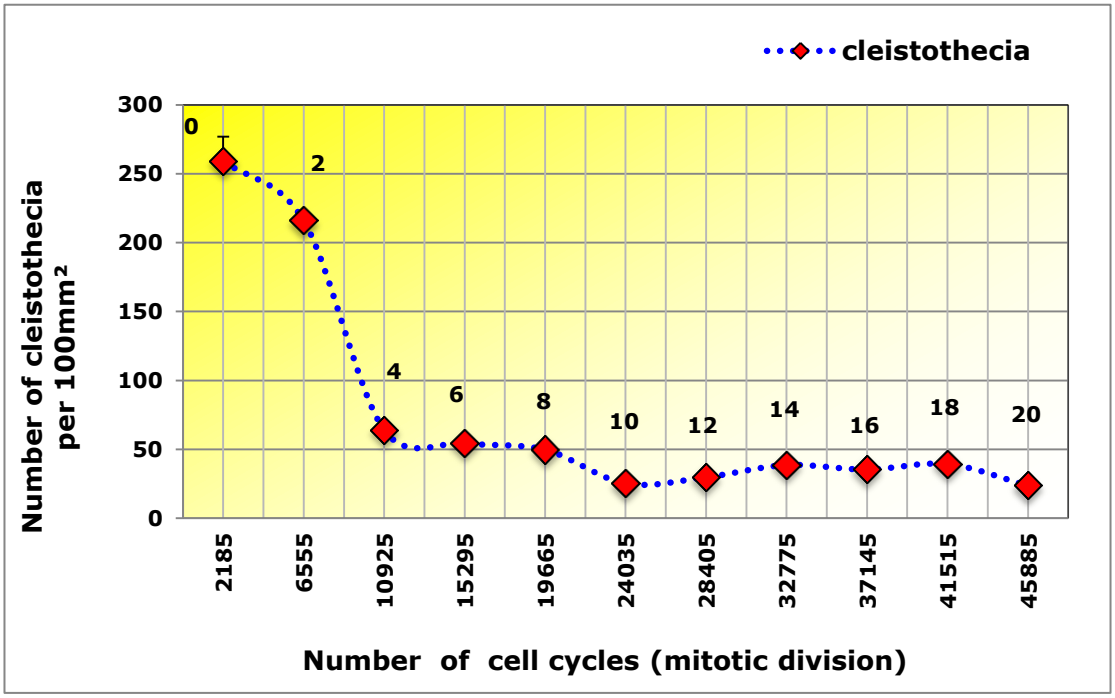

Figure 5-17 The effect of propagation by conidia (0-20 transfers) on production of cleistothecia in *E. repens* isolate 51-5. Error bars indicate ±SEM.

Finally, **Figure 5-18** shows that there was a dramatic effect of conidial cultural transfer on sexual fertility of isolate 51-6, which exhibited an almost total loss of fertility by the end of the transfer period (over 45,000 mitoses). However, there was an unexpected increase in the production of cleistothecia at the fourth and sixth rounds of asexual transfers (10,925 and 15,295 mitotic divisions). The sexual fertility rate then declined almost continuously during further stages of transfer. A one-way ANOVA revealed a high significant variation in number of cleistothecia produced [F (df) = 27.377 (10, 55); p <0.001] over the transfer period. Using ascospore transfer had no clear effect at the T_{12} stage, but the sexual fertility of this isolate was slightly restored when ascospores were used especially at the last rounds of sexual transfers (45,885 mitosis), with a 7-8 fold increase in fertility although this was still only *ca.* 40% of the starting fertility (**Table 5-3**).

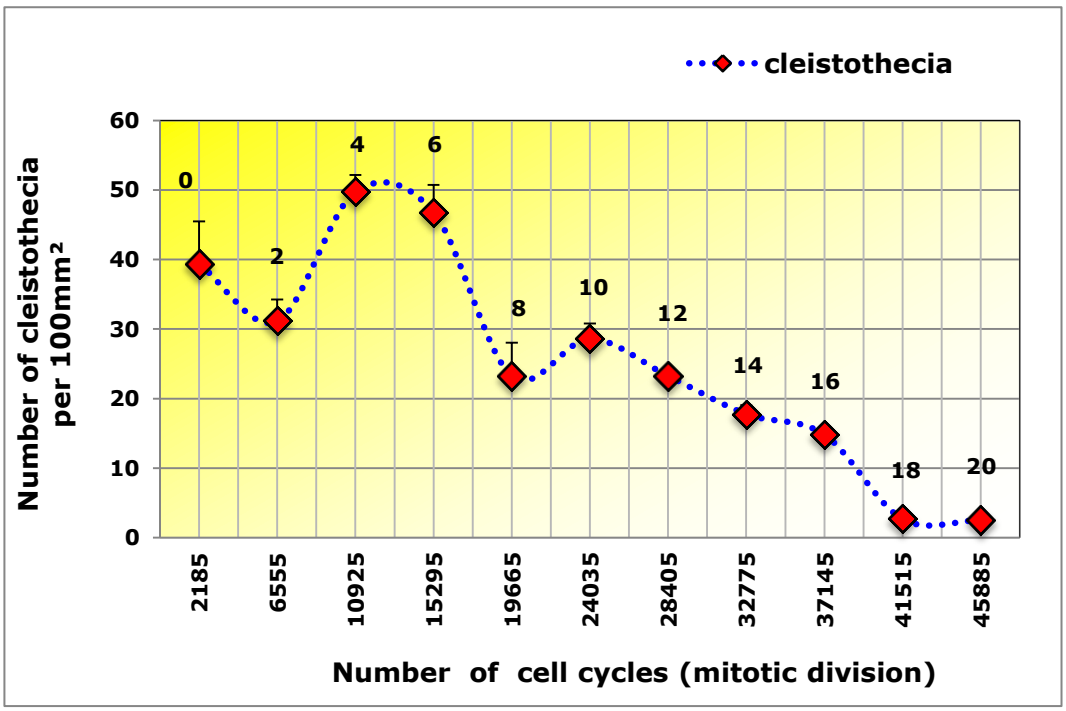

Figure 5-18 The effect of propagation by conidia (0-20 transfers) on production of cleistothecia in *E. repens* isolate 51-6. Error bars indicate ±SEM.

Table 5-3 shows number of cleistothecia (per 100mm²) produced by isolates of *E. repens* that transferred by either conidia or ascospores. Numbers are ±SEM and figures in parenthesis are % fertility of original Transfer 0 cultures.

Isolate	**Propagule Source**	**T_{12}** (7,670) *	**T_{18}** (11,210) *	**T_{20}** (12,390) *
51-3	Conidia transfer	64.0±4.2 (72.1±4.8%)	20.1±1.8 (23.2±2.0%)	13.0±1.4 (15.0±1.6%)**
	Ascospore transfer	33.1±1.5 (38.1±1.7%)	78.0±3.3 (89.0±3.8%)	72.1±3.1 (100.1±3.5%)**
** High significance according to T-test [p <0.001; T-test = 17.0 (df, 10)]				
51-4	Conidia transfer	26.2±2.4 (24.2±2.2%)	33.1±2.9 (31.2±2.7%)	33.3±2.6 (31.3±2.4)**
	Ascospore transfer	28.0±1.8 (26.0±1.6%)	80.1±1.8 (75.2±3.2%)	92.2±4.5 (85.1±4.1%)**
** High significance according to T-test [p <0.001; T-test = 11.5 (df, 10)]				
51-5	Conidia transfer	30.0±2.5 (12.2±0.9%)	39.2±1.4 (15.3±0.5%)	24.1±2.7 (9.3±1.0%)**
	Ascospore transfer	79.1±3.7 (30.1±1.4%)	43.2±0.8 (17.3±0.3%)	42.2±1.9 (16.2±0.7%)**
** High significance according to T-test [p <0.001; T-test = 5.66 (df, 10)]				
51-6	Conidia transfer	23.1±0.8 (59.1±2.1%)	2.7±0.4 (7.3±0.9%)	2.5±0.6 (6.3±1.4%)**
	Ascospore transfer	14.2±1.5 (36.1±3.9%)	8.2±1.3 (21.2±3.3%)	14.2±1.7 (36.3±4.4%)**
** High significance according to T-test [p <0.001; T-test = 6.39 (df, 10)]				

*** Number of mitotic division in parenthesis.**

**** T-tests were only performed for data of T_{20} (last column).**

5.4 Discussion

Fungi can undergo either asexual and/or sexual reproduction. Some so-called 'facultatively sexual' species can undergo both sexual and asexual reproduction (Kondrashov, 1988), whereas other fungi seem to be strictly asexual (Taylor *et al.*, 1999a). Both types of reproduction have advantages and disadvantages as previously discussed (sections **1.3.1.1** and **1.3.4.1**). For example, asexual reproduction has benefits as an evolutionary strategy because it requires only one parent and all of the parent's genes are passed on to its offspring, whereas in sexual reproduction only half of each parent's genes are forwarded to the next generation. However, sex involving outcrossing can increase the genetic variation of progeny (Kondrashov, 1988) and can maintain beneficial and remove detrimental mutations (Kück and Pöggeler, 2009). Understanding the molecular-genetic basis that determine whether species are facultatively sexual or obligately asexual should provide fundamental insights into the evolution of asexuality and have applied consequences if it were possible to restore sexuality to asexual species of commercial importance.

As reviewed earlier in this chapter (section **5.1.2**), there are several possible explanations as to why fungal species appear to have completely lost their sexuality, and it has been suggested that fungi can exhibit multiple transitions to an asexual state (Lobuglio *et al.*, 1993). Various studies have been carried out to investigate the evolution of sex and movement from asexuality to sexuality or *vice versa* in many fungi including *Candida* (Butler *et al.*, 2009), *Aspergillus* (Geiser *et al.,* 1996), *Penicillium* (López-Villavicencio *et al.,* 2010), and *Microsporidia* species (Ironside, 2007). One key hypothesis is that species as a whole may exhibit a 'slow decline' in sexual fertility as a result of the accumulation of various genetic changes such as reduced expression and mutation in sex-related genes, and epigenetic factors (Dyer and Paoletti, 2005). Leslie and Klein (1996) also pointed out that mutations may cause changes in the expression of essential genes leading to a dramatic loss of sexual

fertility. Indeed, there is limited evidence suggesting that the sexual fertility of some species is prone to be lost in prolonged subculture, consistent with such a 'slow decline'. As already described (section **5.1.2**), pioneering work by Jinks (1954) and Mather and Jinks (1958) reported that sexual reproduction in *A. nidulans* and *A. glaucus* could be lost as result of repeated transfer by asexual conidia. Furthermore, sexual fertility could be restored to the initially high levels by transfer via a single sexual ascospore, which was attributed to changes within the cytoplasm (Jinks, 1954; (Mather and Jinks, 1958). If this pattern were repeated in the wild, then this would provide a mechanism for the evolution of asexuality. However, these reports lacked many experimental details to substantiate the findings. Work in the present study was therefore undertaken with three aims. Firstly, to determine whether a slow decline in sexuality can indeed be demonstrated in *Aspergillus* species when isolates are sub cultured by repeated asexual transfer, as a model to assess whether this could be a general phenomenon in fungi. Secondly, to use modern microscopic staining methods to try and correlate any decrease in sexuality with number of mitotic divisions occurring. Thirdly, to investigate whether cultures can be restored to a sexual 'default' state by propagation by sexual ascospores rather than asexual conidia.

5.4.1 Evolution of asexuality by slow decline in sexual fertility

A main experiment was set up using multiple isolates of three homothallic species *A. nidulans*, *N. fischeri* and *E. repens* to determine whether a slow decline in sex could be detected following continued asexual propagation (sections **5.3.1.1**, **5.3.2.1** and **5.3.3.1**). A highly important overall finding was that there was a clear decrease in levels of sexual fertility for all isolates and strains for all three species sub cultured repeatedly by conidial transfer i.e. providing convincing evidence of the possibility of a 'slow decline' in sexual fertility and a clue to understand the evolution of asexuality in many previously sexual species.

Considering the individual species, the four representatives of *A. nidulans* exhibited a 45-90% decrease in sexual fertility (as judged by production of cleistothecia), those of *N. fischeri* showed an 85-90% loss in fertility, whilst those of *E. repens* showed a 70-90% loss of sexual fertility following serial asexual sub-culture. Indeed, for three isolates of *N. fischeri* cleistothecial production was almost entirely lost by the end of this main experiment, and it took very careful searching of all six replicate 'sex plates' to find any cleistothecia at all. It is tempting to speculate that further asexual propagation would have indeed led to a purely asexual state. However, it is noted that there were some anomalies in the results. Firstly, only two isolates, *N. fisheri* 53-16 and *E. repens* 51-5, showed a 'textbook' gradual slow decline in fertility. Instead, all other isolates/strains under study exhibited a fluctuating decrease in fertility, with some unexpected increases in fertility often seen particularly during the initial rounds of asexual sub-culture, despite the initial passage by ascospore to establish a 'default' T_0 state. The reason for this fluctuation was unclear. It was possibly due to inherent stochastic variation in levels of sexual fertility. Alternatively, despite best efforts to minimise environmental fluctuation there might have been slightly different growth (incubation) conditions at different transfer stages. Another possibility relates to the experimental design whereby transfer was via a single morphologically 'representative' isolate at each stage. The chosen single-spore conidial isolate (one chosen out of six) might not necessarily been representative of the majority of asexual conidia at that transfer stage, hence giving an unexpected level of sexual fertility. Elsewhere Schoustra *et al.* (2007); (2010) used transfer via mass conidial transfer, rather than via a single spore, in their experimental evolution work. However, passage via a single spore was chosen here as this might accelerate any evolutionary change and was apparently the method used by Jinks (1954) and Mather and Jinks (1958) in their pioneering work (although this was not fully detailed). Secondly, it is noted that the three species exhibited quite different levels of sexual fertility. The isolates/strains of *A. nidulans* formed a maximum of between 55-130 cleistothecia per 100 mm^2, those of *N. fischeri* formed

between 15-35 cleistothecia per 100 mm^2, whilst isolates of *E. repens* produced between 50-250 cleistothecia per 100 mm^2. It was unclear whether these differing levels of fertility influenced the loss of sexual fertility. Two of the *A. nidulans* isolates, 2-149 and 2-154, contained the *veA1* mutation and were very likely to have already been subjected to multiple asexual transfers before they were obtained for the present study, as these isolates have a long history of laboratory usage. Meanwhile, the fertility of the *N. fischeri* isolates was surprisingly low given the supposed sexual abundance of the species. Indeed, levels of sexual fertility appeared considerably higher when these isolates were first received at Nottingham, suggesting that a major loss of fertility might have already occurred prior to experimental work in this chapter (Ashour and Dyer, personal observations).

5.4.2 Correlation between mitotic divisions and slow decline in sexual fertility

A protocol derived from that of Momany (2001) and Momany and Kaminskyj (pers. comms) was developed to allow staining of cell nuclei and cell walls of the various *Aspergillus* species under study. This involved the combined use of Hoechst 33258 and Calcofluor. Other staining methods, such as use of DAPI, were also tried but these gave much less reliable results (data not shown). Even the optimised protocol gave variable results but sufficient hyphae were appropriately stained in a long series of experiments to allow enumeration of hyphal length and number of nuclei in the apical tips. This allowed the number of mitotic divisions occurring during growth of colonies in a 9 cm diameter Petri dish to be determined following the observations of nuclear divisions by Kaminskyj and Hamer (1998) and the rationale of (section **5.2.4**). This allowed the number of mitotic divisions occurring during growth and serial transfer over 20 such Petri dishes to be estimated. This revealed that this transfer period was correlated with over 12,000, 20,000 and 45,000 mitotic divisions for *A. nidulans, N. fischeri* and *E. repens*, respectively. Therefore, this method allowed a more precise calibration of the period over which sexual fertility had declined as it was apparent that

a far higher number of mitotic divisions had occurred in *E. repens* than the other species, and that *A. nidulans* had a relatively low number of nuclear divisions. This would not have been apparent from simply the number of conidial transfer stages alone (20 for all species). It was surprising to see such variation in rates of mitotic division between species. Almost all cellular/genetic work in the aspergilli has been focussed on *A. nidulans*, and the current work indicates that there might be considerable variation at the cellular level in other aspergilli.

However, there are caveats to the above findings. Firstly, it has been assumed that the nature of nuclear division observed in *A. nidulans* [i.e. only at the apical tips (Kaminskyj and Hamer, 1998)] also applies to *N. fischeri* and *E. repens*. Without extensive extra work this assumption had to be made. Secondly, that data gained from the two representative isolates per species used in the staining work applied to all isolates of the same species. But given that no difference was detected between any of the latter representatives, this seems a reasonable assumption.

5.4.3 Restoration of sexual fertility by ascospore transfer

It was found that transfer of cultures via an ascospore at the final T_{20} stage of the experiment consistently led to an increase in the sexual fertility for all isolates/strains of every species. For *A. nidulans*, a 1.5-3.0 fold increase in sexual fertility (relative to that of the T_{20} conidial transfer) was evident, for *N. fischeri* a 4-14 fold increase in sexual fertility was evident, whilst for *E. repens* a 2-7 fold increase in sexual fertility was evident, according to specific isolates in question. Increases in sexual fertility were also evident when subculture via ascospores was made at many of the T_{18} and T_{20} stages (**Table 5-1, Table 5-2** and **Table 5-3**). Thus, my results overall indicated that it was possible to restore levels of sexual fertility lost via asexual transfer, by passage through an ascospore. This observation could be very important in allowing restoration of sex in asexual species of commercial/medical importance.

However, it is cautioned that only quite rarely was the sexual fertility

restored to the full original T_0 'default' state; this being the case for *A. nidulans* 2-149, *N. fischeri* 53-15 and 53-16, and *E. repens* 51-3. Instead, a lower restoration of fertility was evident for most isolates. For *A. nidulans* ascospore transfer at the T_{20} stage resulted in 25-100% of the original T_0 default fertility, for *N. fischeri* 15-100% of the original default fertility, and for *E. repens* 20-100% of the original default fertility. These observations were consistent with both mutational and epigenetic changes occurring during the serial transfers. It would be predicted that a mutational change(s) decreasing fertility levels would not be effected by passage through an ascospore, resulting in a permanent loss of fertility, as seen in *A. nidulans* 2-154 and *E. repens* 51-5. Whereas epigenetic changes might be 'reset' by passage through an ascospore, considering the ability of sexual recombination to repair mutations (Goddard *et al.*, 2005; Heitman, 2006; Heitman, 2010; Chun and Fay, 2011).

Epigenetic changes have been widely defined as "changes in gene expression that occur without a change in DNA sequence". Epigenetic changes are thought to play key roles in various cellular processes such as recognition of nucleic acid sequence homology, imprinting, RNA turnover and chromosome remodelling in fungi, plants, and animals (Wolffe and Matzke, 1999). Epigenetic changes can occur for example by histone modifications and DNA methylation, but critically these changes are often not permanent and can be reversible. In the case of insects and mammals it has been found that the genomes of these organisms can 'clean' most of the DNA methylation and chromatin modifications occuring in somatic gene regulation during the early germ cell emryogenesis and this finally promotes somatic cell differentiation and post-fertilization development (Kota and Feil, 2010). A similar process occurring in the aspergilli would explain the restoration in fertility levels seen in *A. nidulans* 2-149, *N. fischeri* 53-15 and 53-16, and *E. repens* 51-3.

Other examples of epigenetic changes in fungi include repeat-induced point mutation in *Neurospora crassa* and also methylation induced premeiotically (MIP) in *Ascobolus immerses* that give rise to pairing-

dependent modification of DNA sequence duplications during the sexual reproduction of these species (Martin *et al.,* 2010). These two phenomena illustrate the ability of the eukaryotes to interact with redundant DNA sequences through mechanisms involving the recognition of DNA repeats (Rossignol and Faugeron, 1995). Epigenetic changes may also be involved in the genome defence system in bacteria, fungi and other various organisms. For instance, cytosine methylation, which is the most common epigenetic mechanism that causes repression of gene expression, has been found in bacteria to inhibit the severe effect of bacteriophage infection. This was also observed in various species that have 5-methyl cytosine in their DNA include *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Caenorhabditis elegans* suggesting that methylation is an ancestral epigenetic state (Chandler and Walbot, 1986).

