

Nitric Oxide: A Chemical Effector of Pathogenesis in *Magnaporthe oryzae*

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Abstract

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Research detailed in this thesis investigated the generation of Nitric Oxide (NO) and its role in the pathogenesis of the rice blast fungus *Magnaporthe oryzae*. Two putative nitric oxide synthase genes and single copy nitrate and nitrite reductase genes were cloned as potential sources of NO in *M. oryzae*. Single and double gene disrupted mutants were generated and their phenotypes assessed.

Detection of NO is problematic. Herein, a fluorescent plate reader assay was developed, exploiting the NO sensitive dye DAR-4M AM and the NO scavenger PTIO, to compare wildtype NO generation with the mutant strains. All strains were assessed for infection-related development on an artificial surface inductive to appressorium formation and maturation in the wildtype strain. Appressorium formation in the presence of PTIO and the NO donor DETANONOate was recorded for all strains on this surface. The pathogenicity of the wildtype and mutant strains were assessed, in terms of their ability to infect rice and barley plants. Finally, the capacity of each strain to metabolise nitrogen was evaluated to confirm the disruption of the nitrate and nitrite reductase genes.

Collectively, the data demonstrate that the plate reader assay provides robust evidence for the generation of NO in *M. oryzae*. However, none of the various mutant strains showed a reduction in NO emission during germling morphogenesis. However, they exhibited significantly different infection-related development on an inductive artificial surface as compared with the wildtype strain. Moreover, exogenous application of PTIO to the wildtype strain provided evidence for NO and its involvement in germination and appressorium development. No significant differences in the infection of rice and barley leaves were observed between the wildtype and mutant strains, indicating their disrupted genes are dispensable for pathogenesis. The nitrate and nitrite reductase genes were found to be essential for nitrate assimilation.

In summary, this work provides the most robust evidence for the generation of NO in fungi to-date, but the molecular mechanism underpinning the generation of NO in *M. oryzae* remains elusive.

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Abbreviations

AA	Ascorbic Acid
ABA	Absciscic Acid
Ack	Acetate Kinase
AddA	Acetoaldehyde Dehydrogenase
Ald	Alcohol Dehydrogenase
ATP	Adenosine Triphosphate
BH ₄	Tetrahydrobiopterin
BIC	Biotrophic Interfacial Complex
BODIPY	Boron-dipyrromethene
CaM	Calmodulin
cAMP	Cyclic Adenosine Monophosphate
CFEM-GPCR	Conserved Fungal Extra Membrane - G Protein Coupled Receptors
cGMP	Cyclic Guanosine Monophosphate
CM	Complete Media
CN	Cyanide
CO	Carbon Monoxide
cPTIO	Carboxy-2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide
DAF-2 DA	4,5-Diaminofluorescein Diacetate
DAF-2	4,5-Diaminofluorescein
DAF-FM DA	Diaminofluorescein-FM Diacetate
DAF-FM	4-Amino-5-methylamino-2',7'-difluorescein
DAG	Diacylglycerol
DAN	2,3-Diaminonaphthalene
DAR-2 T	4,5-Diaminofluorescein Triazole
DAR-4M AM	Diaminorhodamine-4M AM
DAR-4M T	Diaminorhodamine-4M Triazole
DAR-4M	Diaminorhodamine-4M
DAR-4M-DHA	Diaminorhodamine-4M-Dehydroascorbic Acid
dCTP	Deoxycytidine Triphosphate
DEPC	Diethylpyrocarbonate
DETANONOate	3,3-Bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene
DETAPA	Diethyltriamepentaacetic Acid
DMSO	Dimethyl Sulfoxide
D-NAME	N ω -Nitro-D-arginine Methyl Ester
D-NMMA	NG-Methyl-D-arginine
D-NNA	N ω -Nitro-D-arginine
dNTPs	Deoxyribonucleotides
dpi	Days Post Inoculation
DSB	Double Strand Break
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethyleneglycoltetraacetic Acid
EIHM	Extra Invasive Hyphal Membrane
eNOS	Endothelial Nitric Oxide Synthase
EPR	Electro-paramagnetic Resonance
ETI	Effector Triggered Immunity
ETS	Effector Triggered Susceptibility
FAD	Flavin Adenine Dinucleotide
FDA	Fluorescein Diacetate
Fe-S	Iron sulphur cluster
FMN	Flavin Mononucleotide

FNOCT	Fluorescent Nitric Oxide Cheletropic Traps
gDNA	Genomic DNA
GPCR	G Protein Coupled Receptors
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
iNOS	Inducible Nitric Oxide Synthase
L-NA	N α ,N ϵ -di-Cbz-L-lysine N-hydroxysuccinimide ester
L-NAME	N ω -Nitro-L-arginine methyl Ester
L-NMMA	NG-Methyl-L-arginine
L-NNA	N ω -Nitro-L-arginine
MA	Melanised Appressoria
MAMPs	Microbial Associated Molecular Patterns
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
MES	2-(N-morpholino)ethanesulfonic Acid
MM	Melanised Mycelia
MN	Minimal Media
Mo-MPT	Molybdenum-Molybdopterin
mtNOS	Mitochondrial Nitric Oxide Synthase
N ₂ O ₃	Nitrous Anhydride
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
Nap	Periplasmic Dissimilatory NR
Nar	Membrane Bound Respiratory NR
Nas	Cytoplasmic Assimilatory NR
NAT	2,3-naphthotriazol
NHEJ	Non-Homologous End Joining
Nia1	<i>M. oryzae</i> Nitrate Reductase
Nii1	<i>M. oryzae</i> Nitrite Reductase
NIR	Nitrite Reductase
NJ	Neighbour Joining
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOHA	N ω -hydroxy-L-arginine
NOR	Nitric Oxide Reductase
NOS	Nitric Oxide Synthase
NR	Nitrate Reductase
ODQ	1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one
PAMPs	Pathogen Associated Molecular Patterns
PAPANONOate	(Z)-1-[N-(3-aminopropyl)-N-(n-propyl)amino]diazen-1-ium-1,2-diolate
PKA	Protein Kinase A
PTI	PAMP Triggered Immunity
PTIO	2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide
QTL	Quantitative Trait Loci
RNS	Reactive Nitrile Species
RNS	Reactive Nitrile Species
ROS	Reactive Oxygen Species
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscope
sGC	Soluble Guanylate Cyclase

SNAP	S-Nitroso-N-acetyl-DL-penicillamine
SNP	Sodium Nitroprusside
SULFONONOate	N-nitrosohydroxylamine-N-sulfonic acid
TAE	Tris base, acetic acid and EDTA
TM	Total Mycelia
WA	Water Agar
XOR	Xanthine Oxidoreductase

Chapter 1: Introduction

1.1 The global importance of rice and rice blast fungus

1.1.1 Rice

Rice (*Oryza sativa*), wheat (*Triticum* sp.) and maize (*Zea mays*) are grown on 40% of the available global cropland, and represent the three most important food crops; of these, cultivated rice is the most important (Khush 2005). Rice imparts on average 35-75% of the daily calorific intake for over three billion people, the majority of which live in Asia (Goff 1999; Khush 2005).