It is also cautioned that there was some fluctuation in the effect of ascospore transfer, with propagation sometimes having no effect, or even resulting in a decrease, in sexual fertility. The reasons for this were unclear. But as explained above (section **5.4.1**) it was possibly due to inherent stochastic variation in levels of sexual fertility, slightly differences in the growth conditions at different transfer stages, and/or an artefact of the experimental design whereby transfer was via a single morphologically 'representative' isolate at each stage. In the latter case, the chosen single-spore ascospore isolate (one chosen out of six) might not necessarily been representative of the majority of sexual ascospores at that transfer stage, hence giving an unexpected level of sexual fertility. If time had allowed, in retrospect it might have been better to measure the fertility of a series of different ascospore lines and take their mean at this stage.

5.4.4 Context of wider scientific literature

The results overall concerning a loss of sexual fertility were consistent with those of Jinks (1954) and Mather and Jinks (1958) who reported that sexual reproduction in *A. nidulans* and *A. glaucus* gradually decreased as a result of repeated transfer by asexual conidia (see section

5.1.2 and **Figure 5-1**). Indeed, Jinks (1954) found that sexual reproduction could be lost entirely in *A. nidulans* by the '14th transfer' of conidial propagation, although the length of the transfer period was not defined. By contrast, sexual fertility was never lost in *A. glaucus*, more in line with results from the present study. Elsewhere, it was observed that the sexual fertility of two strains of *Cryptococcus neoformans* was completely lost when the strains were subjected to prolonged asexual propagation *in vitro*. However, the reason (s) of this loss have not yet been determined whether an epigenetic and/or mutational effect (Xu, 2002). Similar investigations also revealed that the sexual fertility of two other species *Histoplasma capsulatum* and *Blastomyces dermatidis* could be lost under laboratory conditions (Heitman, 2010). Most recently, a study very similar to the present study has been published. Loss of sexual fertility (in terms of perithecium production) in a female isolate of the rice pathogen *Magnaporthe oryzae* was investigated (Saleh *et al.*, 2012). The results showed that female fertility was rapidly lost *in vitro* in the absence of sexual reproduction over 10 to 19 asexual generations (estimated to correlate with at least 20,000 to 38,000 mitotic divisions, although a detailed microscopic investigation was not performed). It was argued that the loss of fertility might be related to genetic and/or epigenetic changes, but the fact that female-sterile mutants increased in abundance over successive asexual generations (when sexual reproduction was absent) suggested that this change occurred primarily due to mutation (Saleh *et al.*, 2012).

Meanwhile, the later 'sex restoration' experimental work in the present study was again overall consistent with the results of Jinks (1954) and Mather and Jinks (1958) who reported that a decline in levels of sexual fertility in *A. nidulans* and *A. glaucus,* as a result of repeated transfer by asexual conidia, could be reversed by propagation through an ascospore (see section **5.1.2** and **Figure 5-1**). However, whereas Mather and Jinks (1958) found an apparent complete restoration in *A. glaucus*, in the present study this was not the case for many isolates/strains. Indeed, Jinks (1954) state that it took "several generations" of propagation by ascospores to restore original levels of perithecial production in *A.*

nidulans. This suggested both genetic and/or epigenetic changes. Kelly and Aramayo (2007) suggested that epigenetic effects could be involved in many sterile phenotypes of *Neurospora crassa* and *Caenorhabditis elegans.*

5.5 Conclusion

Although considerable research has already been devoted to investigate the loss of sexual fertility in various asexual fungal species, the mechanism and reason(s) behind this effect, whether genetic and/or environmental are still not yet fully understood. Therefore further investigations are needed in this area in order to advance our understanding of how fungal species shift from sexual to asexual state, helping to study the evolution of asexuality more comprehensively in fungi. This might lead to a better understanding of how diversity in fungi has evolved. Moreover it may have fundamental applications of economical industrial, and medical importance. One main important question that has not yet been precisely answered is which mechanism(s) are behind the loss of sex? There are various possibilities such as: cryptic sex, oversights, deleterious mutations, epigenetic effects and unavailability of appropriate environmental conditions for sex. Data from the current chapter confirmed that repeated asexual transfer for prolonged could also cause a loss of sexual fertility in all strains of the homothallic *Aspergillus* species investigated, namely *A. nidulans*, *N. fischeri* and *E. repens* confirming earlier data of Jinks (1954) and Mather and Jinks (1958). A new discovery was that this loss of sexuality was correlated with approximately 10,000 to 40,000 mitotic divisions for these species, which is arguably a relatively short time frame might finally lead to asexuality if this were repeated under field conditions.

Chapter 6 Identification of Novel Genes Regulating Sex in *Aspergillus nidulans*

6.1 Introduction

It has been estimated that at least 400, and probably many more, genes are required for sexual reproduction in filamentous ascomycete species (Dyer *et al.*, 1992). However, so far only approximately 200 genes have been identified from genome studies (Galagan *et al.* 2005) and only 70-80 'sex-related' genes have actually been experimentally characterised, many of these from *Aspergillus* species (Dyer and O'Gorman, 2011). Of the genes that have been identified, arguably the most important are the mating-type (*MAT*) genes, as described in (section **1.3.4**). In the case of *A. nidulans,* the *MAT1* (alpha-domain) and *MAT2* [high-mobility (HMG) group domain] genes are located on different chromosomes and deletion of either of the *MAT* genes has been found to result in formation of small, sterile cleistothecia (Paoletti *et al.*, 2007). There was no obvious effect on asexual sporulation or vegetative growth resulting from *MAT* gene deletion (Paoletti *et al.*, 2007).

In addition to the *MAT* genes, a variety of other genes have been identified with roles in sexual reproduction (**Figure 6-1**; see also section **1.3.6.1**). These include genes encoding proteins involved with initial detection of environmental conditions suitable for sex, mating processes (e.g. pheromone precursor and pheromone receptors), fruit body development and final ascospore development. In the case of the aspergilli full details of these genes are provided in the reviews of Dyer (2007), and for brevity will not be described further due to the number of genes involved.

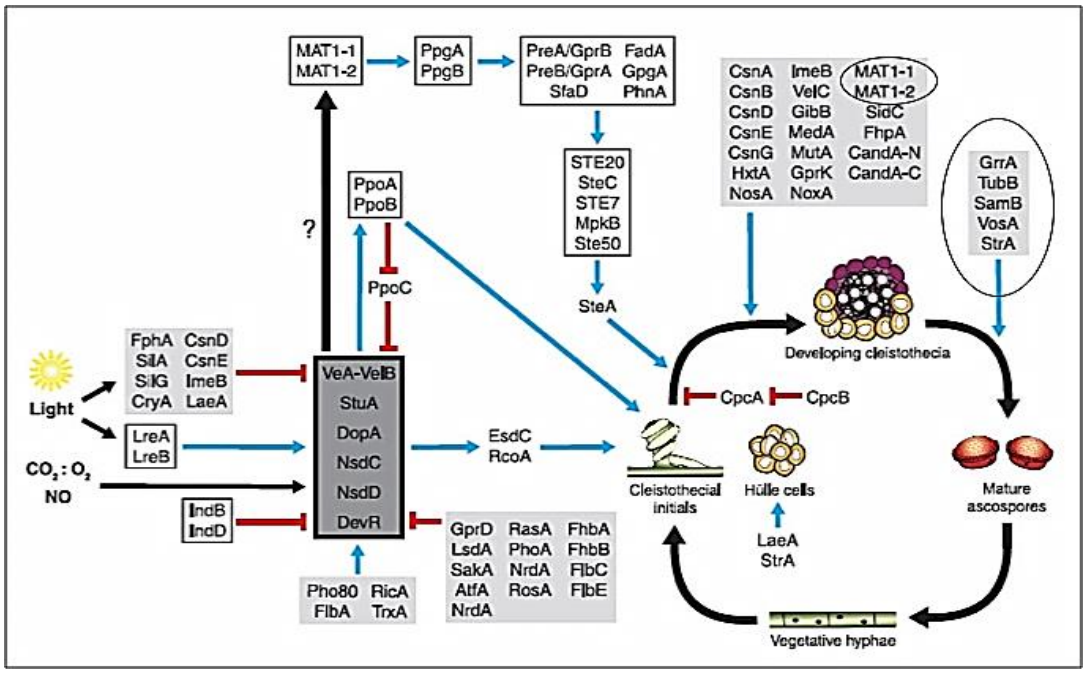

Figure 6-1 A schematic network displaying characterised genes which orchestrate particular stages of sexual reproduction in *Aspergillus* species. Most studies have focussed on *A. nidulans*. Proteins involved at a similar developmental stage are boxed together. Blue arrows: gene activation. Red lines: gene repression/inhibition. [Adapted from Dyer and O'Gorman (2012)].

As well as these characterised genes, a variety of genes involved with governing meiotic processes have been identified from *Saccharomyces cerevisiae*, and it is likely that these will also be essential for correct sexual development in filamentous ascomycetes. For example, genes required for chromosome segregation (Wang *et al.*, 1987), chromosome recombination (Klapholz *et al.*, 1985) and ascospore formation (Klapholz and Esposito, 1980). Also extra genes beyond those listed by Dyer and O'Gorman (2011) have been identified more recently from studies of *A. nidulans* and other filamentous ascomycete species, which are likely to be needed for sexual development in the aspergilli in general. For example, a striatin homologue *strA* was needed for ascosporogenesis in *A. nidulans*, with gene deletion strains producing odd, small cleistothecia and uncharacteristic ascospores which were unable to separate and could be abnormally shaped (Wang *et al.*, 2010). Fungal striatin orthologs (which act as scaffold proteins) were also found to be involved in

perithecium development of Sordariomycete species (Pöggeler and Kück, 2004; Shim *et al.*, 2006; Simonin *et al.*, 2010).

Thus, many genes are essential for correct sexual development. But it is noted that genes which are believed to act as transcription factors or other proteins localized to the nucleus appear to have particularly key roles, with mutation in genes such as *nosA, rosA, nsdD, steA*, *medA*, *MAT1, MAT2* and *veA* resulting in considerable change in the normal programme of sexual development (Vallim *et al.*, 2000; Han *et al.*, 2001; Kim *et al.*, 2002b; Vienken *et al.*, 2005; Vienken and Fischer, 2006).

For comparison, a series of genes regulating sexual reproduction have also been identified from *Neurospora*, *Sordaria* and *Podospora* species, which have been widely used as model fungi in parallel with *Aspergillus* species. Many of these genes are homologues of genes found to be involved with sexual reproduction in the aspergilli. For example, Debuchy and Coppin (1992) identified two mating-type genes regulating sex in *Podospora anserina*, which were named FPR1 and FMR1 (fertilization plus and minus regulators). These two genes were found to be sufficient to allow fertilization in this species. Further work identified two more genes, named SMR1 and SMR2, which were found to be present in the mat– idiomorph. The combined action of FMR1, SMR1 and SMR2 was found to be required to promote the normal development and maturation of perithecia in *P. anserine* (Debuchy *et al.,* 1993). Meanwhile, in *Neurospora crassa* two mating -type genes mat α-1 and mat A-1 have been found to play a key role in determining α and A mating specificity, respectively (Staben and Yanofsky, 1990; Glass *et al.*, 1990a). Further investigations using reverse transcription-PCR successfully amplified two cDNAs from the mat A-1 region in *N. crassa*. Mating-type and other developmental genes have also been described from the homothalic species *Sordaria macrospora* (Debuchy *et al*., 2010).

6.1.1 Aim of this chapter

Fully annotated genome sequences are now available for various species of *Aspergillus*. But over 50% of the annotated genes have no known function. It is possible that many of these genes might be involved with sexual development. As previously discussed, one key reason why species might be asexual is due to mutation in an important gene needed for sexual development (section **5.1.2**). Therefore there is an ongoing need to fully identify the suite of genes involved with sexual reproduction in the aspergilli, so that putative asexual species can be screened fully for such genes to determine whether such a mutation exists.

The main aim of this chapter is therefore to identify new gene(s) involved with sexual reproduction in the aspergilli. This was made possible by the deposition at the start of PhD studies of a series of gene disruption (knockout) cassettes at the Fungal Genetics Stock Centre (FGSC), USA. These cassettes were produced at Dartmouth Medical School (USA) following a previous model based on gene knockout in *N. crassa* (Colot *et al.*, 2006) . Each cassette contains the *A. fumigatus pyrG* gene as a selectable marker, flanked by approximately 1 kb of sequence upstream and downstream of the target gene, to facilitate efficient homologous integration (http://www.fgsc.net/Aspergillus/ko_cassettes/Strategy.htm). However, only the disruption cassettes were available, i.e. it was still necessary for the end user to undertake the necessary transformations for production of gene knockout strains.

Given that transcription factors might have a particularly important role in sexual development, studies in the present chapter are focussed on a series of putative high mobility group (HMG) domain transcription factor genes in *A. nidulans*, which had been identified during prior BLAST analyses (P. Dyer personal communication). This particular family of transcription factors was chosen as HMG proteins are generally known to be involved with sexual development in higher eukaryotes, e.g. the fungal MAT-2 proteins and SRY sex determinant in humans (Debuchy *et al.*, 2010; Martin *et al.,* 2010).

6.2 Materials and Methods

6.2.1 Materials

A series of chemicals needed to be prepared specifically for work in this chapter as follows.

1000X Trace element solution 10 g EDTA, 4.4 g $ZnSO_4 \cdot 7H_2O$ (zinc sulphate), 1.01 g $MnCl_2$ (manganese chloride), 0.32 g $CoCl_2 \cdot 6H_2O$ (cobalt chloride), 0.315 g $CuSO_4 \cdot 5H2O$ (copper sulphate), 0.22 g $(NH4)_6Mo_7O_{24} \cdot 4H_2O$ (ammonium molybdate), 1.47 g $CaCl_2 \cdot 2H_2O$ (calcium chloride) and 1.0 g $FeSO_4 \cdot 7H_2O$ (ferrous sulphate) were added in order and dissolved completely into 900 ml ddH_2O, allowing each to dissolve completely before adding the next, and the pH adjusted to 6.0 with 1.0 M NaOH after each component was dissolved. After all components were dissolved the pH was adjusted to 4.0 with 1.0 M HCl. The solution then was made up to the final volume of 1.0 L with distilled water and was initially green, but turned purple with time. The solution was autoclaved and stored at room temperature.

25X (MN) salts 37.5 g of $NaNO_3$, 3.25 g of KCl and 9.5 g of KH_2PO_4 were dissolved fully into 200 ml dH_2O, and then the solution was made up to 250 ml with distilled water and autoclaved.

50X $MgSO_4$ 6.5 g of $MgSO_4.7H_2O$ was dissolved into 250 ml of distilled water then autoclaved and stored at 4°C.

50X AspA solution 75 g of 3.5 M $NaOH_3$, 6.5 g of 350 mM KCL and 19 g of 560 mM KH_2PO_4 were dissolved completely into 250 ml of distilled water then adjusted to pH 5.5 with KOH, autoclaved and stored at room temperature.

PABA stock solution 1 g of para-amino benzoic acid (PABA) was dissolved in 100 ml of distilled water, filter sterilised then 1.0 ml of this stock was added per litre of ACM or AMM where necessary.

Pyrodixine HCL stock solution 0.1 g of Pyrodixine HCL was dissolved into 100 ml of distilled water, filter sterilised then 50 µl of this stock was added per litre of ACM or AMM where necessary.

***Aspergillus* minimal media agar (AMM)** 12.5 g glucose, 1 g casamino acids, 1 g uridine, 0.8 g uracil were dissolved in 800 ml of distilled water, adjusted to pH 6.5 with conc. NaOH, then 15 g agar was added, this solution was made up to around 920 ml with distilled water and autoclaved at 117°C. After autoclaving 20 ml of 50X AspA, 20 ml of $MgSO_4$, 40 ml of 25X MN salts and 1 ml of 1,000X trace element solution were added aseptically. Finally, after cooling down, PABA and/ or pyrodixine HCL was also added as required (other chemical stocks as described above).

Sorbitol-containing minimal agar (SCM) 40.0 ml of 25X MN salts, 1.0 ml trace element solution, 10 g glucose, 218.64 g of sorbitol (= 1.2 M) and 15 g agar were dissolved into 900 ml of distilled water (ddH2O), and then adjusted to pH 6.5 with conc. NaOH. This solution was made up to final volume of 980 ml with distilled water; autoclaved and aseptically 20 ml of 50X $MgSO_4$ stock was added prior to pouring plates (other chemical stocks as described above).

Mycelium wash 44.4 g of 0.6 M $MgSO_4.7H_2O$ was dissolved into 300 ml distilled water, autoclaved and stored at 4°C.

MM Buffer 59.15 g of 1.2 M $MgSO_4.7H_2O$ and 0.8 g of 20 mM MES were dissolved into 150 ml dH_2O then the solution was made up to final volume of 200 ml with distilled water, adjusted to pH 5.8, autoclaved and stored at room temperature.

NM Buffer 11.7 g of 1.0 M NaCL, 0.8 g of 20 mM MES were dissolved into 150 ml dH_2O, then the solution was made up to final volume of 200 ml with distilled water, adjusted to pH 5.8, autoclaved and stored at room temperature.

STC solution 65.6 g of 1.2 M sorbitol, 0.36 g of 10 mM Tris base, 2.2 g of 50 mM $CaCl_2.2H_2O$ were dissolved fully into 250 ml of distilled water then adjusted to pH 7.5 with conc. HCL, the solution was made up to the final volume of 300 ml with distilled water, autoclaved and stored at 4°C.

Sodium acetate (3M pH5.2) 40.0 g of $NaOAc.3H_2O$ was dissolved in 80 ml of distilled water (ddH2O), and then adjusted to pH 5.2 with glacial acetic acid. This solution was made up to the final volume of 100 ml with distilled water and filtered to sterilise.

1.1 M KOH solution This solution always had to be made fresh before use because KOH reacts with CO_2 in the atmosphere. 200 ml of distilled water was boiled in a 500 ml glass beaker, then left to cool down for a few minutes to 35-40ºC. A small beaker and 100 ml graduated cylinder were rinsed with this boiled, deionized distilled water (bddw), then 6.17 g KOH was dissolved thoroughly into approximately 70 ml of bddw in the small beaker. The KOH solution was then poured into the graduated cylinder and made up to 100 ml with bddw, then covered with Parafilm and mixed by inversion. Great caution was exercised because KOH is a strong base; therefore both eye and skin protections were used to prevent any contact of the solution.

KCl, citric acid solution 8.2 g KCl and 2.1 g of citric acid monohydrate (MW 210) were added and dissolved into 50 ml ddH_2O. The solution was adjusted to pH 5.8 with 1.1 M KOH solution (as described above) and made up to a final volume of 100 ml with distilled water. The solution was sealed carefully with Parafilm before storage for long periods to prevent evaporation. The solution was stored for short periods (less than 2 weeks) in a refrigerator, or sterilised by autoclaving (ensuring that the volume was not reduced during autoclaving) and stored indefinitely at room temperature.

PEG solution 4.47 g KCl and 0.74 g $CaCl_2.2H_2O$ were dissolved fully in 50 ml of distilled water in a graduated cylinder, then 0.802 ml of 1 M Tris–HCl and 0.196 ml of 1.0 M Tris base were also added and mixed up thoroughly. This gave a pH of 7.5, then 25 g PEG (average molecular weight 3,350) was gradually added and dissolved thoroughly (NB. The PEG dissolved slowly with mixing), then approximately 30 ml of distilled water was added and the cylinder covered with Parafilm and mixed by inversion. After the PEG was in solution, the cylinder was filled up to 100 ml with distilled water, mixed by inversion and poured into small vials and sterilised by autoclave ensuring that the volume was not reduced during autoclaving. Bottles were finally sealed to prevent evaporation and stored indefinitely at room temperature. N.B. If the PEG solution is cooled to 4 °C or frozen, the PEG will precipitate and it must then be warmed and mixed thoroughly before use to allow the PEG to dissolve.

2× protoplasting solution Vinoflow FCE is a commercially available inexpensive enzyme used in winemaking, which is reportedly an excellent protoplasting enzyme, having pectinase and beta 1,3-1,6 glucanase activity. It does not work well with germinating spores, but efficiently protoplasts hyphae (Berl Oakely, cited from http://www.fgsc.net/Aspergillus/Vinoflow_protoplasting.pdf). A 2x protoplasting solution was made by dissolving 1.28 g Vinoflow FCE (Novo Nordisk) in 10 ml KCl, citric acid solution (as described above). The solution was sterilised by filtering it through a sterile low protein binding Millex GV filter (0.22 µm pore size). To eliminate any residual detergent

that might be present in the filter, the first millilitre or so of the solution that comes out through the filter was discarded. N.B. This solution had to be made fresh, being stored for less than 30 min before use.

6.2.2 Methods

6.2.2.1 Disruption cassette and transformation protocol

BLAST searching of the *A. nidulans* genome with consensus high-mobility group (HMG) sequences allowed the identification of number of candidate HMG genes (P Dyer, personal communication). These genes were checked against a list of gene deletion cassettes and amplification primers available at the Fungal Genetics Stock Centre (FGSC), USA (http://www.fgsc.net/Aspergillus/KO_Cassettes.htm.).

Each gene disruption cassette contained the *A. fumigatus pyrG* wild-type gene as a selectable marker, flanked by approximately 1 kb of sequence upstream and downstream of the target gene, to facilitate efficient homologous integration. Studies in the present study focussed on disruption (knock-out) cassettes of *A. nidulans* available and ordered from the FGSC for the following five genes: ANID_01631.1, ANID_03667.1, ANID_03784.1, ANID_05279.1, ANID_06578.1 (see the AspGD website for full gene details: http://www.aspergillusgenome.org/). On receipt cassettes and primers were stored at 4ºC. The effect of gene deletion, via the use of these knock-out cassettes, on sexual reproduction in *A. nidulans* was then determined. This involved the following laboratory experimental procedures.

6.2.2.2 Amplification of disruption cassettes

The disruption cassettes were supplied with 40-50 bp primers for their amplification, which annealed to the 5’ and 3’ ends of the cassettes (http://www.fgsc.net/Aspergillus/KO_Cassettes.htm.).

Following instructions provided, cassette templates were diluted 20 and 100 fold by TE buffer and amplified used the following PCR cycling

parameters with high fidelity (Sigma-Aldrich): 1.0 min at 94ºC; then 35 cycles of 30 sec at 94ºC, 30 sec at 60ºC, 5 min at 72ºC; finally 10 min at 72ºC. The components for LA Taq in a 50-µl reaction were 1.0 µl of the 5f/3r primer mix (2 µM final each primer) and 400 µM each dNTP. Amplicons were resolved by agarose gel electrophoresis (section **2.2.6.1**) to ensure that sizes were as predicted from the FGSC (http://www.fgsc.net/Aspergillus/KO_Cassettes.htm.).

In general sufficient DNA product for later transformation work could be generated by pooling the contents of two 50 µl PCR tubes. DNA was purified (section **2.2.6**) and cleaned up using different columns for each 50 µl reaction, then products were pooled together in a 1.5 µl Eppendorf tube. To concentrate the DNA, 0.1 volumes of 3.0 M sodium acetate and 2.0 volumes ice-cold 99.9% ethanol were added and left at -20ºC for 30 min, then centrifuged at 12,000 rpm for 10 min at 4ºC and finally the upper (aqueous) phase was removed. The resulting pellet was washed twice with 70% ethanol (adding 500 µl each time), vortexed, centrifuged and then pellets air dried for 10 min. Finally the DNA pellet was re-suspended in TE buffer (e.g. 5-10 µl volume) and the concentration was verified on Nanodrop (ND-100 V3, 1.0. users manual). Ideally a final DNA concentration of 0.5-1.0 µg per µl (500-1000 ng/µl) was obtained. DNA products were stored at 4ºC for later transformations (section **6.2.2.4**).

6.2.2.3 Protoplasting

Seven day-old cultures of *A. nidulans* strain 2-258 (*pyrG89-, pyro A4-, nkuA⊿::argB, veA*+; gift of O. Bayram, original code number AGB551) were harvested gently with 5.0 ml of 20% Tween. Spores were filtered through a sterile Miracloth filter and diluted to make 1×10^8 spore suspension (sections **4.3.1.1** and **4.3.4**), and 8-10 ml was inoculated into 20 ml of *Aspergillus* complete medium (ACM broth supplemented with uracil and uridine, section **5.2.1**) and pyridoxine which was incubated with shaking for 13-14 hr at 28ºC to allow germination. Freshly germinated mycelia were filtered through a double layer of sterile Miracloth (Calbiochem) and washed with about 5.0 ml cold mycelium wash buffer. Mycelia were then placed in a sterile 100 ml flask and re-

suspended in 8.0 ml of complete medium (ACM broth supplemented with uridine and uracil). 8.0 ml of filter sterilised 2X protoplasting solution was also added into the flask [the 2X protoplasting solution consists of 1.1 M KCl, 0.1 M citric acid (the KCl, citric acid solution is made first and the pH is then adjusted to 5.8 with freshly made 1.1 M KOH)]. The final concentration of Vinoflow FCE was, thus, 64 mg/ml. The protoplast mixture solution was mixed evenly, and mycelia were incubated gently at 30°C at 50-80 rpm (80 rpm initially, then reduced to 50 rpm after about 30 min when the viscosity of the cell suspension decreased) for 2-4 h. The protoplasting process was monitored microscopically after 2 h and then at 30 min intervals until sufficient protoplasting was achieved. To aid visual examination, a small sample (10~ul) was diluted with an equal volume of water, which causes the protoplasts to swell up and become disentangled from the background mycelium (some lysis may also occur). At this stage, the protoplasts were purified from mycelial fragments and cell debris by purification through sterile polyallomer wool (SUPA AQUATIC SUPPLIES LTD, UK) in two purification colums, prepared by loosely packing two sterile 20 ml syringe barrels with pre-autoclaved polyallomer wool (one with the wool more tightly packed). The loosely packed column was set up above the other on a clamp stand. The protoplast mixture was slowly poured on to this column and separation allowed to occur by gravity. If the column blocked up, the suspension was stirred with a blue tip and washed with a 5.0 ml of cold NM buffer. The protoplasts were collected in fresh 50 ml tubes (15 ml per tube) and 30 ml cold NM buffer added. The protoplast solution was gently mixed by inversion and spun at 2,500 rpm at 4°C for 10 min in a swing out rotor and the supernatant was poured off. The residual pellet may have a black lower region (due to black spore contamination) with a white/grey upper layer. The white/grey layer was scraped gently with a sterile blue pipette tip and re-suspended in 1.0 ml cold STC. The procedure was repeated twice when it was necessary to recover more protoplasts. The re-suspended white/grey layer was pooled in a fresh, cold, sterile 50 ml tube and topped up with cold STC to 50 ml. The solution was mixed gently by inversion. The protoplasts were spun at 2,000 rpm at 4°C for

10 min in a swing out rotor. The supernatant was poured off, leaving the protoplast pellet, which was ideally creamy grey in colour with very little trace of black spore contamination. The pellet was gently re-suspended in 1.0 ml cold STC. A sample of a 10^{-2} dilution (5 µl + 495 µl STC) was counted in a haemocytometer and the protoplasts diluted to 5 x 10^{7}/ml in cold STC. The protoplasts were spherical or slightly "crinkled" in appearance. N.B. Fresh protoplasts gave the best transformation frequency but cells stored overnight on ice could also be transformed although their viability was found to reduced by about 50%.

6.2.2.4 Transformation

Approximately 100 µl of the concentrated protoplast suspension from above (section **6.2.2.3**) were transformed with 2-3 µg of a given DNA disruption cassette in a sterile 15 ml centrifuge tube by PEG-mediated transformation (Fitzgerald *et al.*, 2003; Paoletti *et al.*, 2007). The transforming DNA (5 µl) was added to the protoplast suspension and tubes were mixed gently and incubated at room temperature (RT) for 25 min. Also a minus DNA control was included in transformations i.e. protoplasts with PEG added but no transforming DNA (i.e. STC in place of DNA). The protoplast suspension mixture was mixed gently again and 200 µl of PEG solution was added gradually (drop by drop with gentle mixing as the PEG solution was highly viscous). Then a further 200 µl of PEG was added gradually (with mixing) and finally another 850 µl of PEG (with mixing). Tubes were incubated at RT for 20 min. Tubes were then filled with cold STC up to 10 ml and mixed gently by inversion until PEG has been completely diluted. Tubes were spun at 2,000 rpm at 4°C for 10 min in a swing out rotor and the supernatant was poured off. Cells were re-suspended in 600 µl STC and then 75 µl aliquots of this suspension was plated out directly onto sorbitol-containing minimal agar plates (section **6.2.1**) making 8 replicate plates for each transformant experiment. These including: sorbitol-containing minimal media + pyrodixine, or sorbitol-containing minimal media + pyrodixine + uracil/uridine (latter as a control) according to separate protocol. Plates were incubated at 28ºC for 3-5 d until transformants appeared (ideally

yielding 10-20 colonies per plate). 12 transformants were collected and sub-cultured onto *Aspergillus* minimal media (AMM) + pyridoxine. Also 4 protoplast 'controls' were also collected and sub-cultured onto *Aspergillus* minimal media (AMM) + pyridoxine + uracil/uridine. The remaining transformation plates were stored in a fridge at 4ºC. N.B. High MW or concentrated DNA may precipitate or cause the protoplasts to clump when the PEG solution is added and this may reduce transformation frequencies.

6.2.2.5 Transformant screening and sexual fertility test

Eight transformants were collected and screened for sexual fertility on ACM medium supplemented with both pyridoxine + uracil/uridine (Robellet *et al.*, 2010). A protoplast control (no DNA added) was included and also the original parental strain 2-258 as a further control. A standard method for *A. nidulans* sex was used, i.e. inoculating 5.0 cm Petri dish with a spore suspension (1 x 10^5 conidia) spread over the surface. Plates were incubated at 32ºC in the dark and sealed with two layers of parafilm after 15-18 hr of incubation and then were left for further 30 d to obtain mature cleistothecia. Six replicate plates were inoculated, and then scored for the presence/absence, number and size of cleistothecia, and also presence of ascospores. Results for all deletant mutants were compared to the controls.

After screening of sexual fertility, further characterisation of the transformants was carried out using PCR to confirm the loss of original target gene of the mutants and correct homolgous integration by positional PCR (Paoletti *et al.,* 2007). Primers (TG1 and TG2) within the target gene and TP3 and TP4 inside the PyrG (transforming DNA) were designed (**Figure 6-2** and **Appendix 1**). Further PCR primers (FP1 and FP2) slightly outside the flanking regions of the target gene for the transforming DNA were also designed (**Figure 6-2** and **Appendix 1**). Transformants and controls were grown up and DNA was extracted from all isolates using a phenol-chloroform procedure (section **2.2.5.2**) and then the primer pairs TG1 and TG2, FP1 and TP3, and TP4 and FP2 used in PCR of both controls and putative deletants. PCR was performed using

a High-Fidelity DNA Polymerase (Finnzymes, Finland). Each 25 µl reaction contained ~50 ng genomic DNA, 5 µl 5X PCR Buffer (containing 7.5 mM $MgCl_2$), 0.2 µl (25 mM each) dNTPs, 1 µl (50 µM) forward primer, 1 µl (50 µM) reverse primer, 0.25 µl DNA polymerase and ultrapure water to a final volume of 25 µl. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: an initial denaturation step at 98°C for 5 min; 35 cycles consisting of 30 s at 98°C, 1 min at 60°C and 30 s at 72°C; followed by a final extension step at 72°C for 5 min, all steps used a ramp rate of 70°C/min. The PCR screening was planned in such a way that if heterologous gene integration had occurred in transformants then a product would be produced with primers TG1 and TG2 (and also in the parental control), whereas if gene replacement had occurred then no product would be expected. Instead, in the latter case if the transforming DNA had homologously integrated at the correct locus then PCR products would be generated with the primer pairs FP1 and TP3, and TP4 and FP2. Amplicons were analysed by gel electrophoresis in 1.2% agarose gels and products visualised using ethidium bromide staining and a BioRad Chemidoc XRS (section **2.2.6.1**).

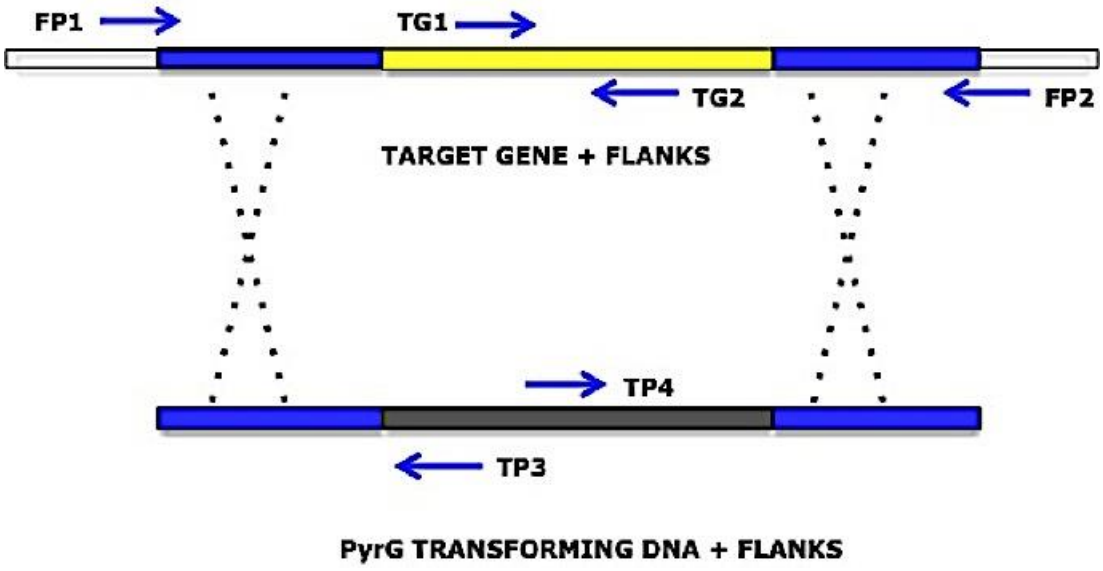

Figure 6-2 Location of primers used inside and outside of the target gene and also inside PyrG for screening of DNA transformants.

6.2.2.6 Transformant complementation

In order to confirm any loss of sexual fertility was due to loss of the deleted gene rather than some other artefact(s), the gene was re-inserted back by sexual crossing i.e. gene complementation. It was then expected that sexual fertility would be restored to the original parental levels. Original gene transformations were made with strain 2-258, with transformants exhibiting *pyrG*+ prototrophy, but these still contained the *pyroA4*-, wild-type green spore colour and *paba*+ markers. This allowed a complementation strategy by crossing to strain 2-259 [*paba A1-, yA2* (yellow spore), *veA*+, *pyro*+; gift of O. Bayram] (**Figure 6-3**).

The crossing protocols of Todd *et al.* (2007) were followed. 10 µl spore suspensions of concentration 1 x 10^5 conidia from each parent were inoculated opposite each other in a 9.0 cm diameter Petri dish containing ACM supplemented with pyrodixine, uracil/uridine and PABA and were incubated at 28ºC in the light for 2 d. By this time the colonies had met, and a small agar plug was then cut out from the zone of mixed hyphae where the colonies met, using a sterile wire. Agar plugs were then transferred to selective media (non-supplemented *Aspergillus* minimal media (AMM; section **6.2.1**) to promote heterokaryon formation for 64 hr at 32ºC. Putative heterokaryon plates (2-258 x 2-259) were incubated at 32ºC in the dark for 64 hr, then sealed with two layers of parafilm and incubated for further two weeks to produce mature cleistothecia on AMM. 50-100 relatively large cleistothecia were selected randomly from the edge of a Petri dish, which are more likely to be the result of outcrossing than self fertilisation (Todd *et al.,* 2007). Cleistothecia were transferred with a sterile wire to a 4% agar plate and individual cleistothecia rolled across the agar plate to remove any aerial hyphae, Hülle cells, conidia and conidiophores. The cleaned cleistothecia then were transferred to a sterile microfuge tube containing 500 µl of 20% Tween and heated at 55ºC for 30 min to remove any adhering conidia or hyphae. Finally cleistothecia were ruptured against the wall of the tube with a wire to

release the asci. The asci were also broken by a sterile loop in the bottom of the microfuge tube and disrupted evenly by vortexing to generate an ascospore suspension.

Ascospore suspensions were initially sampled for outcrossing by plating to complete medium (ACM) to determine whether they arose from a hybrid cleistothecium or from selfing of either parent. A mixture of green and yellow conidia was observed in ascospores progeny as an indication of crossing (versus all green or all yellow if from self fertilisation). An ascospore suspension arising from a hybrid cleistothecium was then spread plated and colonies were transferred to an ACM master plate using a standard single-spore approach. Ascospores were grown up and green-spored offspring that were *pyro A4*-, *paba*+ (i.e. resembling the 2-258 parent) were selected. Then 32 colonies from the master plate of those green-spored offspring were transferred and grown on three different *Aspergillus* minimal media (AMM or selective media). The first plate contained only AMM and the second plate contained AMM supplemented with pyrimidines (uridine/uracil), but not pyrodixine and the third plate contained AMM supplemented with pyrodixine, but not pyrimidines. Single ascospore colonies that failed to grow on all three different media (circled in black, **Figure 6-3**) were selected, whose phenotype was consistent with reinsertion of the target gene of interest as a result of outcrossing, and restoration of the original host parent 2-258 auxotrophic markers and colony colour (i.e. green spored, *PyrG-, PyroA4-, HMG*+). These colonies were subcultured on ACM (complete media) supplemented with pyrimidines (uridine/uracil) and pyrodixine and incubated at 28ºC for 5-7 d in the light to produce asexual spores, then conidia were harvested and spore suspension was made to test for sexual fertility as described above (section **6.2.2.5**). Six replicate plates were set up for sex, and scored for presence/absence of cleistothecia; also size and number; and were also tested for presence of ascospores. Results of those 'restored' complementation strains were compared relatively to sexual control. Finally successful 'restored' green strains were grown up and the DNA was extracted then tested for presence (restoration) of the target gene by PCR using primers TG1 and TG2 as described above.

6.2.3 Statistical analysis.

Statistics software (SPSS, version 21) for Macintosh (Mac OS X version 10.6.8) was used. ANOVA (analysis of variance) was used to analyse the data and to determine whether the results were significant at a 5% confidence level according to Barnard *et al.* (2007).