Between 1966 and 2000 major advances were made in rice production, resulting mainly from the adoption of green revolution technologies. These advances led from using disease-resistant and high yielding rice varieties, coupled with increased irrigation and fertiliser application (Khush 2005). In real terms, this represented a 343 million tonne increase in rice yields between 1966 and 2000 (Khush 2005). However, despite increases in yields, the number of malnourished individuals continues to exceed one billion (Barrett 2010). Unfortunately, over the past decade the rate at which rice yields have increased has slowed, whilst the global population continues to rapidly expand (Strange & Scott 2005). Currently, each hectare of rice plantation is required to feed 27 mouths per year. However, in order to meet the predicted global population of 9.2 billion by 2050, each hectare of rice will be required to feed 43 mouths per year (Bongaarts 2009).

Rice is considered the most diverse food crop and is grown in a remarkable spectrum of different environmental conditions ranging from arid to submerged locations, and from below sea level to high altitudes (Khush 2005). However, the amount of arable land available for rice production is decreasing as urbanisation and desertification increase, and as land is turned over

for biofuel production or protected under conservation acts (Barrett 2010; Strange & Scott 2005). The increasing cost of non-renewable fuels and reduction in fresh water availability place increasing pressure on the remaining arable land to feed the global population (Baulcombe 2010). This problem will likely be further compounded by climate change, which is predicted to reduce rice yields as environmental conditions become less suited for rice growth, such as, reduced rainfall and extreme fluctuations in temperature (Luck et al. 2011).

1.1.2 Rice blast disease

Currently 114 phytopathogens have been identified which infect rice. Of these, *Xanthomonas oryzae* and *Magnaporthe oryzae* are the two most devastating pathogens. *X. oryzae* causes bacterial blight of rice and severely affects rice yields in India (Strange & Scott 2005). *X. oryzae* has been identified in multiple continents including Asia, Australasia, Africa, and North America, and can result in >50% yield losses if seedlings become infected (Chen et al. 2011; Petty 2008).

The Ascomycete fungus *M. oryzae* is the causal agent of rice blast disease and represents the most significant pathogen of rice. *M. oryzae* has been isolated from 85 countries and is able to infect all aerial parts of the plant, causing neck and panicle rot, leaf blast, node blast and collar rot, meaning that, in the absence of disease control, infection of mature rice plants results in 10-30% yield loss, whilst infection of seedlings can lead to 100% yield losses (Talbot 2003; Ebbole 2007; Skamnioti & Gurr 2009). A 10% loss in the global rice yield represents a significant amount of rice, sufficient to feed the population of the UK (>60 million) for a year (Talbot 2003). *M. oryzae* is also able to infect other economically important crops including barley (*Hordeum vulgare*), finger millet (*Eleusine coracana*) and wheat (*Triticum aestivum*) (Igarashi et al. 1986; Ekwamu 1991; Zhan et al. 2008). *M. oryzae* is therefore considered to be amongst the most important threats to global food security.

The importance of *M. oryzae* as a plant pathogen is well illustrated by the 1995 epidemic in Bhutan. In this year favourable environmental conditions combined with an increase in the endemic pathogen population, resulted in an outbreak of rice blast disease which affected over 700 hectares and caused the loss of >1000 tons of rice; individual farms experienced yield losses of between 22-78% (Thinlay et al. 2000). If we are to successfully feed the current malnourished population and the predicted additional three billion people by 2050, then understanding and preventing rice blast disease is essential.

1.1.3 *Magnaporthe* Taxonomy

Tharreau et al. (2009) recently identified the Southern foothills of the Himalayas as a candidate location for the origin of *M. oryzae* (Tharreau et al. 2009). This area remains one of the few locations where fertile *M. oryzae* mating types, Mat 1.1 and Mat 1.2, have been isolated and the potential for sexual recombination exists (Tharreau et al. 2009). *M. oryzae* predominantly follows an asexual life-cycle in the wild (see 1.1.6 for further details). *M. oryzae* falls within the *Magnaporthe* species complex, which also contains *Magnaporthe grisea*. Phylogenetic analyses of actin, β -tubulin and calmodulin genes from morphologically indistinguishable lineages within the *Magnaporthe* species complex revealed they separate into two distinct clades (Couch & Kohn 2002). The first clade contains lineages associated with the infection of Crabgrass (*Digitaria*) and retains the *M. grisea* nomenclature. The second clade contains lineages associated with the infection of rice, but also millets and other grasses including barley, and was termed *Magnaporthe oryzae* (Couch & Kohn 2002).

1.1.4 Rice blast control

Rice blast disease is controlled through various methods including crop rotation, burning of infected plant material, sowing of non-infected seed, planting of resistant rice cultivars and fungicide application.

Currently, the most widely used method for controlling rice blast is the planting of resistant rice cultivars (Skamnioti & Gurr 2009). Resistant rice cultivars contain a single dominant resistance (R) gene or *Pi*-gene. To date over 80 *Pi* genes have been identified in rice; 11 of these genes have been cloned, including the well documented *PI-TA* resistance gene (Bryan et al. 2000; Dai et al. 2010). *Pi* genes encode proteins which belong to the nucleotide-binding site–leucine-rich repeat family; they function by detecting the products of avirulence (AVR) genes carried by *M. oryzae*, of which 25 have so far been identified. The products from the *Pi* and AVR genes directly interact to trigger disease resistance in the rice (Yu et al. 1998; Bryan et al. 2000; Dai et al. 2010). The protection conferred by *Pi* genes is total, so long as the *M. oryzae* strain carries the corresponding avirulence gene. This resistance however is not durable, and in the field is frequently overcome by *M. oryzae* within several seasons. An alternative option to single resistance genes is the use of broad spectrum non-race-specific resistance genes, quantitative trait loci (QTL) (Skamnioti & Gurr 2009). QTLs confer partial resistance to multiple *M. oryzae* strains and resistance is generally more durable than that conferred by monogenic resistance genes. For example, a moderate-resistant rice cultivar called Acuce has been grown for over 100 years in the Yunnan Province, China, and is to date still being grown within regions endemic to rice blast (Dai et al. 2007; Liu et al. 2010). Nine QTLs have been defined using molecular markers, however, it is not known if each QTL is recognised by single or multiple AVR genes, or by what mechanism they initiate plant defences following infection (Liu et al. 2010).

Plants also protect themselves against pathogen invasion via a basal defence process termed innate immunity, which is activated when the host recognises specific microbial/pathogen associated molecular patterns (MAMPs/PAMPs) e.g. chitin, using pattern identification receptors, the end result of which is PAMP triggered immunity (PTI) (Bari & Jones 2009). Pathogens overcome PTI by secreting effectors which prevent host resistance and cause effector triggered susceptibility (ETS), disease is only prevented if the host recognises the pathogen effectors and activates effector triggered immunity (ETI) (Bari & Jones 2009).

The application of fungicides is an effective method for controlling rice blast disease. Fungicides function by targeting specific biochemical pathways within *M. oryzae*, including melanin synthesis (appressorium formation) and phosphatidylcholine synthesis (membrane formation) (Kurahashi 2001; Uesugi 2001; Skamnioti & Gurr 2009). However, fungicide use is biased towards wealthy developed countries. As the majority of rice is grown in poorer nations, the use of fungicides is often not an option for farmers. Japan, for example, grows only 2% of the world's rice but represents 46% of the Probenazole fungicide market. In comparison, China and India use only 9 and 6% of Azoxystrobin and Isoprothiolane fungicide markets respectively, but grow 50% of the world's rice (Skamnioti & Gurr 2009). However, similar to monogenic resistance cultivars, the protection conferred by a single fungicide is often ephemeral, with resistant *M. oryzae* strains emerging within a short time period following application. The speed at which fungicide resistance emerges depends upon the fungicide's mode of action: resistance is more likely to emerge against a fungicide which has only one site of action relative to a fungicide which disrupts physiological processes at multiple sites. Fungicides which inhibit *M. oryzae* melanin biosynthesis have remained highly effective, with no resistant strains emerging following 30 years of use (Kurahashi 2001). Aside from their cost, fungicides are also notoriously bad for the environment (Komárek et al. 2009).

To maintain rice blast resistance, new durable control strategies must be developed. Durable resistance strategies effectively spread selection pressures across multiple genes within *M. oryzae*, therefore reducing the likelihood of a spontaneous mutation(s) overcoming resistance. The simplest durable resistance method involves inter-cropping both blast-resistant and non-resistant rice cultivars within a single field; this avoids susceptible rice monocultures and increases resistance to rice blast (Skamnioti & Gurr 2009). Zhu et al. (2000) demonstrated rice inter-cropping to be highly effective by conducting large scale field trials in China designed to specifically involve local rice farmers (Zhu et al. 2000). Farmers planted multiple rice cultivars per field and the resulting reduction in rice blast disease was so substantial that within two

years rice was being successfully grown without fungicide application (Zhu et al. 2000). However, rice inter-cropping is not always favoured by farmers due to the mixed seed harvest, hence this effective rice blast prevention technique is rarely used.

The pyramiding of resistance genes within a single rice cultivar is an alternative durable resistance strategy. Gene pyramiding generates single high yielding rice cultivars which contain multiple resistance genes (Hittalmani et al. 2007). For resistance to be overcome mutations must occur in each corresponding *M. oryzae* avirulence gene; the likelihood of this is significantly less than the chance of a single mutation in one avirulence gene. Hittalmani et al. (2007) observed increased rice blast resistance following the pyramiding of three resistance genes, *Pi1*, *Piz-5* and *Pita*, in a single rice cultivar compared to the same cultivar containing only a single resistance gene (Hittalmani et al. 2007). Similarly, the Jefferson rice cultivar which carries the *PIK* and *PIZ* genes has successfully been grown in the USA since 1997, without the emergence of resistance (McClung 1997; Fjellstrom et al. 2004).

Understanding *M. oryzae* epidemiology is a further important aspect of controlling rice blast disease. The development of specific and sensitive diagnostic tools allows *M. oryzae* populations to be monitored specifically with regard to i) identifying new virulent strains; ii) monitoring *M. oryzae* population structures; iii) identifying potential horizontal gene transfer, and iv) identifying rice blast disease from other pathogens (Skamnioti & Gurr 2009). To-date multiple techniques are used to distinguish separate *M. oryzae* populations, such as RFLPs and rep-PCR DNA fingerprinting, which separate *M. oryzae* lineages based on the abundance of the transposons *MGR586* and *POT2* (Levy et al. 1991; George et al. 1998). Techniques such as TaqMan provide more sensitive and specific results at the cost of time and money (Martin et al. 2000). Currently there is no single method which combines specificity, sensitivity, ease of use and cost effectiveness together (Skamnioti & Gurr 2009). However, as progress in *M. oryzae* identification techniques improves, and the speed and efficiency of genomic sequencing

increases, a greater wealth of information regarding *M. oryzae* epidemiology will become available, aiding the safeguarding of rice.

The ideal defence against rice blast disease would be a single rice cultivar containing multiple monogenic resistance genes and QTLs, coupled with up-to-date information regarding global and regional rice blast epidemiology.

1.1.5 *Magnaporthe oryzae* a model phytopathogen

Aside from its importance as the premier pathogen of rice, *M. oryzae* has emerged as a model appressorium-forming fungi in the study of plant disease. The development of new protocols and techniques along with the complete genome sequence has enabled rapid gene identification and comparative genomics studies (Dean et al 2005). For example *M. oryzae* is readily cultured *in vitro* and germinates on artificial surfaces: from a research perspective this makes it an ideal subject to study. *M. oryzae* (wildtype strain Guy11) infects rice and barley, allowing its pathogenesis and pathogen-host dynamics to be studied (Parker et al. 2008). The generation of targeted gene replacement mutants has become relatively straight-forward following the development of transformation techniques such as split marker gene replacement and the deletion of the Ku70 and Ku80 dimers, responsible for non-homologous end joining (NHEJ) (Balhadère et al. 1999; Villalba et al. 2008). *Agrobacterium*-mediated transformation has generated a mutant collection which covers 61% of the *M. oryzae* genome and RNA mediated gene silencing has recently been developed, allowing any gene of interest to be silenced through the generation of a double stranded RNA molecule (Jeon et al. 2007; Nguyen et al. 2008) The use of temperature sensitive promoters allows gene function to be studied without requiring targeted gene replacement (Wilson & Talbot 2009). Thus *M. oryzae* provides an excellent model to study appressorium forming fungi.