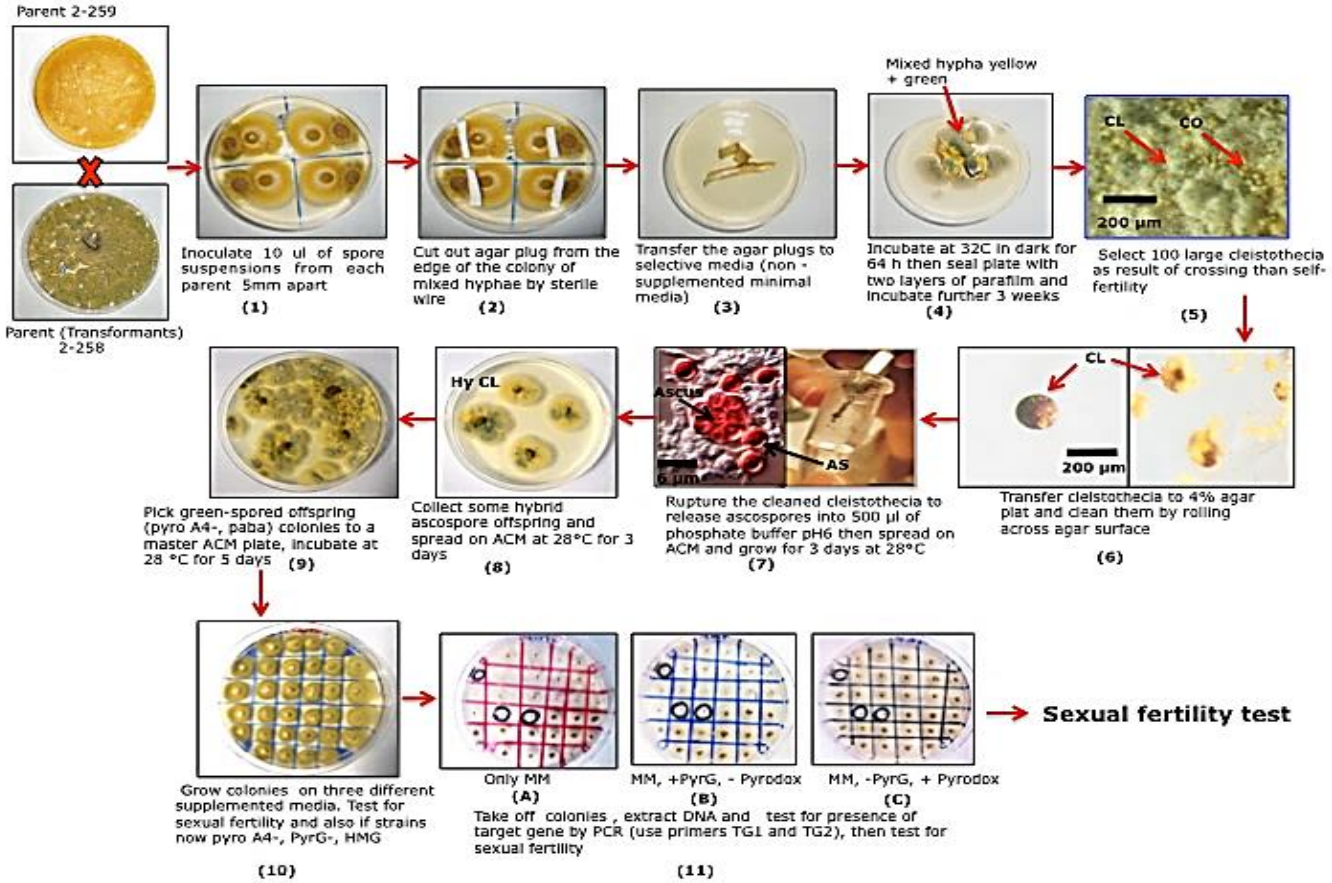

Figure 6-3 Sexual crossing protocol of *A. nidulans* between 2-258 transformant gene deletants (green spores) and 2-259 (yellow spores) for complementation of transformants.

6.3 Results

6.3.1 Identification, protoplasting and transformation of disruption cassettes

Five *A. nidulans* disruption cassettes were obtained from the Fungal Genetics Stock Centre (FGSC), which were designed to knockout putative high mobility group (HMG) genes in *A. nidulans*. Full sequences of these five genes (ANID_01631.1, ANID_03667.1, ANID_03784.1, ANID_05279.1, ANID_06578.1) were available from the *Aspergillus* Genome Database (AspGD) website at http://www.aspergillusgenome.org/. Although these were all stated to be uncharacterised, most of the genes were listed as having orthologues with DNA binding activity and/or nuclear localisation, consistent with action as transcription factors (**Figure 6-1**). The FGSC provided further details about the size of the upstream and downstream flanking regions (from 701-964 bp in size) and the sequence of the amplification primers provided (**Table 6-2**).

All disruption cassettes were successfully amplified using Faststart High-Fidelity enzyme and specific primers following the cycling parameters mentioned earlier (section **6.2.2.2**), and the linear DNA used to transform fresh protoplasts of strain 2-258 (*pyrG-, pyro A4-, nkuA∆::argB, veA*+) as described (section **6.2.2.4**). Twelve transformants for each disruption cassette, together with 4 protoplast 'controls', were collected and sub-cultured onto *Aspergillus* minimal media (AMM) + pyridoxine and uracil/uridine. Eight morphologically 'typical' colonies of the transformants, together with two controls (no DNA added protoplast control and original parent strain 2-258) were then screened for sexual fertility (presence and/or absence, number and size of cleistothecia, and also presence of ascospores) on ACM + pyridoxine and uracil/uridine (section **6.2.2.5**). Overall results showed that disruption of all of the putative HMG genes had a dramatic negative effect on the sexual fertility of *A. nidulans (***Figure 6-3, Figure 6-4** and **Figure 6-5**).

Two patterns were evident. Four of the gene deleted strains (Δ01631.1, Δ03784.1, Δ05279.1 and Δ06578.1) produced significantly lower numbers of mature-purple-black cleistothecia than the control strains, and instead exhibited an increased amount of yellow-gold Hülle cells (**Figure 6-4 A-G**). On the other hand, Δ03667.1 (three transformants analysed) failed totally to produce any cleistothecia, and instead produced yellowish-golden Hülle cells in abundance, which covered the whole surface of Petri dishes (**Figure 6-4 H**).

In terms of the number of cleistothecia that were formed, the four deletant mutants, Δ01631.1, Δ03784.1, Δ05279.1 and Δ06578.1, generally produced fewer cleistothecia, which were also larger in size, compared with the controls.

Figure 6-5 shows that the controls (host and transformation strains) produced higher numbers of cleistothecia, around 130 cleistothecia per 100 mm^2, than all the deletant strains, which formed between 24 to 68 cleistothecia per 100 mm^2 (i.e. 18-52% fertility levels of the controls) with abundant golden Hülle cells. One-way ANOVA revealed that there was an overall significant variation in number of cleistothecia formed by all strains [F (df) = 50.587 (6, 245); p <0.001]. Although the three transformants of Δ03667.1 deletant also formed Hülle cells in abundance, no cleistothecia were observed at all when triplicate plates of these strain were incubated in appropriate sexual conditions indicating that the strain had completely lost the ability to produce cleistothecia and had become fully sterile due to gene loss (**Figure 6-5**).

With regard to the size of cleistothecia, **Table 6-3** shows that cleistothecia produced by the gene deletion mutants were significantly larger than those formed by the control strains (based on measurement of diameter of 120 cleistothecia per strain). Statistically, one-way ANOVA revealed that there was an overall significant variation in size of cleistothecia formed by strains [F (df) = 274.181 (6, 832); p <0.001].

Table 6-1 Details of putative *A. nidulans* HMG genes for which knock-out cassettes were obtained from the Fungal Genetics Stock Centre (FGSC).

Gene Alias	Systematic Name	Feature type	Feature Qualifier	Description
ANID_01631	AN1631	ORF	Uncharacterized	Ortholog(s) have L-methionine secondary active transmembrane transporter activity and role in cysteine transport, methionine transport
ANID_03667	AN3667	ORF	Uncharacterized	Has domain(s) with predicted DNA binding activity
ANID_03784	AN3784	ORF	Uncharacterized	Ortholog(s) have damaged DNA binding, zinc ion binding activity, role in nucleotide-excision repair, DNA damage recognition and nucleotide-excision repair factor 1 complex localization
ANID_05279	AN5279	ORF	Uncharacterized	Has domain(s) with predicted sequence-specific DNA binding RNA polymerase II transcription factor activity, zinc ion binding activity, role in regulation of transcription, DNA-dependent and nucleus localization
ANID_06578	AN6578	ORF	Uncharacterized	Ortholog(s) have role in positive regulation of gluconate transmembrane transport and cytosol, nucleus localization

Table 6-2 Details of length of flanking regions and sequences of amplification primers for gene disruption cassettes provided from the Fungal Genetics Stock Centre, USA.

Target Gene Locus	Primer	Primer Sequence	5' flanking length (bp)	3' flanking length (bp)
ANID_01631.1	5' forward	GTAACGCCAGGGTTTTCCCAGTCACGACGCTACTCCTCGATGAACGAC	964	735
	3' reverse	GCGGATAACAATTTCACACAGGAAACAGCGTTATCCAGACGCTAAGACC		
ANID_03667.1	5' forward	GTAACGCCAGGGTTTTCCCAGTCACGACGCTCACTAGAGCAGCGTTGTA	804	784
	3' reverse	GCGGATAACAATTTCACACAGGAAACAGCTATCTGAGGAGGCGAGTATC		

ANID_03784.1	5' forward	GTAACGCCAGGGTTTTCCCAGTCACGACGGAAGGATCTGAACCTGTGAC	701	889
	3' reverse	GCGGATAACAATTTCACACAGGAAACAGCCCATACACAGCACCACTATC		
ANID_05279.1	5' forward	GTAACGCCAGGGTTTTCCCAGTCACGACGGACCTGAATGGAGAACTGAG	746	919
	3' reverse	GCGGATAACAATTTCACACAGGAAACAGCCTATAGGCCGTTCTCAGTTC		
ANID_06578.1	5' forward	GTAACGCCAGGGTTTTCCCAGTCACGACGGTCCAGTCCAGTCCTTATCT	854	839
	3' reverse	GCGGATAACAATTTCACACAGGAAACAGCAATCTAGACAGACCCAGTCG		

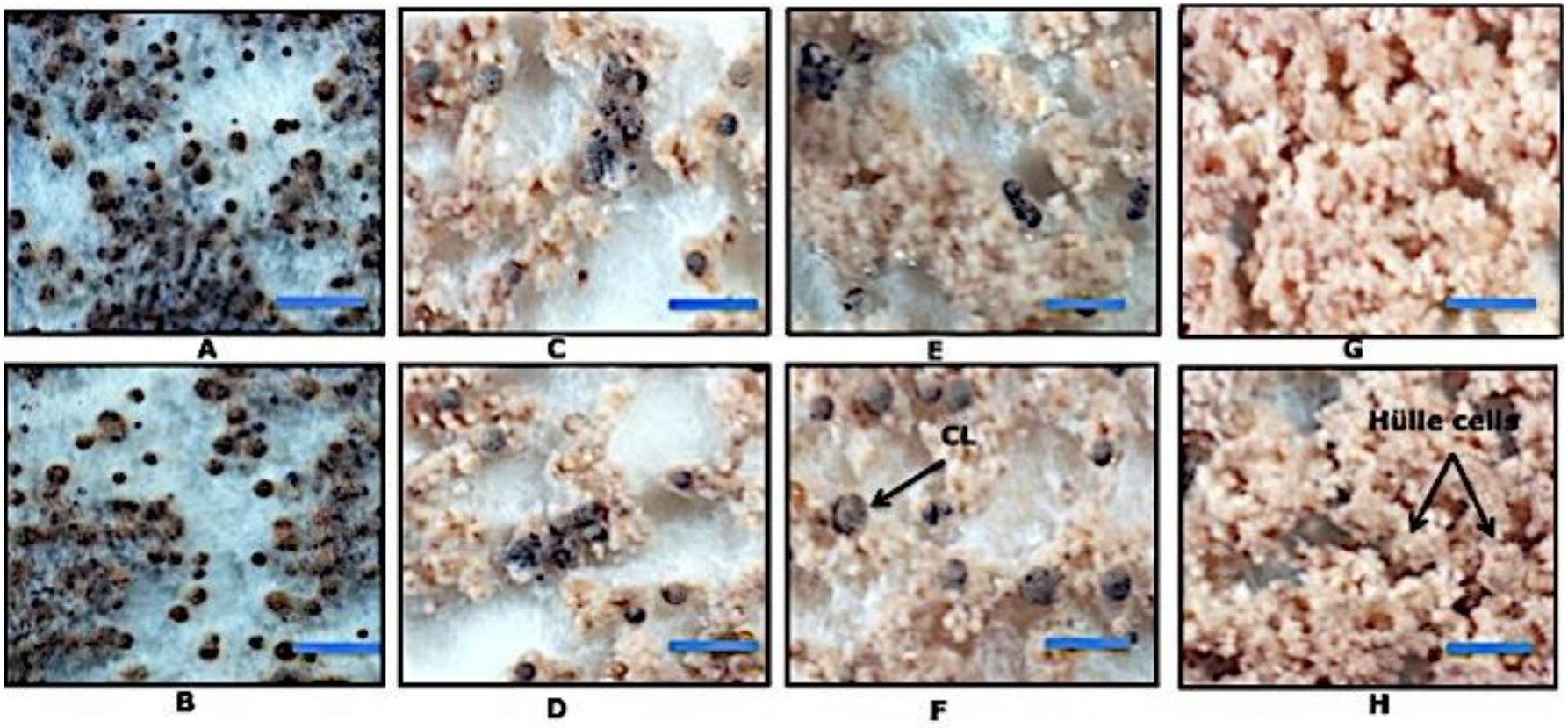

Figure 6-4 Development features of controls (host parent and transformation strains) and five gene deletion strains after 4 weeks of incubation on ACM supplemented with pyridoxine + uracil/uridine in the dark at 32ºC. (A, B) dark small-mature cleistothecia of host parent and transformation control, respectively (C, D, E, F) few cleistothecia (arrowed CL) with abundant Hülle cells produced respectively by the mutants Δ01631.1, Δ03784.1, Δ05279.1 and Δ06578.1 (G, H) only golden Hülle cells formed via Δ 03667.1. Scale bars indicate 200 µm.

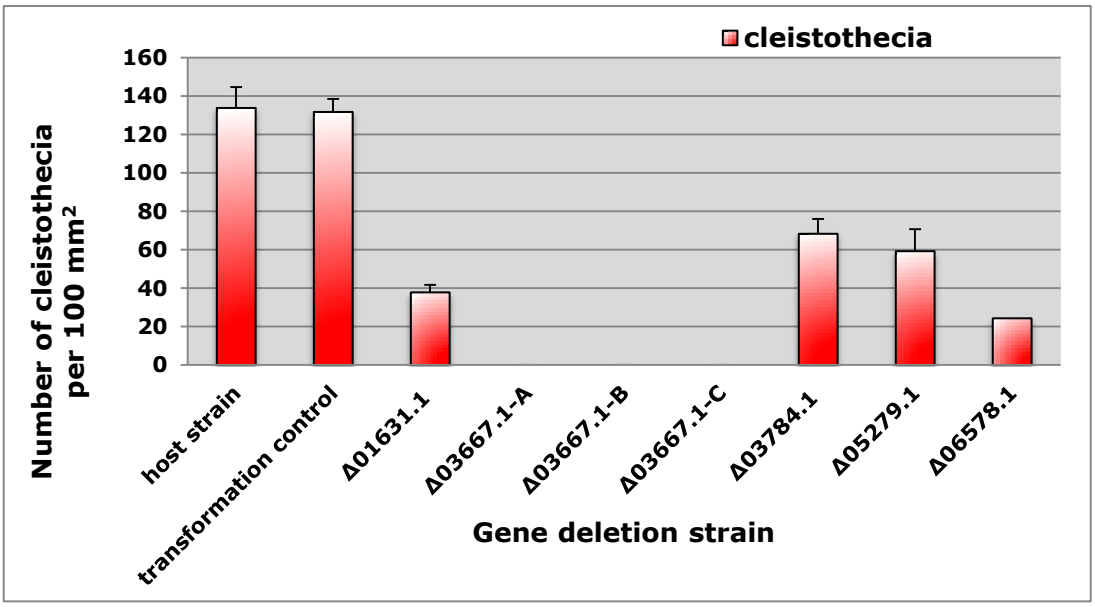

Figure 6-5 Number of cleistothecia formed by host parent and transformation control strains and other seven putative HMG gene deletion strains of *A. nidulans*. Error bars indicate ±SEM.

Table 6-3 Size of cleistothecia formed by host and transformation control strains and seven HMG gene deletion strains of *A. nidulans*. Error bars indicate ±SEM.

Strain	**Cleistothecia diameter (μm)**
Host control parent 2-258	90.2 ±(3.5)
Transformation control	90.0±(3.1)
Δ01631.1	167.5±(4.4)
Δ03667.1-A	No cleistothecia
Δ03667.1-B	No cleistothecia
Δ03667.1-C	No cleistothecia
Δ03784.1	133.0±(3.2)
Δ05279.1	225.7±(4.3)
Δ06578.1	249.2±(5.4)

Based on this aforementioned data, it was apparent that sexual development in all of the gene deletant strains was defective. Therefore, further characterisations were made of the transformants to confirm that the original target gene had been replaced during the transformation process. PCR using primers designed to amplify the target genes or the integrating DNA was performed (section **6.2.2.5; Figure 6-2**). Target genes were successfully amplified by PCR from the control strains (host parent and –DNA protoplast strains) using the primers TG1 and TG2 designed within the target gene. However, as was expected no DNA product was produced when attempts were made to amplify target genes from the putative gene deletants using the same primers (**Figure 6-6**). Finally, to confirm the integration of the replacement *pyrG* gene at the correct site, further PCR was performed using primers FP1 and FP2 and primers TP3 and TP4 (section **6.2.2.5** and **Figure 6-2**). This led to the production of amplicons of the predicted size from both mutants and controls (**Figure 6-7**).

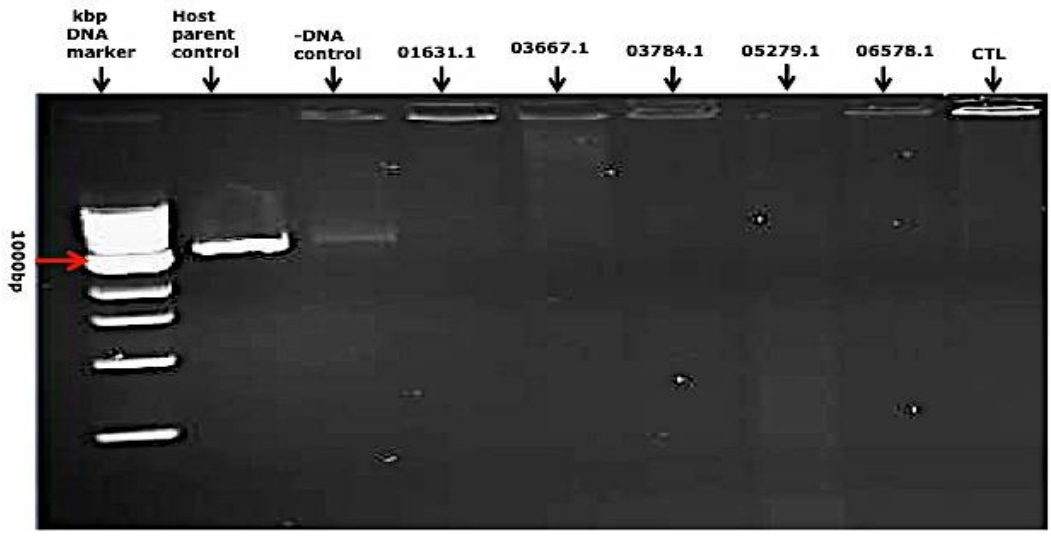

Figure 6-6 A 1.2% agarose gel showing representative results of PCR amplification using primers within the target gene (TG1 and TG2) of both *A. nidulans* controls (host parent and –DNA protoplast) and gene deletant strains (see arrows above gel from left to right). In this case amplification from the host parent and –DNA protoplasting control was for the AN03667 gene. Molecular weight marker (1 kbp), water control (CTL); arrow beside gel shows the 1000 bp band.

Figure 6-7 A 1.2% agarose gel showing representative results of PCR amplification using the primers outside the flanking regions (FP1 and FP2) of the target gene and inside the transforming DNA for gene deletion mutants (TP3 and TP4) or the target gene for controls (TG1 and TG2) (see arrows above gel from left to right). Molecular weight marker (100 bp), water control (CTL); arrow beside gel shows the 500-517 bp bands.

6.3.2 Transformant Complementation

Crosses were set up between *A. nidulans* 2-258 putative transformant gene deletants (*pyrG*+ but still *pyroA4*- wild-type green spored colour and *paba*+) and *A. nidulans* 2-259 (*paba A1*-, *yA2*=yellow spore) as described by Todd *et al.* (2007). Ascospore progeny were collected and analysed for auxotrophic markers (section **6.2.2.6**). Once outcrossing had been ascertained, strains showing restoration of the original parental genotype were tested for sexual fertility to confirm whether complementation with the original gene had restored sexual fertility.