1.1.6 *M. oryzae* asexual lifecycle

Sexual recombination within *M. oryzae* is uncommon, with the exception of the Uttar Pradesh hills of the Indian Himalayas, where both fertile mating types are found (Kumar et al. 1999). Thus, *M. oryzae* predominantly replicates via its asexual lifecycle. Its predominance for asexual reproduction occurs even among populations which have the potential for sexual recombination (Ebbole 2007). The asexual lifecycle begins with the dissemination of three celled asexual spores, termed conidia, through wind and rain splash, Figure 1.1 (Ebbole 2007). Upon arrival at the host, infection commences with conidia attachment to the host cuticle. The conidia secrete spore tip mucilage which helps them attach to the hydrophobic plant cuticle (Hamer et al. 1988). Following attachment, a single germ-tube emerges (usually from the tapered end of the conidium) and elongates, growing across the host cuticle. Two hours after germination, the tip of the germ tube swells and differentiates into the specialised infection cell, the appressorium (Talbot 2003). Appressorium formation is dependent on germlings sensing specific environmental cues including hydrophobicity and the presence of cutin (*cis*-9,10-epoxy-18-hydroxyoctadecanoic acid) and lipid monomers (1,16-hexadecanediol) (Hamer et al. 1988); (Ebbole 2007). Failure to sense environmental cues results in the abortion of infection-related development. However, following the detection of the requisite environmental cues, a strong turgor pressure is generated due to the osmotic imbalance within appressoria, the result of solute translocation, including glycerol from the conidia (de Jong et al. 1997; Wang et al. 2007). To maintain the high turgor pressure appressoria cell walls are composed of chitin and a 20nm thick melanin layer. The melanin layer prevents solutes from exiting the appressorium but allows water to diffuse inwards, generating osmotic turgor pressures of up to 8MPa. The turgor pressure is focused onto a penetration peg, which is driven through the host cuticle, allowing access to the host epidermal cells (Chumley & Valent 1990; Bourett & Howard 1990). Initial invasive growth follows a biotrophic lifestyle, where hyphae grow through rice cells contained within an extra-invasive hyphal membrane. At this point during host invasion no disease symptoms are visible (Kankanala et al. 2007). Invasive hyphae

enter neighbouring cells by passing through plasmodesmata at the pit field sites. In order to do this the invading hyphae must reduce their diameter to $<0.2\mu\text{m}$ (Kankanala et al. 2007; Kang et al. 2010). Approximately 72 hours post penetration disease lesions appear on the leaves, and at this stage *M. oryzae* switches from a biotrophic to a necrotrophic lifestyle, secreting cell wall degrading enzymes and causing plant cell death (Caracuel-Rios & Talbot 2007). The life-cycle is completed following the dissemination of conidia from disease lesions under humid conditions, Figure 1.1. Each lesion at midnight is capable of releasing 20,000 conidia over several days and *M. oryzae* follows a polycyclic lifecycle, meaning that during one host life cycle *M. oryzae* undergoes multiple infection cycles and a single infected acre of rice plants is capable of releasing over 1×10^{11} spores (Barksdale 1961). The large number of conidia produced by an infected host, coupled with the polycyclic lifecycle of *M. oryzae*, results in the rapid devastation of rice.

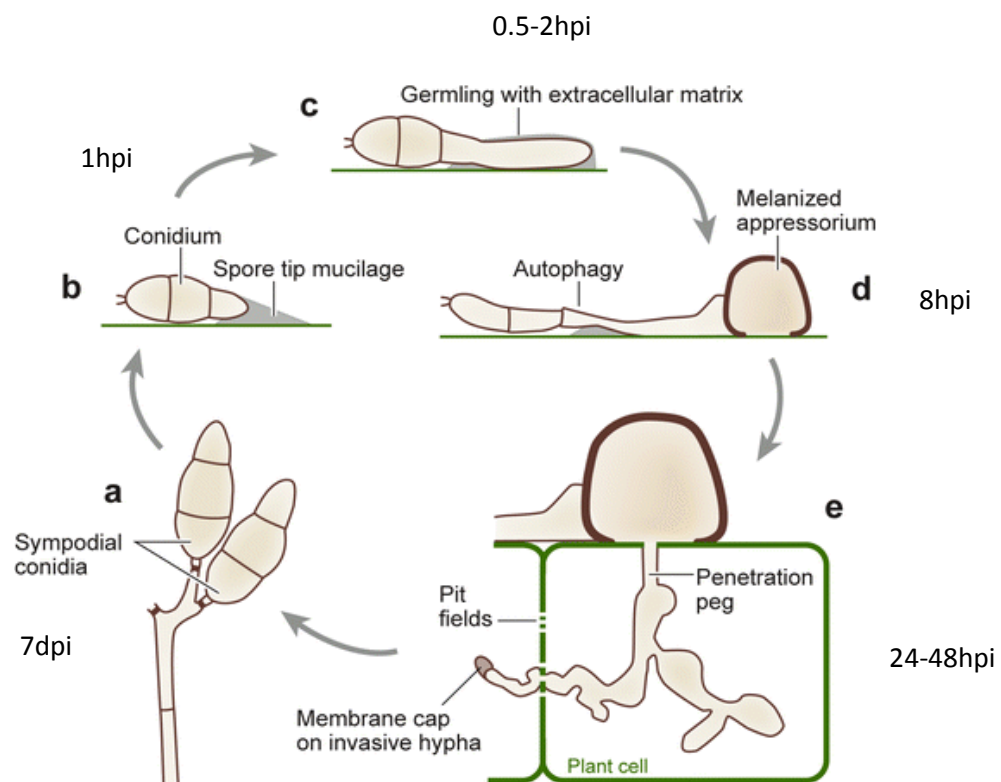


Figure 1.1. *M. oryzae* asexual lifecycle. Taken from Ebbole (2007). a) Conidia disseminate from disease lesions; b) Conidia adhere to the host cuticle by releasing spore tip mucilage; c) After

two hours conidia germinate, with the emergence of a single polarized germ tube; d) Following the detection of specific environmental cues, the specialised infection cell that is the appressorium develops; e) Following appressorium maturation and turgor generation, penetration of the host cuticle leads to invasive hyphae growth. *M. oryzae* growth follows a biotrophic lifestyle for 72 hours, before switching to a necrotrophic lifestyle, characterised by the appearance of disease lesions and conidiophores. The life-cycle is completed within seven days (Ebbole 2007).

1.1.7 The appressorium

Formation of the appressorium is key for successful host invasion and pathogenesis. The appressorium itself is a dome-shaped cell, of which the outer area (with the exception of the area in direct contact with the plant cuticle) contains a complex cell wall structure consisting of chitin and melanin (Talbot 2003). The appressorium pore, located at the base of the appressorium cell in direct contact with the host cuticle, has a thin cell wall and represents the site of penetration (Bourett & Howard 1990). The disruption of genes responsible for melanin biosynthesis in *M. oryzae* resulted in the generation of non-pathogenic mutant strains Δalb^- , Δrsy^- and Δbuf^- . The addition of scyalone, an intermediate in the melanin biosynthesis pathway, restores pathogenicity in Δalb^- (Chumley & Valent 1990). Melanin within the appressorium cell wall prevents carbohydrates from translocating outwards. Internal appressorium glycerol concentrations have been reported to reach 3M, this results in the generation of a substantial osmotic turgor pressure, equivalent to 1200psi (Bourett & Howard 1990). It has been reported that part of the cell wall strengthening process involves the generation of reactive oxygen species (ROS) by NADPH oxidases (Egan et al. 2007). ROS are hypothesised to cross-link proteins in the cell wall, increasing its strength. The disruption of the NADPH oxidases in *M. oryzae* by Egan (2007) resulted in non-pathogenic mutants unable to synthesis superoxide (Egan et al. 2007). Similarly, the GTPase MgRho3 has been identified as a potential regulator of superoxide synthesis: disruption of *MGRHO3* suppressed penetration and diminished virulence (Zheng et al 2007). By contrast, over expression of *MGRHO3* generated a hyper-virulent *M. oryzae* strain, confirming the importance of ROS in pathogenesis (Zheng et al 2007).

The development of appressorium turgor pressure requires careful co-ordination of metabolism with germling morphogenesis. Glycerol is first catabolised within the conidium from lipids, glycogen, mannitol and trehalose, before being transported into the appressorium (de Jong et al. 1997; Wang et al. 2005). Lipid breakdown occurs during appressorium morphogenesis and requires seven intracellular triacylglycerol lipases. Wang et al. (2007) demonstrated that the deletion of any one of the lipases failed to inhibit pathogenesis, indicating a redundancy within the lipolysis pathway (Wang et al. 2007). The translocation of lipid and glycogen into the appressorium were found to be essential for appressoria formation (Wang et al. 2005). Thines et al. (2000) observed that deletion of Pmk1, a mitogen-activated protein kinase (MAPK), prevents the mass movement of glycogen and lipids into the appressorium and leads to the formation of undifferentiated germ tubes (Thines et al. 2000; Ding et al. 2009). Lipid catabolism via peroxisomal β -oxidation has also been shown to be essential for appressorium formation. Acetyl-CoA, the product of peroxisomal β -oxidation, is a precursor for melanin biosynthesis (Wang et al. 2007). The importance of acetyl-CoA was confirmed by targeted gene replacement of both *PEX6*, which encodes a peroxin required for β -oxidation, and *PTH2*, which encodes a carnitine acetyltransferase, required for acetyl-CoA membrane transfer. In both cases the resulting mutants were non-pathogenic and melanin deficient (Wang et al. 2003; Ramos-Pamplona & Naqvi 2006; Bhambra et al. 2006). The role of lipid metabolism in pathogenicity has been shown to be conserved across fungi, with it being identified in other phytopathogenic fungi, *Colletotrichum lagenarium* and *Stagonospora nodorum* (Asakura et al. 2006).

Woronin bodies have been found to have two essential roles in *M. oryzae* pathogenicity. They are required firstly for appressorium development, and secondly, for the maintenance of invasive hyphae during host colonisation (Soundararajana et al. 2004; Galhano & Talbot 2011). The targeted replacement of *TPS1*, which encodes trehalose-6-phosphate synthase and is essential for trehalose biosynthesis, led to a mutant with reduced virulence, conidiation, turgor pressure and an inability to synthesise trehalose (Foster et al. 2003; Fernandez & Wilson 2011).

Tps1 is thought to bind glucose-6-phosphate, a precursor of trehalose, and thereby regulate appressorium physiology through nutritional sensing (Foster et al. 2003; Fernandez & Wilson 2011). Tps1 has also been shown to regulate nitrogen utilisation in *M. oryzae* and is required to regulate the pentose phosphate pathway (Fernandez & Wilson 2011). The $\Delta tps1$ mutant is unable to regulate appressoria physiology, resulting from decreased availability of NADPH, which in turn suppresses NADPH dependent nitrate reductase activity. Therefore $\Delta tps1$ cannot grow in an environment where nitrate is the sole nitrogen source, and represents a key regulator of carbon and nitrogen metabolism in *M. oryzae* (Foster et al. 2003).

Appressorium development has also been shown to be regulated by the cell cycle (Veneault-Fourrey et al. 2006). During development germlings undergo a specific pattern of mitosis and nuclear translocation. Following the transfer of a single nucleus into the appressorium autophagy occurs within the conidium leading to its collapse and appressorium maturation. Suppression of mitosis prevents conidia collapse and appressoria formation (Veneault-Fourrey et al. 2006).

1.1.8 Environmental sensing

As *M. oryzae* progresses through its life-cycle its environmental conditions change dramatically from the hard, hydrophobic host cuticle to the nutrient rich intracellular rice cells. *M. oryzae* must therefore accurately sense its environment. The complete *M. oryzae* genome sequence revealed that *M. oryzae* carries a family of 74 G protein coupled receptors (GPCRs) (Dean et al. 2005). Twelve of these GPCRs carry a conserved fungal specific extracellular membrane spanning domain (CFEM); all twelve CFEM GPCRs were shown to be expressed during pathogenesis (Dean et al 2005; Li et al. 2010). In contrast, *Neurospora crassa* and *Aspergillus nidulans*, non-phytopathogenic fungi, carry only a single CFEM GPCR, suggesting that the additional CFEM GPCRs identified in *M. oryzae* may be necessary for its phytopathogenic lifestyle (Dean et al 2005; Caracuel-Rios & Talbot 2007). Pth11 was the first GPCR identified in

M. oryzae. Following disruption of *PTH11*, its phenotype mirrored that of wildtype conidia germinating on a surface non-inductive for melanised appressoria formation; approximately 10-15% of $\Delta pth11$ germlings form appressoria on an inductive surface, with the majority of germlings not developing beyond elongate germ-tubes. This suggests Pth11 is not required for appressoria development but for surface recognition (DeZwaan et al. 1999; Liu et al. 2009). Subsequent to the identification of Pth11 by DeZwaan et al. (1999) GPCR signalling has been well-studied in *M. oryzae*. The MAPK Pmk1 (see 1.1.10 for further details) is activated through GPCR signalling and responds to environmental cues, such as hydrophobicity. The disruption of the GPCR α -subunit gene *MAGB*, prevents appressoria formation; however the addition of cAMP to the $\Delta magb$ mutant restores appressorium formation. This indicates that MagB activates a cAMP dependent signalling pathway (Liu & Dean 1997). The generation of a dominant activate *MAGB* allele results in a mutant which forms appressoria on both hydrophilic and hydrophobic surfaces, highlighting the role MagB plays in surface sensing (Liu & Dean 1997). Along with surface hydrophobicity, specific plant chemicals are known to strongly induce appressoria formation, for example the cutin monomers cis-9,10-epoxy-18-hydroxyoctadecanoic acid and cis-9-octadecen-1-ol (Liu & Dean 1997). Application of these monomers to $\Delta magb$ induces appressorium morphogenesis, demonstrating a *MAGB* independent pathway for cAMP synthesis; therefore the activity of adenylate cyclase is regulated via more than one pathway (Gilbert et al. 1996; Liu & Dean 1997; Ebbole 2007).