These sexual fertility tests yielded two contrasting results. Notable success was achieved with gene complementation of the three complements of Δ03667.1 strain, (A, B and C) which had previously lost the ability to form cleistothecia (**Figure 6-4** and **Figure 6-5**). Reinserting the AN03667.1 gene by sexual crossing restored levels of

sexual fertility back to those similar to the parental and protoplast controls, indicating a fundamental involvement of the deleted AN03667.1 gene in sexual fertility of this strain (**Figure 6-8 and Figure 6-9**). By contrast, the sexual fertility levels of the other four-transformants (Δ01631.1, Δ03784.1, Δ05279.1 and Δ06578.1) could apparently not be restored back to the original control levels, and formed numbers of cleistothecia similar to the original gene deletant strains (**Figure 6-5**). However, as 'sex tests' with the original parent and protoplast controls were unfortunately not included in this series of tests with the complementation strains, it is difficult to draw firm conclusions.

In the limited time remaining studies were then focussed on the putative gene complementation strains of Δ03667.1 because these had shown the most dramatic effect. Regarding the presence of ascospores, when cleistothecia were ruptured they were found to contain a large number of red-colored ascospores. The ascospores were tested for viability by checking for germination on ACM at 28ºC in unsealed plates under light for further 3-5 d. Consequently, all ascospores were found to produce green asexual colonies similar to the control parental strain 2-258. The size of cleistothecia formed by the three complemented Δ03667.1 strain(s) was also measured (measuring the diameter of 20 cleistothecia per replicate plate, 6 plates in total) and data compared with values of the original controls. Perhaps surprisingly, the deletant strain produced cleistothecia which were significantly larger than those of the controls (**Table 6-4**). One-way ANOVA illustrated that there was a significant variation in size of cleistothecia formed [F (df) = 96.898 (2, 357); $p < 0.001$]. However, it is again cautioned that unfortunately no controls were included in this subsequent assay; thus even the parental and protoplast controls might have formed larger cleistothecia in this subsequent round of sex tests.

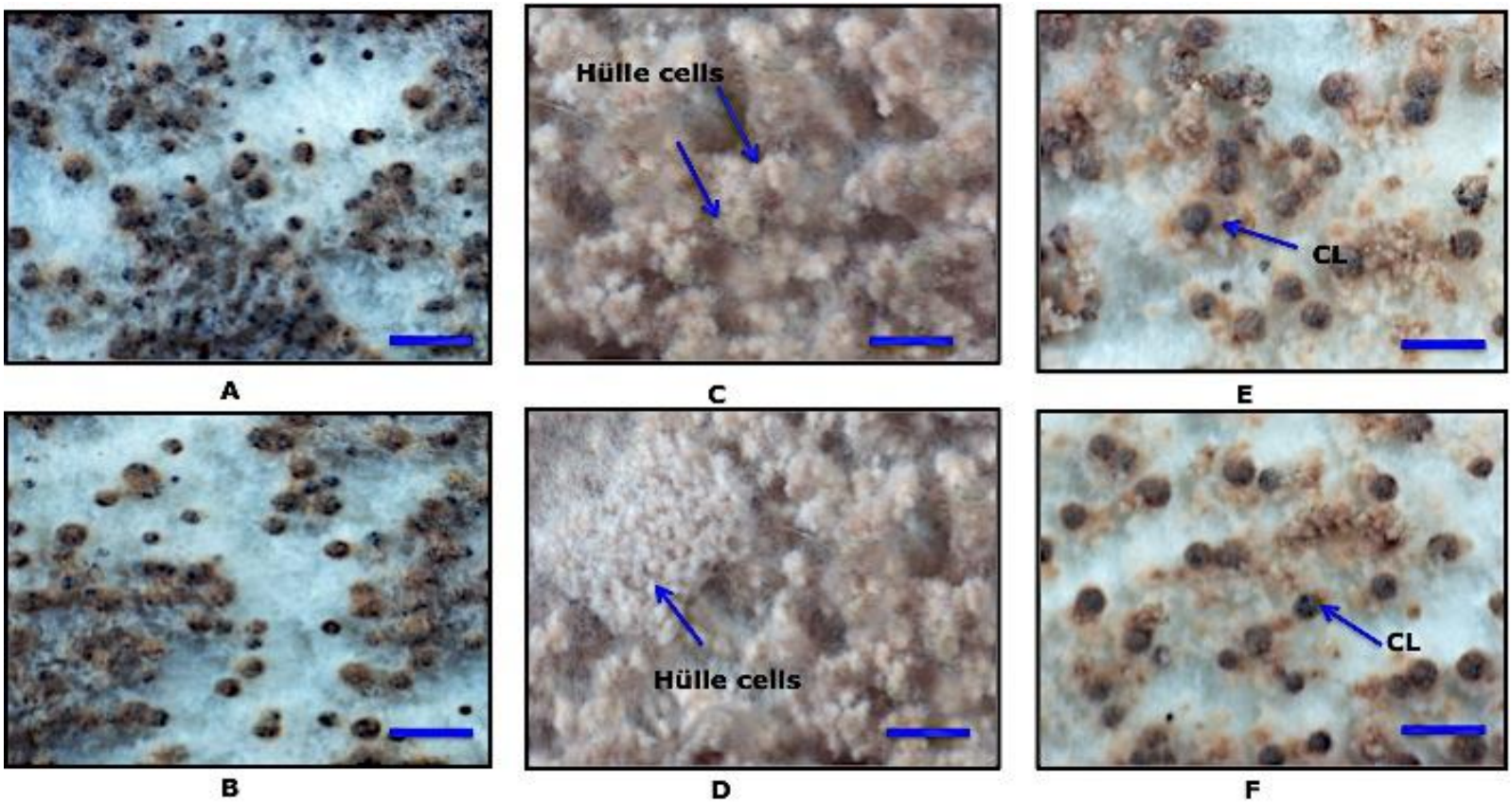

Figure 6-8 Developmental features of Δ03667.1 strain after the complementation (restoration of sexual fertility) compared with controls. All strains grown on ACM supplemented with pyridoxine + uracil/uridine in sealed plates, incubated in darkness at 32ºC for 4 weeks. (A, B) dark small-mature cleistothecia produced by host parent and transformation control, respectively; (C) yellow- golden Hülle cells and (D) aggregation of Hülle cells formed by the gene deletion strain before complementation; (E, F) dark-shelled cleistothecia covered by salmon-gold colored Hülle cells formed after complementation. Scale bars indicate 200 µm.

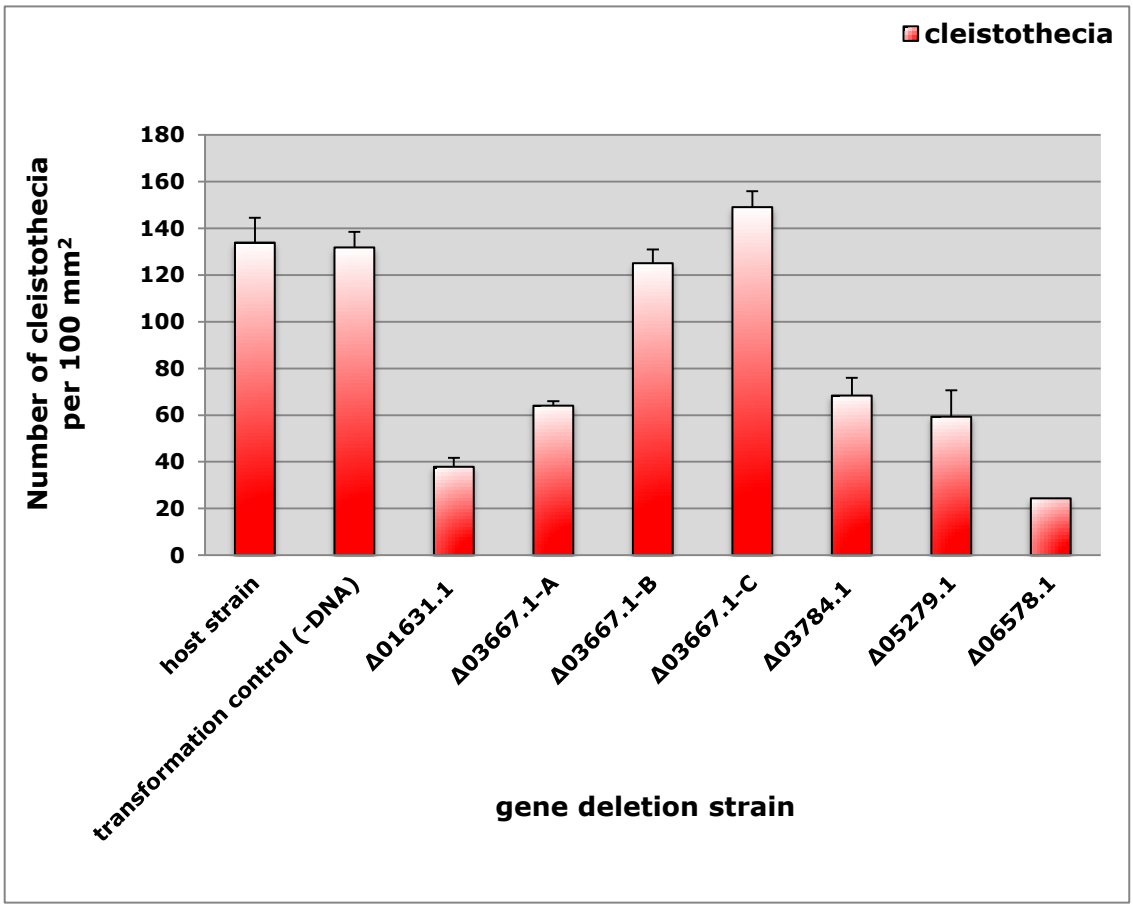

Figure 6-9 Number of cleistothecia formed by Δ03667.1 gene complemented strain (A, B and C) compared with parental and transformant control strains and other four gene deletion strains. Error bars indicate ±SEM.

Finally, successful 'restored' green strains were grown up and DNA was extracted then tested for the presence of target gene by PCR using primers TG1 and TG2 (within the target gene) from earlier (**Figure 6-2**). Accordingly, the target gene was successfully amplified by PCR showing a clear product in both controls (host parent and transformation strains) and also the complemented Δ03667.1 strain (**Figure 6-10**). The presence of target gene in this mutant was used as further confirmation and evidence for the success of the complementation. Unfortunately, restriction of time meant that similar PCR tests were not performed for the other gene complementation strains of Δ01631.1, Δ03784.1, Δ05279.1 and Δ06578.1.

Table 6-4 Size of cleistothecia formed by Δ03667.1 complementation strain compared to those of the parental and transformation protoplast control strains. Values are ±SEM.

Strain	Cleistothecia diameter (µm)
Host control parent 2-258	90.2 ±(3.5)
Transformation control	90.0±(3.1)
Δ03667.1-A	64±(4.8)
Δ03667.1-B	142.5±(2.3)
Δ03667.1-C	149±(3.0)

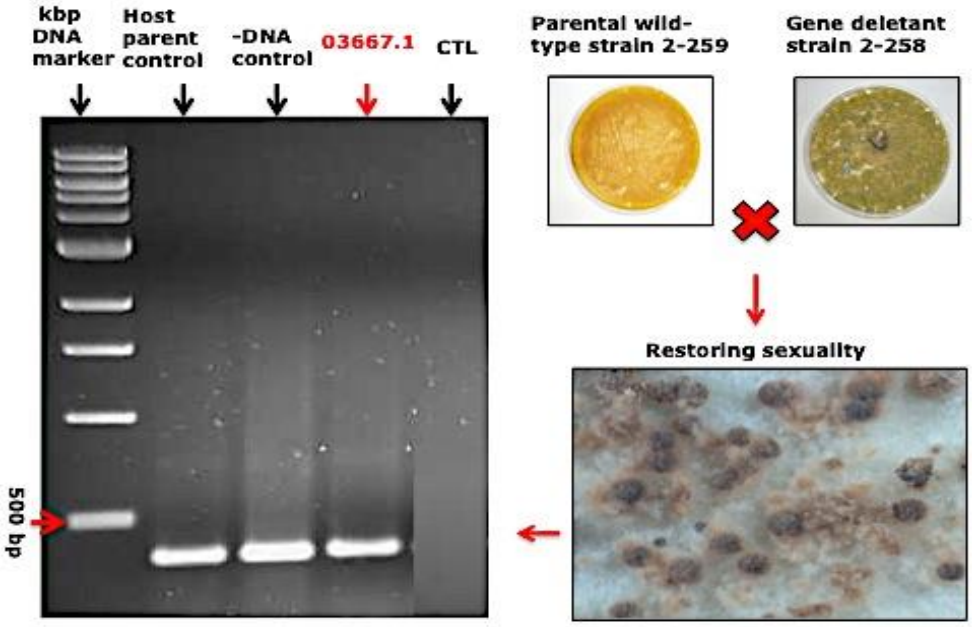

Figure 6-10 A 1.2% agarose gel shows representative results of PCR amplification using the primers TG1 and TG2 within the target gene of both *A. nidulans* controls and complemented strain Δ03667.1 (arrows above from left to right) compared with molecular weight marker (1 kbp), water control (CTL), arrow beside gel shows 500 bp band.

6.4 Discussion

Aspergillus nidulans has long been used as a model filamentous fungus for studies of cell biology and gene regulation (Todd *et al.,* 2007). One particular area of study with the fungus has concerned the genetics of sexual development. During sexual reproduction cleistothecia are formed which are encased in special cells termed 'Hülle' cells. These cells have a unique structure and indicate the commencement of sexual reproduction in *A. nidulans* (Wang *et al.*, 2010). Cleistothecia host many asci, each ascus containing 8 haploid ascospores formed as a result of meiotic division (Sohn and Yoon, 2002; Dyer, 2007). Over 200 genes with a possible role in sexual development have been identified from *A. nidulans* (Galagan *et al.,* 2005) and over 70 of these 'sex-related' genes have been experimentally characterized and shown to be correlated with various stages of the sexual cycle (Dyer, 2007).

Although many genes have already been identified as being involved with the sexual development of *A. nidulans*, it is important to continue to attempt to identify novel gene(s) involved with sex, as such genes might have been mutated during the evolution of asexuality, and thus provide a functional explanation for asexuality. Therefore, the purpose of work in the present chapter was to attempt to discover a novel gene(s) participating in sexual fertility of the model species *A. nidulans* (teleomorph *Emericella nidulans*). Work was facilitated by the availability of a series of gene disruption cassettes from an American genomics project. Studies focussed on five putative high-mobility group (HMG) family genes. This was because HMG proteins have been shown to be involved with sexual development in many higher eukaryotes, including of especial note the fungal *MAT1-2* family proteins (Martin *et al.*, 2010; Debuchy *et al.*, 2010) and the *A. nidulans* genome was found to contain at least five putative HMG genes listed as function unknown. Experimental work involved amplification of the knock-out cassettes, integration by PEG-mediated protoplast transformation, screening of transformants for sexual fertility, and final gene complementation by

sexual crossing, together with molecular checks by PCR at various stages to confirm correct integration.

A major finding was that sexual fertility was reduced dramatically as a result of deleting for all of the five putative HMG genes AN01631.1, AN03784.1, AN05279.1, AN06578.1 and AN03667.1. Deletion strains of the first four genes still continued to produce fertile cleistothecia, but these were significantly lower in abundance and larger in size than the parental and protoplast control strains and showed a high amount of yellow-gold colored Hülle cells under sexual conditions (**Figure 6-4**; not quantified). By contrast, deletion of the AN03667.1 gene produced an even more extreme phenotype, with the three ∆03667.1 transformants strains studied being completely sterile (in terms of lack of production of cleistothecia and ascospores), and instead showing prolific production of Hülle cells (**Figure 6-4**; not quantified). These observations show some similarities but also differences with other sexual development gene deletion mutants of *A. nidulans*. For example, deletion of the *MAT1* (alpha domain) and MAT2 (HMG domain) mating-type genes of *A. nidulans* similarly gave rise to mutants producing significantly lower numbers of cleistothecia than control strains, and the occasional proliferation of Hülle cells to form ornate macroscopic structures in the ∆MAT2 strain (Paoletti *et al.,* 2007), the latter in accordance with the ∆03667.1 deletion strain. However, the cleistothecia were smaller than the controls, unlike those in the present study, and were sterile with no ascospore formation (Paoletti et al., 2007). Wang *et al*. (2010) found that the ∆strA strain of *A. nidulans* formed smaller cleistothecia with no ascospores compared with the wild type. On the other hand, the ∆imeB mutant produced higher numbers of fertile cleistothecia than controls (Bayram *et al.,* 2009). Meanwhile, other *A. nidulans* sexual developmental mutants have been shown to produce fruiting bodies but with no survival of ascospores due to deletion of genes such as samB (Krüger and Fischer, 1998) and grrA (Krappmann *et al.,* 2006). Many sexual developmental mutants have also been described in model filamentous ascomycete species such as *Neurospora crassa*, *Sordaria*

macrospora and *Podospora anserina* (Debuchy *et al.,* 2010); (see section **6-1**). But it is noted that Maerz *et al.* (2009) observed that much lower numbers of small and less developed protoperithecia were produced by ΔNcmob3 (phocein homolog) strains of *N. crassa* compared with the controls, and a variety of protoperithical developmental mutants of *S. macrospora* have been identified with various defects in the transition from protoperithecia to perithecia (Pöggeler and Kück, 2004).

Complementation of the putative gene deletant transformants of *A. nidulans* was then attempted by crossing between the 2-258-transformant gene deletant strains (*PyrG+, PyroA4-,* green spored colour and *paba*+, *veA*+) with isolate 2-259 (*paba A1-, yA2*=yellow spore, *veA*+). This allowed the restoration of sexual fertility to the three transformant strains of the Δ03667.1 strain, which produced characteristic cleistothecia coated in typical salmon-gold colored Hülle cells and that contained viable red-coloured ascospores. These observations are in line with the previous literature in which the complementation of the sexual developmental genes was attempted. *MAT*-gene deletants of *A. nidulans* could be restored to sexual fertility by crossing Δ*MAT1* and Δ*MAT2* strains together (Paoletti *et al.*, 2007), with similar observations made from outcrossing of *Gibberella zeae MAT* mutants (Lee *et al.*, 2003). Likewise, crossing between the grrAD strain AGB259 and the csnDD mutant AGB195 resulted in production of cleistothecia by a heterokaryic mycelium of *A. nidulans* (Krappmann *et al.*, 2006). Furthermore, sexual fertility (cleistothecia and ascospores) was complemented in a ΔstrA background by expression of strA from its endogenous promoter and by over-expression of strA using the OEstrA construct. The OEstrA triggered the development of both cleistothecium and Hülle cells respectively in shacking media with 0.6 M KCl (Wang *et al.*, 2010). Parallel research demonstrated that the overexpressing of both fundamental sexual inducers nsdD and veA enhanced the production of cleistothecia and Hülle cells in shaking liquid culture (Han *et al.*, 2001; Kim *et al.*, 2002b). Additionally, Bayram *et al.* (2009) illustrated that imeBΔ and over-expression of *imeB* (the protein kinase ImeB)

significantly promoted the production of cleistothecia and reduced the production of conidia in light and in dark.

However, there were some anomalies in the findings of the present chapter. Surprisingly, cleistothecia formed by two of the three complements (B and C) of Δ03667.1 strain were significantly larger than the parental and protoplast control strains. It is unclear why this was the case. It might have been a genuine effect, but this was alternatively perhaps due to the fact that the sex assays with the complemented strain were performed at a different time from the original controls so there might have been other incubation factors influencing cleistothecial development. Meanwhile, complementation with the other four genes (AN01631.1, AN03784.1, AN05279.1, AN06578.1) failed to restore the sexual fertility of the mutants to those of the parental and protoplast control strains. This was also difficult to explain. But it is possible again that the sex assays were performed at a different time from the original controls so there might have been other incubation factors influencing cleistothecial development resulting in a lower number of cleistothecia. Ideally a second round of sex assays for the parent and protoplast control should have been included in this subsequent round of sex assays. Alternatively, the gene complementation by outcrossing for the latter four genes might not have been successful despite the selection of apparent parental phenotypes. Unfortunately there was insufficient time to perform the PCR checks to confirm reintegration of the target gene for these four strains, this work only being conducted with the 'successful' complemented Δ03667.1 strain.

6.4.1 Conclusions

The discovery of a novel gene regulating sexual development in *A. nidulans* was a considerably important finding in the present study. This gene is currently simply termed 'ANID_03667' on genome databases arising from the genome annotation of *A. nidulans* (Galagan *et al.,* 2005) and is listed as an uncharacterized gene which has a domain(s) with

predicted DNA binding activity (**Table 6-1**). However, this gene has now been verified to be involved with the regulation of sexual development because gene deletion resulted in a sterile phenotype lacking the production of cleistothecia or ascospores. It is therefore recommended that this gene be given a new name '*steD*', in accordance with a sequence of previous sterility genes (*steA, steB* and *steC*) identified from *A. nidulans*, disruption of which leads to sterility in all cases, as described by Dyer and O'Gorman (2011) and listed at http://www.fgsc.net/Aspergillus/gene_list/locis.html. The fact that complementation (by outcrossing) restored the sexual fertility in the Δ*steD* mutant confirmed the major role of *steD* as a sexual regulator.

It was especially interesting to note that *steD* encodes a putative HMG family protein. Elsewhere HMG proteins have been shown to have major roles in determining sexual identity in higher eukaryotes and are widespread throughout the fungal kingdom (Martin *et al.,* 2010). Very recently a series of 12 HMG proteins were identified from *P. anserina,* and 11 out of 12 shown to form a an interacting network regulating sexual development (Benkhali *et al.*, 2013). Results from the present study indicate that a similar network might be present in *A. nidulans* and more investigations remain to be undertaken to identify the significance of other HMG genes in sexual development and how they might interact with *steD.*

Chapter 7 General discussion

The overall aim of the current project was to investigate physiological and genetic factors that influence the evolution and control of sexual reproduction in *Aspergillus* species, in order to better understand the processes governing sexual and asexual reproduction in ascomycete fungi as a whole. It was hoped that results gained would give insights into the evolution of sex and asexuality in ascomycete fungi. This would provide both fundamental and applied scientific knowledge, of use for instance in further studies to induce sexuality in supposed 'asexual' species.

Studies focussed on the genus *Aspergillus*, which includes species that are of significant importance in economical, agricultural, medical and industrial applications (Albers-Schonberg *et al.*, 1980; Eaton *et al.*, 1994; Alberts, 1998; Latgé, 1999; Calderone *et al.*, 2002; Magnuson and Lasure, 2004; Bentley and Bennett, 2007). Three homothallic (self fertile) species *Aspergillus nidulans, Neosartorya fischeri* and *Eurotium repens* were chosen as model species for the present study. The first two species have publically accessible genome sequence data and much molecular information is available from studies of cell biology, gene recombination, and manipulation and gene regulation in *A. nidulans* (Todd *et al.*, 2007). These species were also selected as they have multiple other benefits such as they grow rapidly on different media under laboratory conditions, and they all have a homothallic breeding system, meaning that both sexual and asexual reproduction can be induced relatively easily, which is helpful for studies of the evolution of asexuality. And overall it can be argued that these proved suitable model organisms for study, because insights were gained into various aspects of the evolution of sex and asexuality as follows, although there were some caveats as will also be discussed below.

7.1 Identification of conditions to reliably induce asexual and sexual development

In order to ensure progress of future studies, it was first necessary to establish a culture collection of isolates/strains and devise protocols for the reliable induction of the asexual and sexual cycle in the three species *A. nidulans, E. repens* and *N. fischeri.* Where possible recently isolated fungi were used to avoid any artefacts that might have occurred due to prolonged laboratory subculture [such as the *veA1* mutation present in most laboratory strains of *A. nidulans,* which has been propagated after initial mutation and selection in laboratory culture (Dyer, 2007)]. It was possible to obtain field isolates of *A. nidulans, E. repens* and *N. fischeri* which were thought to have only been subcultured perhaps 5-6 times, but the strains of *A. nidulans*, which were needed for their genetic markers, had been subject to numerous laboratory subcultures. It was interesting to note that only 4 of 19 putative specimens of *N. fischeri* were actually proven to be *N. fischeri* by DNA phylogenetic analysis. This suggested that there is unexpectedly high global diversity of *Neosartorya* species.

Experiments set up using several media and temperatures were able to identify suitable conditions for inducing asexual and sexual reproduction fairly reliably for all three species, as described in **chapter 3**. This helped to confirm that certain *Aspergillus* species are very amenable to laboratory studies. Some results were particularly noteworthy. Firstly, the ability to of *N. fischeri* to produce asexual spores at relatively high temperatures between 28ºC-37ºC might be because this species is thermophilic, preferring slightly higher temperatures to grow perhaps relating to a natural ecological niche. Secondly, the best conditions to cultivate and induce asexual and sexual reproduction in *E. repens* were, on the sugar rich M40Y media, which again is consistent with a natural ecological niche on high sugar substrates (Raper and Fennell, 1965; Kozakiewicz, 1995). *E. repens* was found to prefer slightly lower temperatures than *N. fischeri*, such as 25ºC and 28ºC, consistent with this being a mesophilic species. Finally, some surprising insights were

made regarding the optimisation of sexual development in *A. nidulans.* The sexual cycle has traditionally been induced at 37ºC since the original work of Pontecorvo (1953) and this temperature is used as standard in labs worldwide (Todd *et al.,* 2007). However, in this study was found that greater levels of sexual development were possible at the lower temperature of 32ºC, confirming earlier undergraduate project student work (P. Dyer, personal communication). Thus, incubation at 32ºC might become the new standard for *A. nidulans.* In addition, it was found that the method in which plates were stored in the incubator had a significant impact on sexual development. It was found that plates that were kept as a single layer depth underwent higher levels of sex more reliably than those stored in stacks, suggesting different requirements of oxygen or possibly drying of top plates first may affect physically the amount of sex in bottom plates. This again might become a new standard procedure for studies of sex in *A. nidulans* where it is critical to accurately assess numbers of cleistothecia. However, the effect of the time of sealing of plates was more variable and strain/isolate dependent, though on the whole sexual fertility was generally prompted when plates were sealed at 15-18 hr after inoculation.

7.2 Different survival of spores (conidia vs. ascospores)

Homothallic (self fertile) species such as *A. nidulans, N. fischeri* and *E. repens* can reproduce by asexual or sexual means producing conidiospores or ascospores, respectively. Sexual reproduction requires more time and has additional costs compared with asexual development (Bell, 1982; Otto, 2009). Given that there is rarely any increased genetic variation in ascospores progeny of homothallic species (through rare outcrossing events), it is apparently surprising that sex is maintained in homothallic species. It has been suggested that even in homothallics there might be some benefits to sex such as selection of 'fit' nuclei in a selection arena and repair of DNA by recombination (Kondrashov, 1988). One extra benefit might be that sexual ascospores have increased resistance to environmental stresses than asexual conidia. It is well known that some species such as *N. fischeri* have highly heat resistant

ascospores (Mcevoy and Stuart, 1970), but at the onset of studies it was widely assumed for species such as *A. nidulans* that conidia and ascospores exhibit similar resistance against the adverse environmental conditions (Braus *et al.* 2002). However, a very careful comparison of the survival of conidia and ascospores in the present study clearly showed that even for species such as *A. nidulans* and *E. repens* there was also differential survival of the different spores types. For both species ascospores in general had an elevated resistance to heat shock, although levels of resistance were not as high as those seen in *N. fischeri* and other species with highly temperature resistance ascospores (Splittstoesser *et al.*, 1970; Splittstoesser and Splittstoesser, 1977; Scott and Bernard, 1987; Scholte *et al.*, 2004; Dijksterhuis and Samson, 2006). Meanwhile studies with *A. nidulans* showed that ascospores exhibited considerably higher levels of resistance to UV exposure than conidia. When data were converted to *D*-values, conidia had consistently lower *D*-values compared with ascospores of the same isolate/strain. The increased levels of environmental resistance of ascospores over conidia were suggested to be due to various possible factors such as the thickness of cell wall, a binuclear state, a high amount of trehalose and mannitol and the water activity effect.

Overall these observations were highly important as they help answer the question why homothallic sex is retained in species which can otherwise reproduce asexually to form a greater number of spores with less metabolic costs and in a shorter time. These results, using *A. nidulans* and *E. repens* as model species, indicate that the production of ascospores could be of broader evolutionary and ecological benefit as they would allow increased survival of harsh environmental conditions. This also has industrial implications for food and drinks production and packaging as it might be the case that contaminating ascospores might unexpectedly survive sterilisation procedures designed to kill fungal hyphae and conidia. Finally, an extra finding of practical use in the laboratory was that heating ascospores of *A. nidulans* within entire cleistothecia at 68ºC for 30 min provided a method to obtain much purer ascospore suspensions than the traditional agar rolling system (Todd *et*

al. 2007). This again might become a standard procedure for sexual studies in *A. nidulans.*

7.3 Loss of sexual fertility of *A. nidulans*: the "slow decline"

A key question at the onset of studies was how do asexual species arise from sexual ancestors? There are many possible routes (see section **5.1.2**) but one possibility is that sexual reproduction may be lost in some species by a 'slow decline' in fertility rather than an abrupt change (Dyer and Paoletti 2005). There is previous published work to support this hypothesis, showing that sexual fertility was lost when species were subcultured for period of time by asexual spores (Jinks, 1954; Mather and Jinks 1958). However, the standard of the latter work could be criticised on various grounds and experimental detail required in modern science was lacking. Therefore, studies were undertaken to test whether the work of Jinks (1954) and Mather and Jinks (1958) could be repeated, and the work extended to add extra experimental detail. Three different aspects were considered. Firstly, it was confirmed that a 'slow decline' in sexual fertility could be shown in *A. nidulans, N. fischeri* and *E. repens* as a result of long-term subculture by asexual spores. There was a *ca.* 50-90% decrease depending on the isolate and species in question. Secondly, the number of vegetative cultural transfers was correlated with the number of mitotic divisions by using Hoechst 33258 and Calcofluor solutions to stain the hyphae and nuclei in apical tips following the logic of Schoustra *et al.* (2007) and Schoustra *et al.* (2010). This revealed that sexual fertility in *A. nidulans, N. fischeri* and *E. repens* gradually decreased following asexual sub-culturing over around 10,000–30,000 mitotic divisions (corresponding to 20 serial asexual transfers), the first time that this process has been quantified in such a way. Thirdly, it was shown that sexual fertility could be partially or fully restored to a sexual 'default' state using transfer by a single sexual ascospore as was partly observed by Jinks (1954) and Mather and Jinks (1958). These results overall are therefore highly significant in confirming that asexuality might evolve by a gradual 'slow decline' in sexual fertility and that such a change might occur in a relatively short period of time. The fact that sex

could be partly or fully restored by ascospore transfer is consistent with both mutational and epigenetic effects.

It is also noted that different levels of sexual fertility were seen in the three target species *A. nidulans, E. repens* and *N. fischeri* even at the offset of these fertility studies. It had observed at the start of PhD studies that newly obtained isolates of *N. fischeri* were very sexually prolific, and that even after only 1 or 2 subcultures (before the start of these experiments) that levels of fertility had dropped very significantly. So such 'slow decline' experiments with freshly obtained isolates of *N. fischeri* might have shown an even greater proportionate reduction in fertility. It is further observed that despite the best efforts to implement the 'sex assays' that there was some 'noise' in the data rather than a smooth progression, possibly indicating the stochastic nature of sex given that so many genes are likely to be involved.

7.4 Discovery of a novel gene regulating sexual fertility in *Aspergillus nidulans*

Aspergilus nidulans has long been used as a classical genetic model, and one way in which used is to investigate the molecular background of reproductive modes in fungi (Lee *et al.*, 2010). It has been suggested that at least 400 genes are involved in sexual fertility ((Dyer *et al.*, 1992), and over 200 putative genes involved with sex have been identified and over 70 of these characterised experimentally (Dyer and O'Gorman, 2012). Therefore, it is likely that many other genes that have a role in regulating sex still remain uncharacterised and their functionally unknown. It is possible that changes in such genes could be linked to the evolution of asexuality. Thus, work was undertaken to explore and characterise a new gene(s) that may have a principle role in sexual development of *A. nidulans*. Use was made of disruption (knock-out) cassettes available from an American source. And excitingly, gene deletion of five putative high-mobility group (HMG) genes led either to a significant decrease in fertility or total loss of sexual fertility. Studies focussed on the AN03667.1 gene, in which the gene deletion resulted in a

total loss of cleistothecial development, whilst re-insertion of the gene back by outcrossing (i.e. complementation) resulted in restoration of levels of sexual fertility. According to these observations, the deleted putative HMG gene was therefore shown to be functional, having a role in regulating sexual fertility of *A. nidulans*. The gene finally was given a new name '*steD*' following the list of sterility mutant names of *A. nidulans* gene loci. This finding was of great significance as it now adds an important extra gene in the suite of sexual genes known in *A. nidulans* (**Figure 7-1**), likely to interact with the other HMG genes based on data of Benkhali *et al.* (2013), which might be screened for and/or genetically manipulated in putative asexual species. The relative ease with which this gene was identified suggests that more 'sex-related' genes remain to be described.

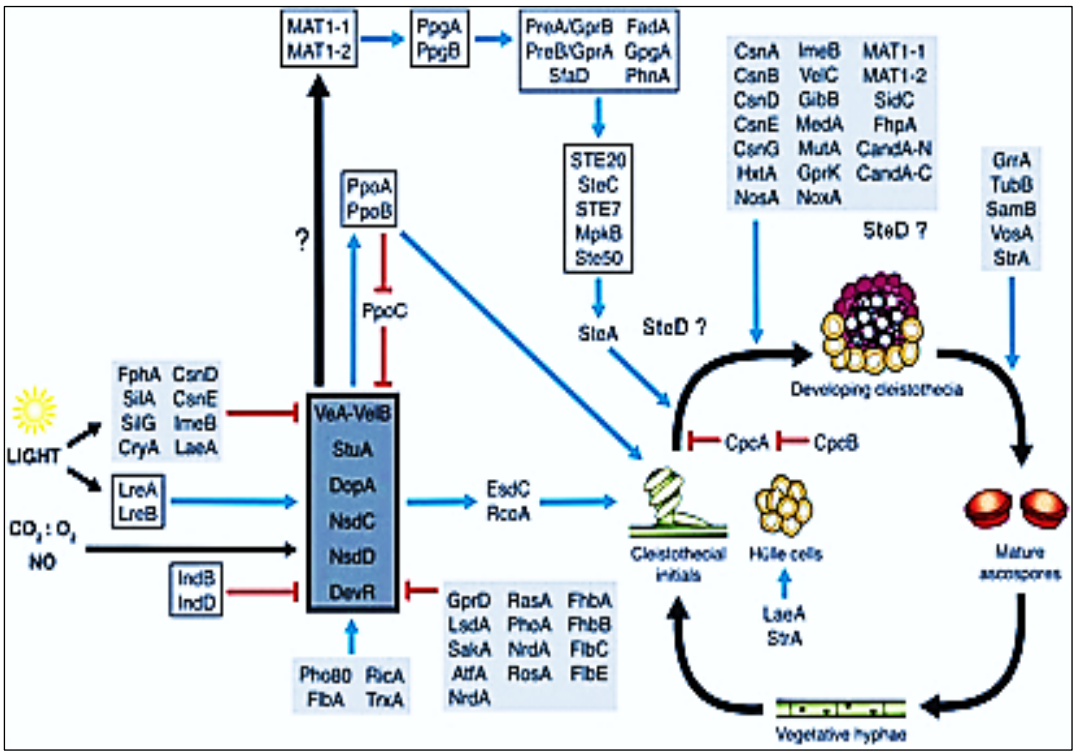

Figure 7-1 Suggested placement of *SteD* gene relative with other sex related genes in Aspergilli.

7.5 Future work

Despite the valuable insights obtained in this project into the control and evolution of sexual fertility in the three homothallic species *A. nidulans*,

N. fischeri and *E. repens*, many further studies have to be undertaken to better understand the process of sexual development and fertility in these species. Furthermore studies need to be broadened to include other homothallic and other heterothallic aspergilli, and other taxa. Therefore various forthcoming investigations are recommended as listed below.

1. Although the sexual fertility of all strains of homothallic species investigated generally declined as result of repeatedly asexual sub-culturing for prolonged confirming the classical finding by (Mather and Jinks, 1958), no accurate mechanistic reason (s) for the 'slow decline' of sex in these species has yet been determined. Therefore the molecular-genetic basis of this decrease in sexual fertility should be investigated, which might show a correlation(s) with a mutational and/or epigenetic changes i.e. methylation of nuclear DNA and/or associated histone proteins. Such work could involve next generation sequencing of strains before, during and after prolonged asexual transfer. Also it would be intriguing to determine whether the loss of fertility occurs in strains that have had the methylation genes knocked out, which would decrease epigenetic effects. Cultures are already available at different transfer stages from the present study to see if any correlation between expression of key sexual regulator genes and fertility can be observed. Indeed, enquiries were made to see if the cultures of Jinks (1954) and Mather and Jinks (1958) were still available, but these were long since lost at Birmingham (Jim Croft, personal communication).
2. Preliminary work has shown that sexual reproduction in fungi is correlated with changes in lipid metabolism. Addition of the fatty acid 'linoleic acid' has been shown to promote sexual reproduction in certain species such as *Neurospora crassa* and *Nectria haematococca* (Calvo *et al.*, 1999; Brown *et al.*, 2008). But how widespread is this phenomenon? So it is worthwhile to investigate whether this effect is seen more broadly in ascomycete fungi. This needs to be analysed in a wide range of filamentous ascomycete species (both homothallic and heterothallic). Target species could

include other *Aspergillus* species, *Sordaria* species and *Gibberella* species. Then methods of Dyer *et al.* (1993) to treat with linoleic acid and other fatty acids should be followed to assess the impact on sexual reproduction. Results will give insights into the general biochemical basis of sexual reproduction.

3. It has been detected in the current study that the ascospores of both homothallic species *A. nidulans* and *E. repens* have slightly greater levels of resistance to heat and UV shock than conidia. So, parallel work could be undertaken to study the effect of different treatments such as pH, sorbic acid MIC, cold and freezing on spore viability (conidia vs. ascospores), which might provide further reasons why sexual sporulation is maintained in homothallic species which can reproduce asexually. And it would then be of interest to determine the underlying physiological reasons for any differential resistance, e.g. determination of different trehalose levels, different cell wall components, confirmation of the binuclear status of ascospores, and role of different spore pigments.
4. The novel 'steD' gene warrants further investigation. In particular, phylogenetic analysis is needed to determine its exact relationship to other HMG family proteins, overexpression work might provide intriguing effects as regards promotion of sex in asexuals, and work is needed to determine the localisation of the protein within the cell, and whether it a true nuclear transcription factor.
5. Consistent with the discovery of the novel 'steD gene' is the likelihood that many further genes with a similar important role in sexual function remain to be characterised. Indeed the other four putative HMG genes studied require further attention to assess whether they do indeed influence levels of sexual fertility. Thus, this area will remain one of ongoing interest, warranting further study.

Conference presentation from this study

Ashour, A.O., Dyer, P.S and Archer, D.B. (2012). Benefits of sex and ascospore production in *Aspergillus nidulans*. Asperfest 9, The 9th International *Aspergillus* Meeting. Marburg, Germany, March 29 – 30, 2012.

Ashour, A.O., Dyer, P.S and Archer, D.B. (2013). Homothallic sex and the advantages of ascospores formation in *Aspergillus nidulans*. XI International Fungal Biology Conference. Karlsruhe, Germany, September 29 - October 3, 2013.