Regulators of G protein signalling (RGS) negatively regulate GPCR signalling cascades in eukaryotes. Rgs1 identified in *M. oryzae* interacts with each GPCR α -subunit, suppressing their respective activities (Liu et al. 2007). Originally, only *MAGB* was thought to be involved in appressorium formation; however, a more recent study has reported a role for *MAGA* in appressorium formation (Liu & Dean 1997; Liu et al. 2007). $\Delta maga$ mutants develop normal appressoria; however generation of a dominant-activate *MAGA* allele resulted in a strain which

formed appressoria on hydrophilic surfaces, indicating that both MagA and MagB are linked in regulating appressorium development (Liu et al. 2007).

1.1.9 Cyclic AMP signalling in *M. oryzae*

The importance of cAMP in fungi as a regulator of development has been well-documented. Its importance in *M. oryzae* was first identified by Lee & Dean (1993), Figure 1.2 (Lee & Dean 1993; Firtel et al. 1989; Wilson & Talbot 2009; Ebbole 2007). Targeted gene replacement of the adenylate cyclase gene, *MAC1*, in *M. oryzae* led to mutants with reduced vegetative growth, fertility, conidiation, conidia germination and an inability to develop appressoria. cAMP therefore plays an essential role in *M. oryzae* morphogenesis and pathogenicity (Choi & Dean 1997). The addition of cAMP to $\Delta mac1$ restored its appressoria formation and pathogenesis to wildtype equivalent levels. Indeed, the application of cAMP to wildtype conidia induces appressoria generation on non-inductive surfaces (Choi & Dean 1997). cAMP functions as a second messenger activating a cAMP dependent protein kinase A (PKA) by interacting with its regulatory subunit. Mutation of the PKA regulatory subunit, coded by *CPKA*, resulted in the continuous activation of CpkA which was able to restore appressoria formation in the $\Delta mac1$ mutant (Adachi & Hamer 1998; Xu et al. 2007). Interestingly, the deletion of *CPKA* did not abolish appressorium formation: small non-functional appressoria were generated by $\Delta cpka$. Together these findings demonstrate that cAMP activated PKA is required for *M. oryzae* pathogenesis.

1.1.10 Mitogen activated protein kinase pathways in *M. oryzae*

MAPKs function by transmitting environmental and developmental signals to the nucleus inducing gene expression. MAPKs are activated via an upstream kinase (MAPKK), itself activated by a further upstream kinase (MAPKKK). Three MAPKs cascades have been identified in *M. oryzae* *OSM1*, *MPS1* and *PMK1*. $\Delta Osm1$ deletion mutants exhibit sensitivity to osmotic stress and display morphological defects during vegetative growth in hyper-osmotic environments

(Dixon et al. 1999; Caracuel-Rios & Talbot 2007). *MPS1* was found to be essential for pathogenicity; *MPS1* disruption resulted in appressoria which were unable to penetrate the host cuticle. *Δmps1* mutants also displayed sensitivity to cell wall degrading enzymes, reduced conidiation and infertility. Interestingly, *Δmps1* was still able to induce plant defence responses, which indicates that the host is aware of *M. oryzae* prior to its invasion (Xu et al. 1998; Zhang et al. 2011). *PMK1* is also required for pathogenesis. A functional homologue of *FUS3/KSS1* in yeast, it is essential for appressorium formation and invasive hyphal growth (Xu & Hamer 1996; Zhang et al. 2011). *PMK1* deletion mutants fail to form appressoria, but are capable of responding to environmental cues: exogenously supplied cAMP causes *Δpmk1* appressoria germ-tube swelling. *Δpmk1* mutants are also unable to grow *in planta*: inoculation of *Δpmk1* conidia into wounded plants fails to induce blast disease (Xu & Hamer 1996). Homologues of *PMK1* have been identified in several other appressoria forming phytopathogenic fungi including *Cochliobolus heterostrophus*, *Colletotrichum lagenarium*, *Colletotrichum gloeosporioides*, and *Pyrenophora teres* (Zhao et al. 2007; Zhang et al. 2008). The disruption of the *PMK1* homologue in each fungus rendered it non-pathogenic, indicating MAPK conservation between phytopathogenic fungi. Pmk1 MAPK in *M. oryzae* is activated by Mst7 (MAPKK) and MST11 (MAPKKK). Targeted gene replacement of both *MST7* and *MST11* generated mutants unable to develop appressoria (Zhao et al. 2005; Wilson & Talbot 2009). Expression of a dominant activated *MST7* allele in the *MST11* mutant background restored appressoria formation but failed to recover pathogenesis; this observation placed Mst7 downstream of Mst11. In the presence of the dominant activated *MST7* allele Pmk1 was detected in all strains during both vegetative and pathogenic growth, confirming Pmk1 to be downstream of Mst7 (Zhao et al. 2005; Wilson & Talbot 2009). However no interaction between either Mst7 or Mst11 and Pmk1 was detected. An additional gene, *MST50*, was shown via a co-immunoprecipitation assay to directly interact with both Mst7 and Mst11. Deletion of *MST50* suppressed appressoria formation, whilst the expression of a dominant activated *Mst7* allele in

the *Mst50* background restored appressoria formation but not pathogenicity (Park et al. 2006; Li et al. 2011). *Mst50* interacts with *Mst11* via a sterile α -motif (SAM) domain; mutation of its SAM domain prevented appressoria morphogenesis. In contrast to *Mst11*, both SAM and Ras associated domain (RAD) sites were dispensable for the *Mst50* and *Mst7* interaction. Disruption of the *Mst50* SAM and RAD revealed that the SAM but not RAD domain is required for appressorium formation, see Figure 1.2 for details (Park et al. 2006; Li et al. 2011). Further investigations of *Mst50* demonstrated direct interactions between itself and two Ras proteins in *M. oryzae*, *Ras1* and *Ras2*. Deletion of *Ras1* had no impact on appressorium formation or pathogenicity, hence *Mst50* is thought to function downstream of *Ras1* (Park et al. 2006). Interestingly, yeast-two-hybrid analyses confirmed an interaction between *Mst50* and the *Mgb1* GPCR β -subunit, highlighting GPCRs as an activator of the PMK1 MAPK cascade. Deletion of *Mgb1* abolished appressorium formation (Park et al. 2006; Nishimura et al. 2003).

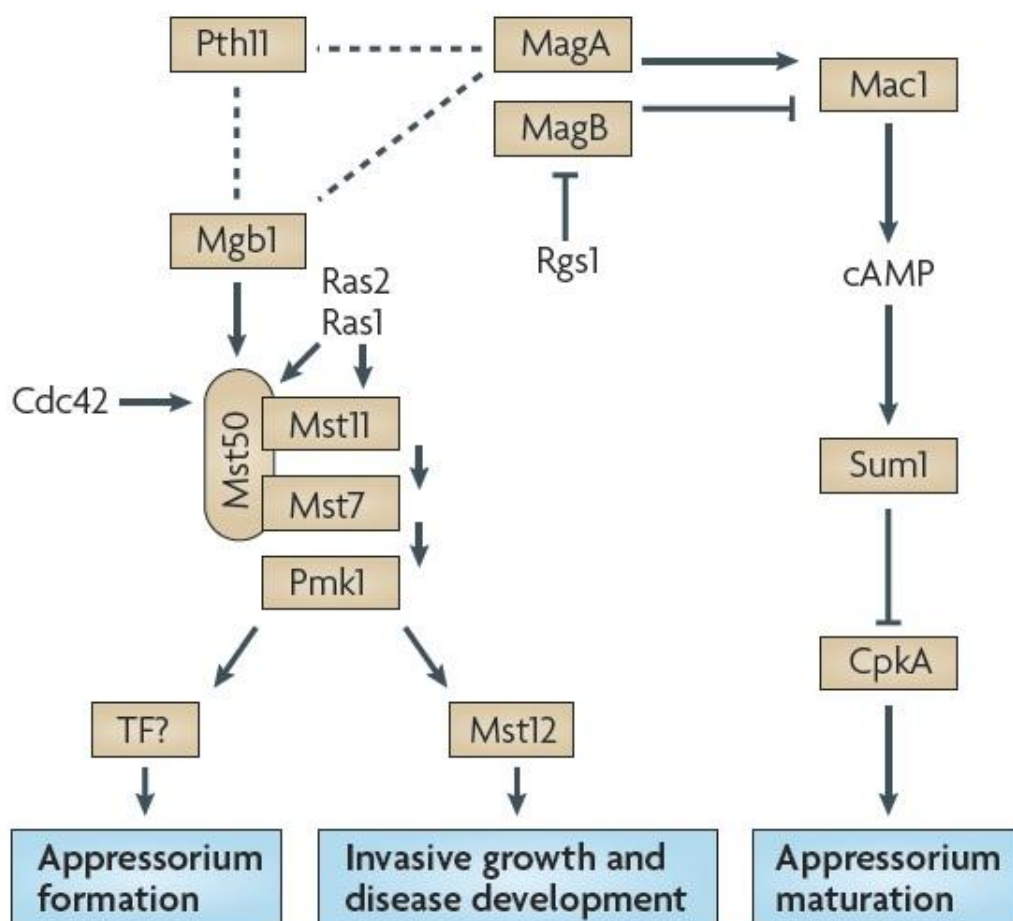


Figure 1.2. Signal transduction pathways required for *M. oryzae* infection related development. Adapted from Wilson & Talbot (2009).

1.1.11 Plant invasion

Penetration through the host cuticle requires the *M. oryzae* MAPK Mps1. Deletion of either *MPS1* or *MCK1* (the upstream MAPKKK associated with Mps1) suppresses penetration and pathogenesis (Xu et al. 1998). The $\Delta mck1$ mutant has several interesting phenotypes; firstly, it has suppressed conidiation, developing only 5% of wildtype levels due to its reduced formation of conidiophores relative to the wildtype (Jeon et al. 2008). Secondly, its cell wall integrity is compromised: $\Delta mck1$ grown on oatmeal agar results in mycelia autolysis, radiating from the centre outwards. Electron microscopy demonstrated mycelia autolysis and abundant intracellular lysates (Jeon et al. 2008). Thirdly, $\Delta mck1$ shows increased susceptibility to cell wall degrading enzymes. $\Delta mck1$ protoplasts form six times faster than wildtype protoplasts, following the treatment of their respective mycelia with cell wall degrading enzymes. Fourthly, $\Delta mck1$ forms atypical appressoria, which appear misshapen and are unable to penetrate the host cuticle. Cytorrhysis analyses confirm $\Delta mck1$ has a significantly different cell wall structure to the wildtype, exhibiting increased solute porosity when germlings are incubated with glycerol solutions at different concentrations. Jeon et al. (2008) hypothesise the increased porosity prevents host penetration by inhibiting generation of appressorium turgor pressure (Jeon et al. 2008).

Host cuticle penetration is thought to require the action of specific cell wall degrading enzymes (Wilson & Talbot 2009). Analysis of the *M. oryzae* genome revealed sixteen putative cutinases (Dean 2005). A study by Sweigard et al. (1992) reported *CUT1* to be dispensable for pathogenicity (Sweigard et al. 1992). However, a more recent study disrupted *CUT2*, shown to be highly expressed during appressoria maturation. The resulting mutant exhibits reduced conidiation, atypical germling morphology and reduced pathogenicity, however, removal of the host cuticle restores full pathogenicity to $\Delta cut2$, confirming its role in plant penetration

(Skamnioti & Gurr 2007). The addition of diacylglycerol (DAG), synthetic cutin monomers, 3-isobutyl-1-methylxanthine and cAMP, also restore the $\Delta cut2$ morphological and pathogenicity defects (Skamnioti & Gurr 2007). Cut2 is therefore thought to be an upstream regulator of DAG/PKC and cAMP/PKA signalling pathways, required for surface sensing.

1.1.12 Invasive plant growth

Two recent studies by Kankanala et al. (2007) and Khang et al. (2010) have significantly advanced our understanding regarding *M. oryzae* and invasive plant growth (Kankanala et al. 2007; Khang et al. 2010). Following appressorium-mediated penetration of the host cuticle, a primary hyphae extends into the first plant cell, whereby bulbous invasive hyphae differentiate filling the cell (Kankanala et al. 2007; Howard & Valent 1996). Primary hyphae which colonise the first plant cell following penetration lack nuclei, which subsequently move through the penetration peg into the hyphae upon establishment of infection, Figure 1.3. The colonisation of surrounding plant cells requires invasive hyphae to ‘search’ for plant plasmodesmata at the pit field sites (Kankanala et al. 2007). Kankanala et al. (2007) failed to observe the infection of guard cells, which lack plasmodesmata, surrounded by heavily infected cells, confirming plasmodesmata to be the sites at which *M. oryzae* spreads into neighbouring cells (Kankanala et al. 2007). For hyphae to pass through the plasmodesmata they must significantly reduce their diameter from 5µm to 0.5µm. However, the invasive hyphal pegs which pass through the plasmodesmata into the neighbouring cells are significantly larger than the diameter of plasmodesmata which suggests hyphae manipulate the plasmodesmata, increasing their aperture allowing the spread of infection (Cook et al. 1997). However, no localised cell wall degradation has been observed; therefore how *M. oryzae* manipulates the plasmodesmata remains unclear.

Upon infection of the first plant cell, colonisation of surrounding cells occurs after approximately 32-36 hpi and neighbouring cells are then colonised every 2-3 hours after

(Kankanala et al. 2007). After entering the adjoining cells, invasive hyphal pegs differentiate into invasive hyphae before developing into bulbous invasive hyphae. Invasive and bulbous hyphae maintain their viability as infection spreads during biotrophic growth and are contained within a plant-derived extra invasive hyphal membrane (EIHM), see Figure 1.3 (Kankanala et al. 2007).