Manuscripts in preparation

Ashour, A.O. and Dyer, P.S (2013). Sex in a model species - benefits of ascospore production in *Aspergillus nidulans* (to be submitted to Fungal Biology).

Ashour, A.O. and Dyer, P.S (2013). Loss of sexual fertility due to a slow decline process in *Aspergillus* species (journal to be decided).

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Appendices

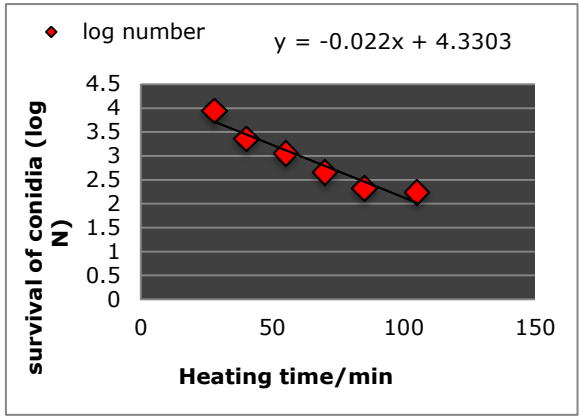
Appendix 1 List of primers used to amplify different genes in the present study.

Primer	Gene	Sequence
Beta-tubulin 2a	beta-tubulin	5¹ GGT AAC CAA ATC GGT GCT GCT TTC 3¹
Beta-tubulin 2b	beta-tubulin	5¹ ACC CTC AGT GTA GTG ACC CTT GGC 3¹
Designed forward	veA	5¹ ACAATTGCGACCTTAGCCAG 3¹
Designed reverse	veA	5¹ TGTCGGTCTTATTGCTGTCG3¹
TG1/ANID_01631.1	Within target gene	5'- TCACTGCTGCGTTCCTGATTCAC -3'
TG2/ANID_01631.1	Within target gene	5'- TGTTAGGGTGGAGGCGACTTACTTG -3'
TG1/ANID_03667.1	Within target gene	5'- GCGATTAGCAGTGATTGAGAAGAGG -3
TG2/ANID_03667.1	Within target gene	5'- CGGGTTGGTTGAGCCAGTTATC -3'
TG1/ANID_03784.1	Within target gene	5'- GCAGTCCTAACGCACGCTTCATT -3'

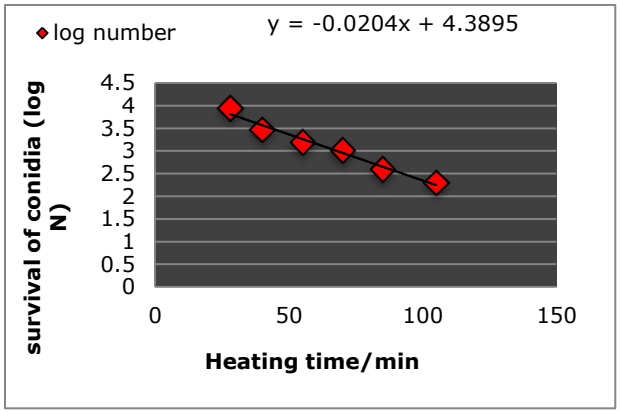
TG2/ANID_03784.1	Within target gene	5'- GGGGATTTGGCTTTTCTATGTGAGG -3'
TG1/ANID_05279.1	Within target gene	5'- CGCAAATGCCAGAAAAAGGG -3'
TG2/ANID_05279.1	Within target gene	5'- AATGGTCTCGCCGACTAACAATAC -3'
TG1/ANID_06578.1	Within target gene	5'- TCGCTGGTGGATTCGTATGG -3'
TG2/ANID_06578.1	Within target gene	5'- CGTTTATCGGGTTCGTCAGAGG -3'
FP1/ANID_01631.1	Outside flanking region	5'- ATTTACGGGGGCTCTCATAC -3'
FP2/ANID_01631.1	Outside flanking region	5'- TCTGCTTCGCTTCCTATG -3'
FP1/ANID_03667.1	Outside flanking region	5'- TTGTAGCCAGATTAGCGTGTC -3'
FP2/ANID_03667.1	Outside flanking region	5'- TCGTGTTCAAAGCGTGTAGTC -3'
FP1/ANID_03784.1	Outside flanking region	5'- GAAATGTCGTTCACCTTAGCC -3'
FP2/ANID_03784.1	Outside flanking region	5'- CTTACCACACCTCCTGAAACC -3'
FP1/ANID_05279.1	Outside flanking region	5'- TAAGTGACGGGTGGGTGTAG -3'
FP2/ANID_05279.1	Outside flanking region	5'- CGTCTCCACCAAAAGATGTG -3'

FP1/ANID_06578.1	Outside flanking region	5'- GGACCTGTCATTTCGCTACTTC -3'
FP2/ANID_06578.1	Outside flanking region	5'- CACCACGCCCTTACTGATTAC -3'
TP4	PyrG transforming	5'- GCTCGCAAATACAAGAACTTCG -3'
TP4	PyrG transforming	5'- ACGGGTGTGAACCTCTCTTC -3'
TP3 (reverse)	PyrG transforming	5'- TTTGCCAGAGGATTGGGGTG -3'
TP3 (reverse)	PyrG transforming	5'- ATCAGCAGAGACGGTAACG -3'
R151	RAPD-PCR	GCTGTAGTGT
UBC90	RAPD-PCR	GGGGGTTAGG
RCO8	RAPD-PCR	GGATGTCGAA
OPAX16	RAPD-PCR	GTCTGTGCGG

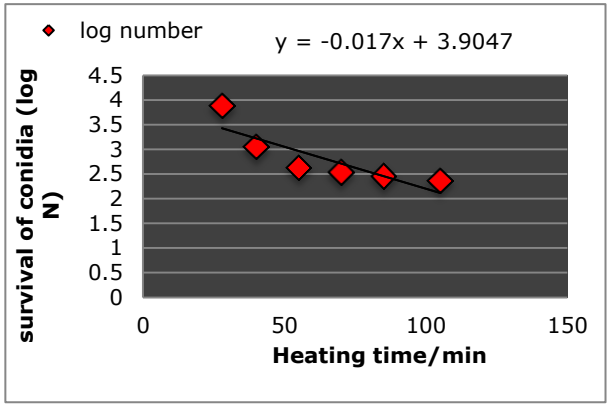
Appendix 2 *D-values* survival curves (log number) of conidia of *A. nidulans* heated at 55 °C for time period 40, 55, 70, 85 and 105 min.



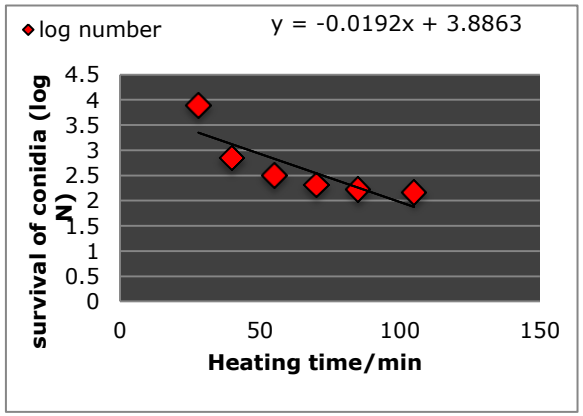
2-37



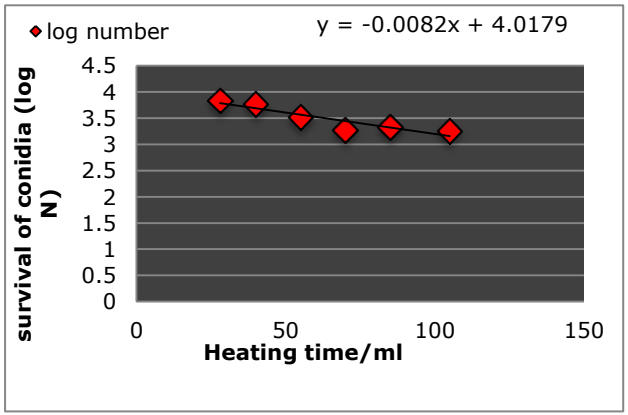
2-139



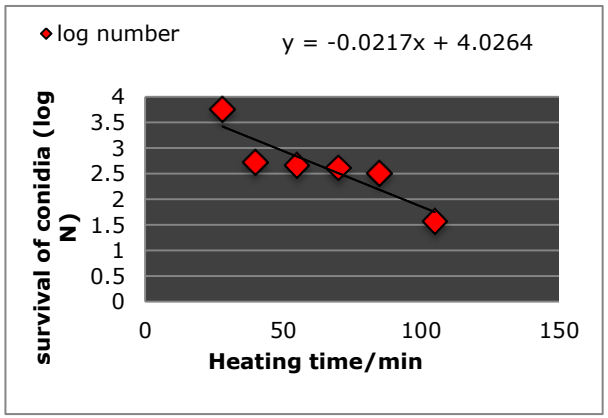
2-149



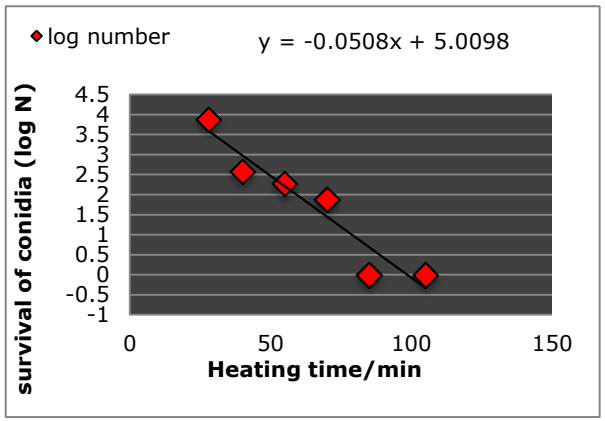
2-154



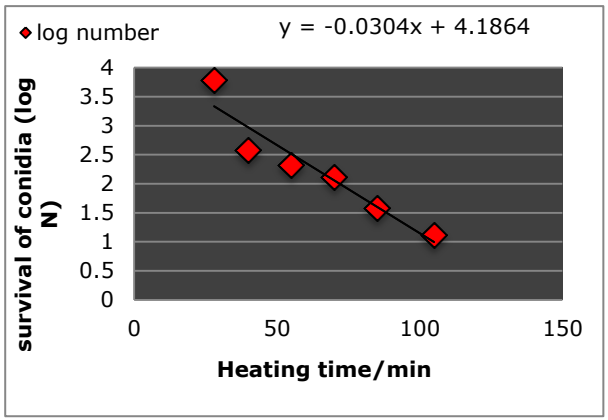
2-155



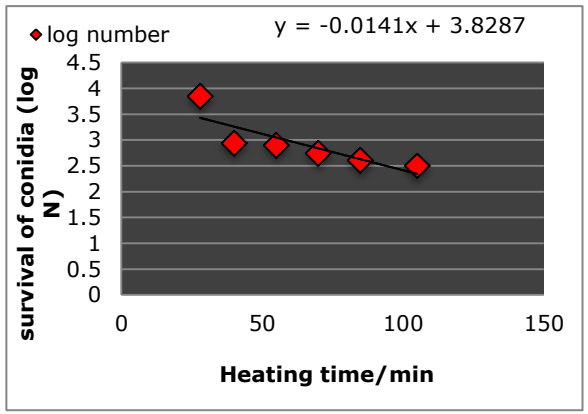
2-224



2-227

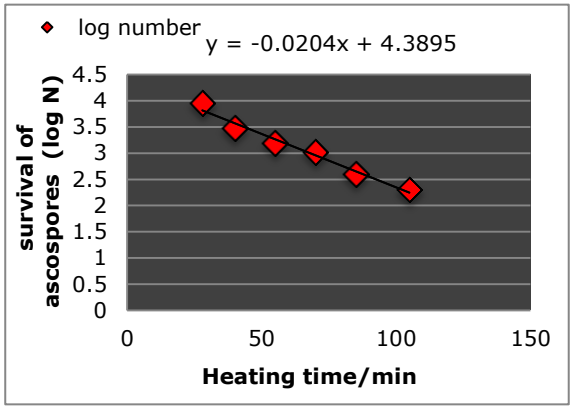


2-232

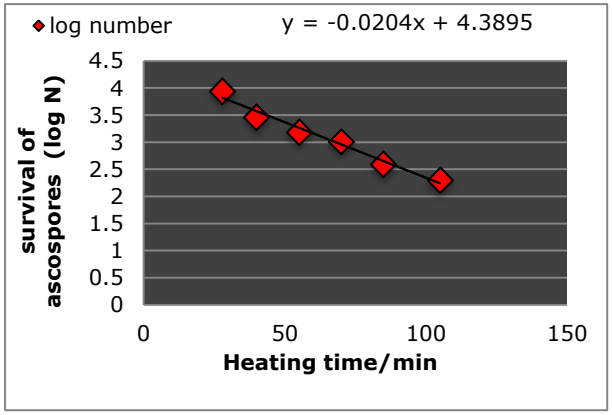


2-250

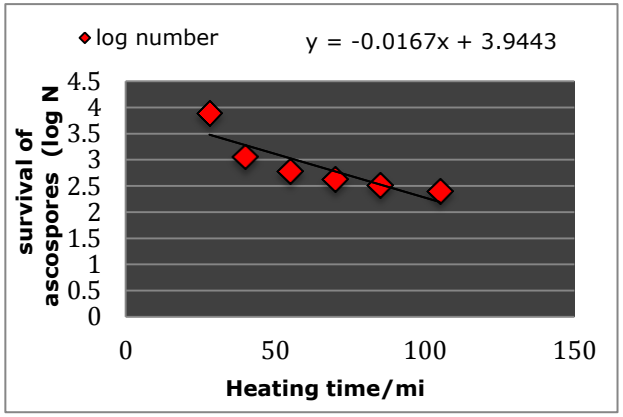
Appendix 3 D-*values* survival curves (log number) of ascospores of *A. nidulans* heated at 55°C for time period 40,55,70,85 and 105 min.



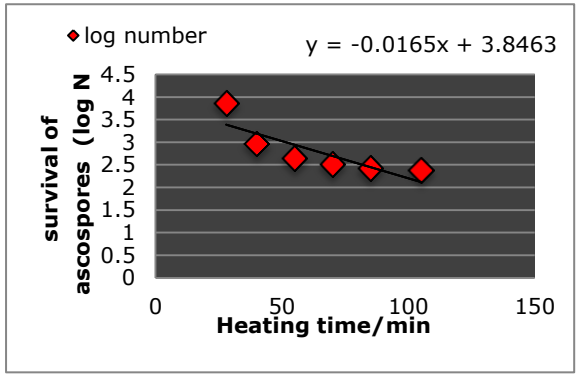
2-137



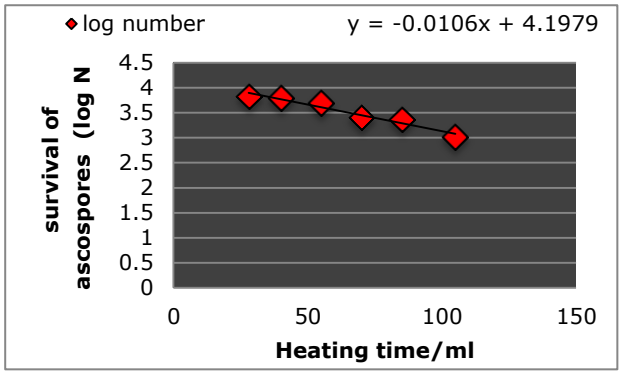
2-139



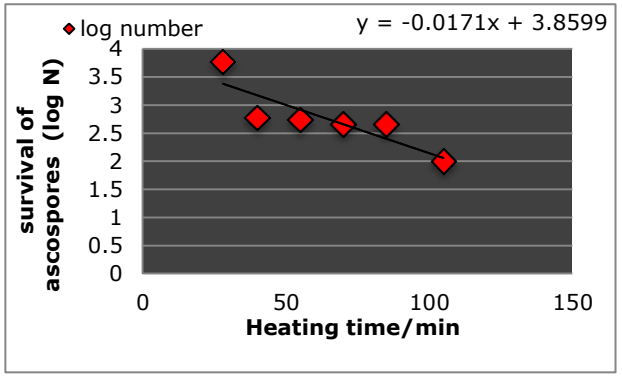
2-149



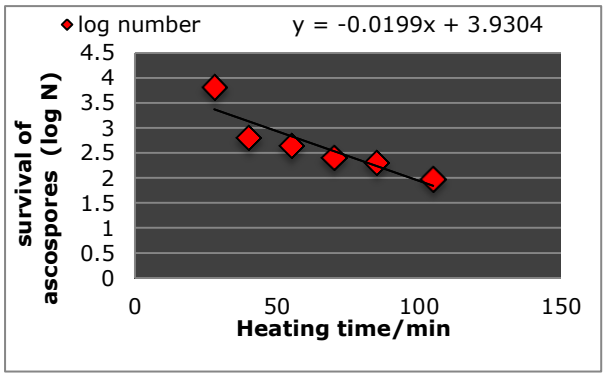
2-154



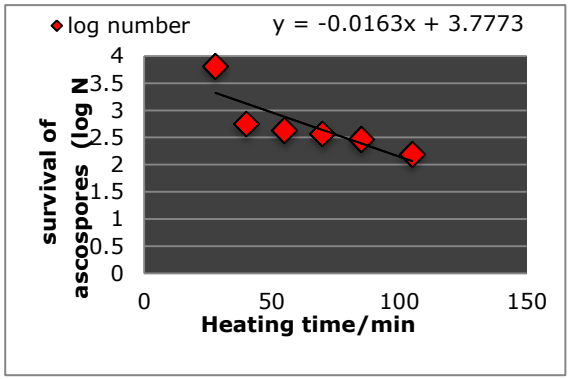
2-155



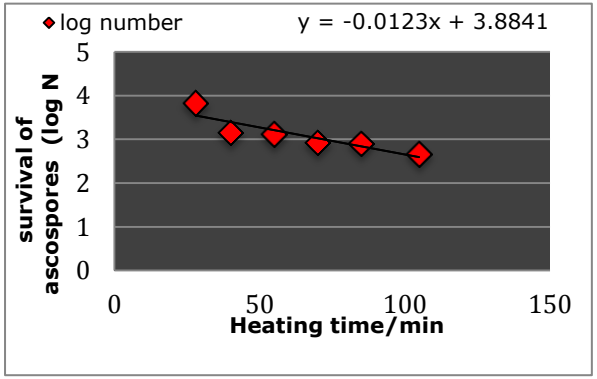
2-224



2-227

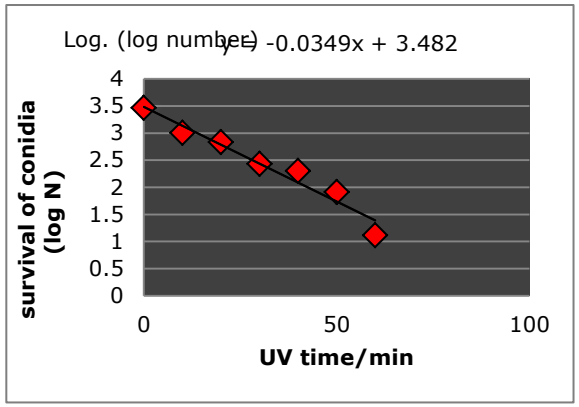


2-232

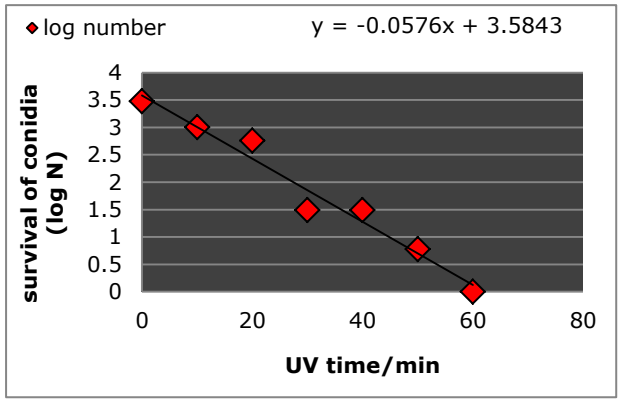


2-250

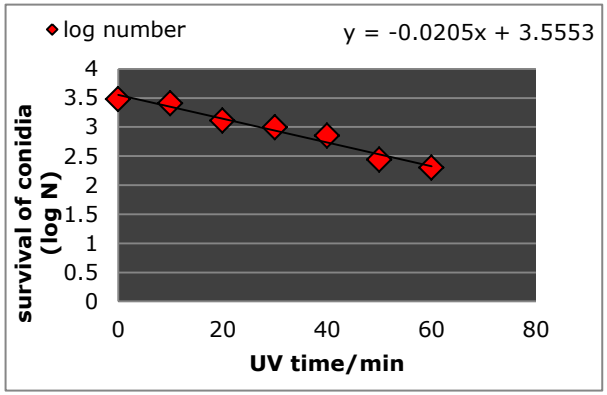
Appendix 4 *D-values* survival curves (log number) of conidia of *A. nidulans* exposed to UV for time period 10, 20, 30, 40, 50 and 60 min.



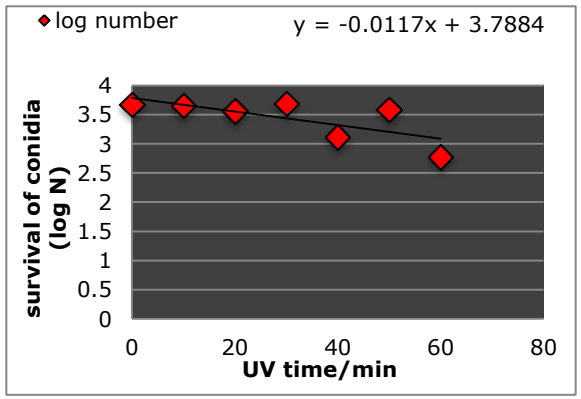
2-137



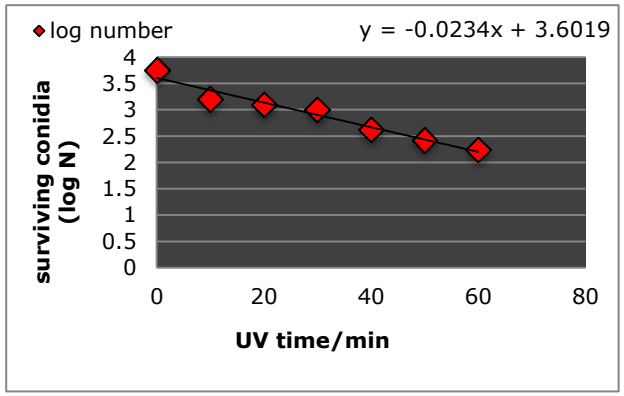
2-139



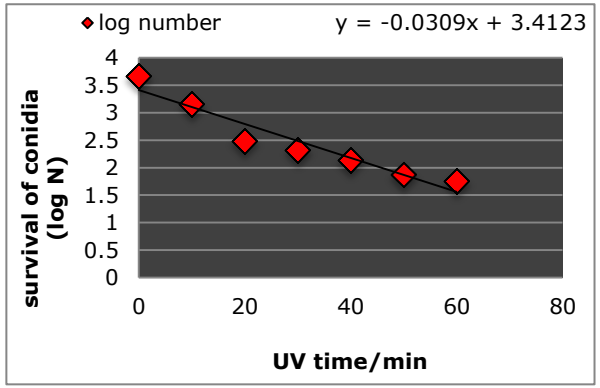
2-149



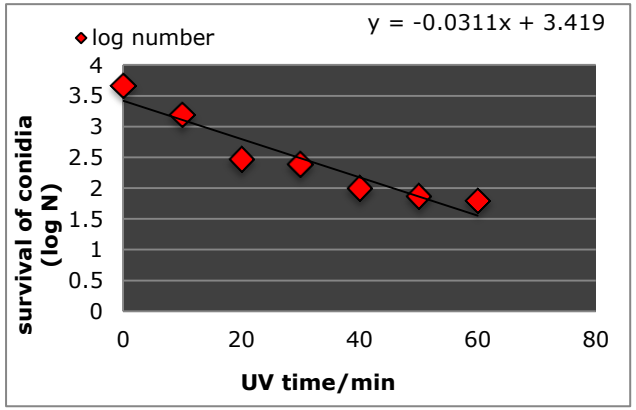
2-154



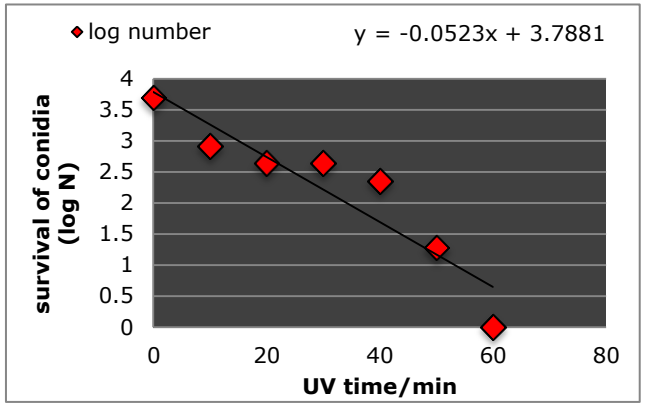
2-224



2-227

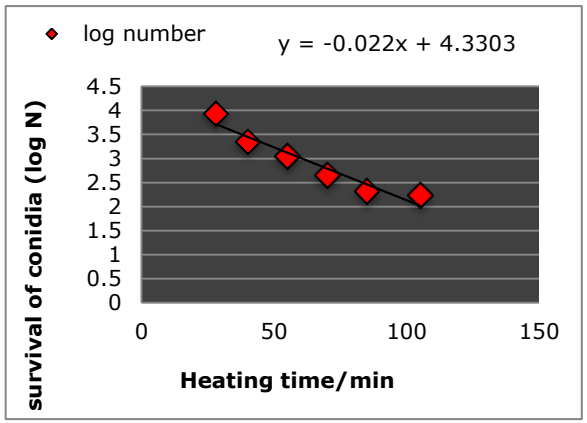

log number
y = -0.0311x + 3.419
survival of conidia (log N)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
UV time/min

2-232

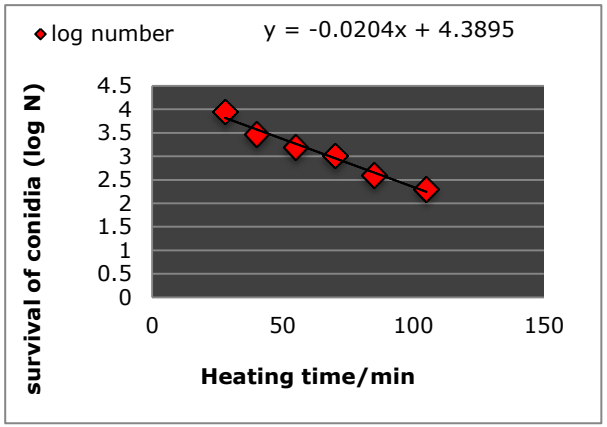

log number
y = -0.0523x + 3.7881
survival of conidia (log N)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
UV time/min

2-250

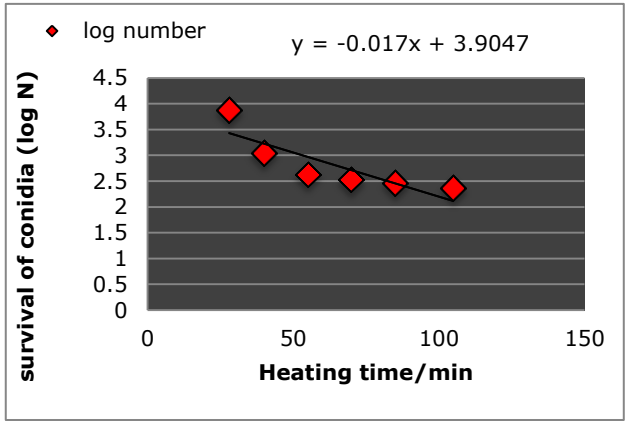
Appendix 5 *D-values* survival curves (log number) of ascospores of *A. nidulans* exposed to UV for time period 10, 20, 30, 40, 50 and 60 min.



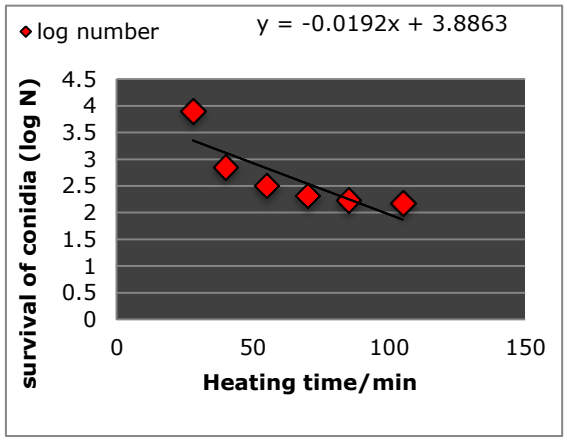
2-137



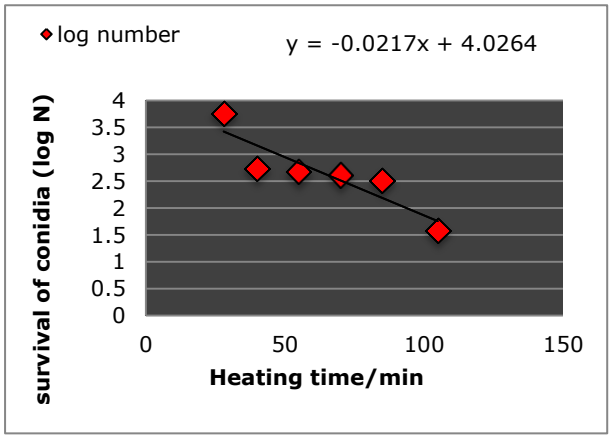
2-139



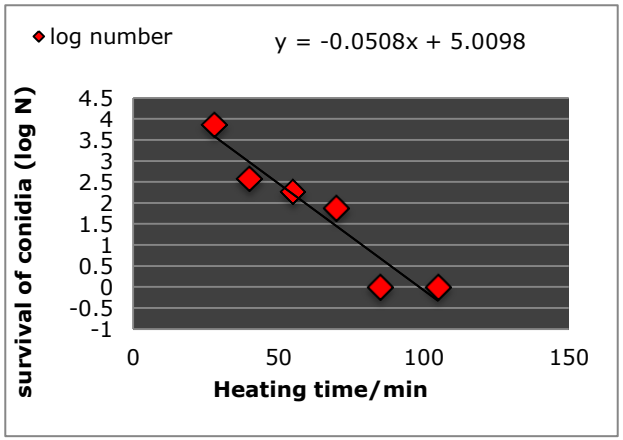
2-149



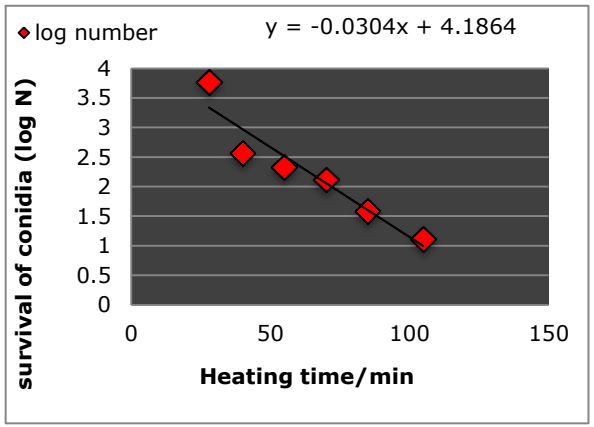
2-154



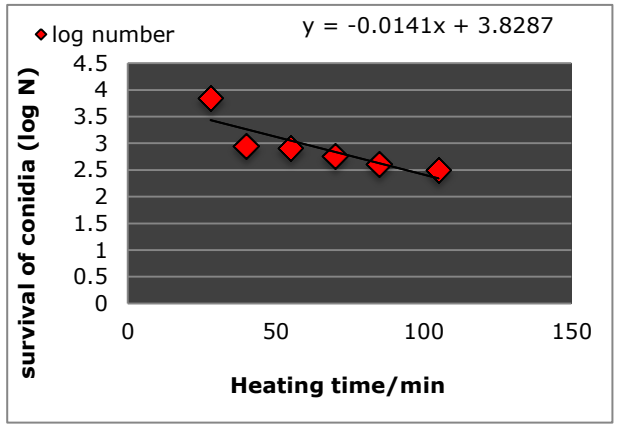
2-224



2-227


log number
y = -0.0304x + 4.1864
survival of conidia (log N)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
50
100
150
Heating time/min

2-232


log number
y = -0.0141x + 3.8287
survival of conidia (log N)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
50
100
150
Heating time/min

2-250

Appendix 6 The sequences of velvet gene for both wild type strains (*ve*A) and mutant strains (*ve*A1).

Strain	Velvet gene	Sequence
2-137	*ve*A	GGATGCACAGTCTTCTAGAGCATTTTGTGTTATCCCATCAAGATGGCTACACTTGCAGCACCACCACCTCCGCTCGG CGAGTCGGGGAACTCGAACTCCGTCAGCCGGATCACTCGCGAGGGAAAGAAGATTACCTATAAATTGAATATTATGC AGCAGCCCAAGCGCGCGAGAGCTTGTGGTCAAGGTTCGAAATGTATGGTGGCAGCACTGGACAGACGTGGAGACA AGGCTAATGTGAGTTGGAACAGCGCACACCGACCGCCGCCCTGTCGATCCTCCGCCAGTCATTGAATTGAACATCTT CGAATCGGATCCCCATGACGACAGCATAAGGACCGACAA
2-139	*ve*A	AGGAAGCACAGTCTTCTAGAGCATTTTGTGTTATCCCATCAAGATGGCTACACTTGCAGCACCACCACCTCCGCTCG GCGAGTCGGGGAACTCGAACTCCGTCAGCCGGATCACTCGCGAGGGAAAGAAGATTACCTATAAATTGAATATTAT GCAGCAGCCCAAGCGCGCGAGAGCTTGTGGTCAAGGTTCGAAATGTATGGTGGCAGCACTGGACAGACGTGGAGA CAAGGCTAATGTGAGTTGGAACAGCGCACACCGACCGCCGCCCTGTCGATCCTCCGCCAGTCATTGAATTGAACATC TTCGAATCGGATCCCCATGACGACAGCAAAAGAACCGACAAT

2-149	*ve*A1	CCTCCTAGCCGTATTGTGTTCATGACTGGCGGAGGATCGACAGGGCGGCGGTCGGTGTGCGCTGTTCCAACTCACATT AGCCTTGTCTCCACGTCTGTCCAGTGCTGCCACCATACATTTCGAACCTTGACCACAAGCTCTCGCGCGCTTGGGCTGC TGCATAATATTCAATTTATAGGTAATCTTCTTTCCCTCGCGAGTGATCCGGCTGACGGAGTTCGAGTTCCCCGACTCGCC GAGCGGAGGTGGTGGTGCTGCAAGTGTAGCAATCTTGATGGGATAACACAAAATGCTCTAGAAGACTTGTTGCATTCCT TGTTAGAAAAGTCTGGCTAAGGCGCAATTGTA
2-154	*ve*A1	TAATATTTGATTAGTGTCGTGCTCCTTACGAGCATTTTGTGTTATCCCATCAAGATTGCTACACTTGCAGCACCACCACCT CCGCTCGGCGAGTCGGGGAACTCGAACTCCGTCAGCCGGATCACTCGCGAGGGAAAGAAGATTACCTATAAATTGAAT ATTATGCAGCAGCCCAAGCGCGCGAGAGCTTGTGGTCAAGGTTCGAAATGTATGGTGGCAGCACTGGACAGACGTGGA GACAAGGCTAATGTGAGTTGGAACAGCGCACACCGACCGCCGCCCTGTCGATCCTCCGCCAGTCATTGAATTGAACAT CTTCGAATCGGATCCCCATGACGACAGCAATAAGACCGACAA

2-155	*ve*A1	TCAAATGCATTCACTAGGTGACAGTCCTTGAGCATTTTGTGTTATCCCATCAAGATTGCTACACTTGCAGCACCACCACC TCCGCTCGGCGAGTCGGGGAACTCGAACTCCGTCAGCCGGATCACTCGCGAGGGAAAGAAGATTACCTATAAATTGAA TATTATGCAGCAGCCCAAGCGCGCGAGAGCTTGTGGTCAAGGTTCGAAATGTATGGTGGCAGCACTGGACAGACGTGG AGACAAGGCTAATGTGAGTTGGAACAGCGCACACCGACCGCCGCCCTGTCGATCCTCCGCCAGTCATTGAATTGAACA TCTTCGAATCGGATCCCCATGACGACAGCAATAAGACCGACAA
2-224	*ve*A	GTTTAGTATGCAAAGTCTTCTAGAGCATCTTGTGTTATCCCATCGAGATGGCTACGCTTGCTGCACCACCACCACCTCCG CTCGGCGAGTCGGGGAATTCGAACTCCGTCAGCCGGATCACTCGCGAGGGAAAGAAGATTACCTATAAATTGAATATTA TGCAGCAACCCAAGCGCGCGAGAGCTTGCGGTCAAGGTTCGAAATGTATGGTGGCAGCACTGGACCGACGTGAAGAC AAGGCTAATGTGAGTTGGTACAGCGCACACCGACCGCCGCCCTGTCGATCCTCCGCCAGTCATTGAATTGAACATCTTC GAATCGGATCCCCATGACGACAGCAAAAGAACCGACAA

2-227	*ve*A	AGGCAAAGGCAAGTCTTCTAGAGCATTTTGTGTTATCCCATCAAGATGGCTACACTTGCAGCACCACCACCTCCGCTCG GCGAGTCGGGGAACTCGAACTCCGTCAGCCGGATCACTCGCGAGGGAAAGAAGATTACCTATAAATTGAATATTATGC AGCAGCCCAAGCGCGCGAGAGCTTGTGGTCAAGGTTCGAAATGTATGGTGGCAGCACTGGACAGACGTGGAGACAAG GCTAATGTGAGTTGGAACAGCGCACACCGACCGCCGCCCTGTCGATCCTCCGCCAGTCATTGAATTGAACATCTTCGAA TCGGATCCCCATGACGACAGCAAAAGAACCGACAA
2-232	*ve*A	GGATGCACAGGTCTTCTAGAGCATTTTGTGTTATCCCATCAAGATGGCTACACTTGCAGCACCACCACCTCCGCTCGGC GAGTCGGGGAACTCGAACTCCGTCAGCCGGATCACTCGCGAGGGAAAGAAGATTACCTATAAATTGAATATTATGCAG CAGCCCAAGCGCGCGAGAGCTTGTGGTCAAGGTTCGAAATGTATGGTGGCAGCACTGGACAGACGTGGAGACAAGGC TAATGTGAGTTGGAACAGCGCACACCGACCGCCGCCCTGTCGATCCTCCGCCAGTCATTGAATTGAACATCTTCGAATC GGATCCCCATGACGACAGCAAAAGGACCGACAAA
2-250	*ve*A	AGATGCACAGTCTTCTAGAGCATTTTGTGTTATCCCATCAAGATGGCTACACTTGCAGCACCACCACCTCCGCTCGGCG AGTCGGGGAACTCGAACTCCGTCAGCCGGATCACTCGCGAGGGAAAGAAGATTACCTATAAATTGAATATTATGCAGC AGCCCAAGCGCGCGAGAGCTTGTGGTCAAGGTTCGAAATGTATGGTGGCAGCACTGGACAGACGTGGAGACAAGGCT AATGTGAGTTGGAACAGCGCACACCGACCGCCGCCCTGTCGATCCTCCGCCAGTCATTGAATTGAACATCTTCGAATCG GATCCCCATGACGACAGCAAAAGGACCGACAA