A novel structure, termed the biotrophic interfacial complex (BIC), has been recently identified and observed to contain fungal effectors (Khang et al. 2010). The formation of BICs is directly linked to plant invasion and colonisation by *M. oryzae*. Following penetration, the primary hyphae secretes effectors into the membrane caps located in the EIHM at the hypha tip. The primary BIC was identified through its accumulation of fluorescent tagged effectors, next to the initial bulbous hyphae cell (Khang et al. 2010). The location of the primary BIC remains constant during colonisation of the first plant cell. After invasion of the surrounding cells the fluorescence of the primary BIC dissipates and fluorescence is detected in the membrane caps of invasive hyphae within neighbouring cells (Khang et al. 2010). These caps form secondary BICs, and in each case a single BIC is located next to the initial bulbous hyphal cell, Figure 1.3. The change in fluorescence between primary and secondary BICs was shown to be the result of effector translocation (Khang et al. 2010). Two specific effectors, Pwl2 (prevents pathogenicity toward weeping lovegrass) and Bas1 (biotrophy associated secreted protein 1), are secreted into the rice cytoplasm and move into un-colonised cells ahead of the invading hyphae. It is thought they potentially prepare the host cells for invasion (Khang et al. 2010). BICs therefore play an essential role in host colonisation.

Infected host cells remain viable until a late stage of colonisation, and in each case significant hyphae growth and neighbouring cell colonisation occurs before the host cell dies. The process of spreading via plant plasmodesmata corresponds with the biotrophic lifestyle phase of *M. oryzae* and is considered to be a defining characteristic of *M. oryzae* pathogenicity (Kankanala et al. 2007). However, the switch between invasive and bulbous hyphae, associated with a

biotrophic lifestyle, to necrotrophic hyphae and the release of cell wall degrading enzymes, associated with a necrotrophic lifestyle, is poorly understood.

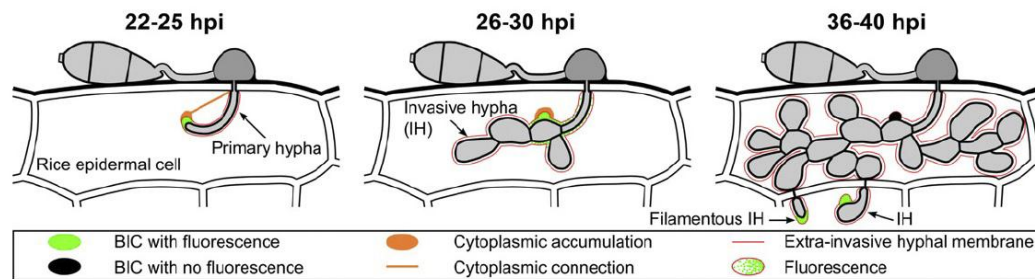


Figure 1.3. Diagram outlining the process of *M. oryzae* plant infection, detailing the location of the BIC and movement of fungal effectors. Adapted from Khang et al. (2010)

1.2 Nitric Oxide

Nitric Oxide (NO) is a universally important signalling molecule which has been well documented within animals, plants and bacteria. However little work regarding this important molecule has been completed within fungi, it therefore, represents a potentially exciting and unexplored area of research.

Nitric oxide (NO) was for many years considered to be a toxic gas, the product of the incomplete combustion of petrol and the oxidation of ammonia (Koshland 1992). It was thought that NO, like CO and CN⁻, bound irreversibly to protein metal centres and was toxic for life. These views were dramatically overturned by the discovery that NO controls mammalian vasodilation, and that it was in fact the elusive endothelium-derived relaxing factor (Ignarro et al. 1987; Palmer et al. 1987). Since its discovery as a signalling molecule, NO has been associated with a vast number of different physiological processes, spanning the Domains of Life.

1.2.1 Nitric oxide chemistry

NO is a stable, diatomic free radical molecule and is gaseous at room temperature. It has an unpaired electron in its anti-bonding π molecular orbital, rendering it highly reactive (McCleverty 2004). NO is redox-active in solution and rapidly undergoes oxidation and reduction forming the nitrosonium (NO^+) and nitroxide (NO^-) ions. NO has an approximately nine-fold greater solubility in hydrophobic solvents than in aqueous solutions, allowing it to act as both an intracellular and intercellular signalling molecule. Its high diffusion constant, calculated at $3300\mu\text{m}^2\text{s}^{-1}$, coupled with its estimated 150-300 μm average diffusion distance, mean that NO has a short half-life in biological systems (<10s) (Malinski et al. 1993; Hughes 2008).

In the presence of oxygen NO reacts to form nitrogen dioxide (NO_2), which, in aqueous solutions, reacts with additional NO to form nitrous anhydride (N_2O_3). N_2O_3 is a powerful electrophilic nitrosating agent, which acts as a mutagen through the nitrosation and deamination of nucleic acids (Caulfield et al. 1998). N_2O_3 is also responsible for post-translational protein modification via nitrosylation (Xu et al. 1998). Following nitrosylation, thiol groups are able to exchange NO between other thiol groups by trans-nitrosation, altering protein function (Bruckdorfer 2005). Nitrosylated proteins, specifically S-nitrosocysteine and S-nitroglutathione, act as NO donors, decomposing to release NO, extending the short half-life of *in vivo* NO from a matter of seconds to minutes or hours (Prince et al. 2010; Singh et al. 1996). The greater *in vivo* stability of nitrosylated proteins relative to NO increases the potential distance over which NO can function as a signalling molecule (McCleverty JA 2004).

In biological systems, NO rapidly reacts with other radical species generating new molecules with increased reactivity and toxicity; its reaction with superoxide (O_2^-) which forms the strong oxidising agent peroxynitrite (ONOO^-) has been well-documented. Peroxynitrite reacts directly with thiols, metal complexes, heme proteins and porphyrins (Radi et al. 1991; Goldstein & Czapski 1995; Ferrer-Sueta et al. 2003). At physiological pH, the protonated form of peroxynitrite (ONOOH) decomposes to form the hydroxyl radical ($\text{OH}^\cdot$), an exceptionally

reactive free radical, and the nitrogen dioxide ($\text{NO}_2^\cdot$) radical (Crow et al. 1994). The high reactivity of peroxynitrite results directly and indirectly in severe biological damage and is a potent inducer of cell death.

NO interactions with transition metals, especially ferrous heme proteins, are of particular biological importance with regards to signalling. The most well-studied of these is the reaction between the Fe(II) centre in the heme prosthetic group of soluble guanylate cyclase and NO, resulting in cGMP formation (Hughes 2008). cGMP initiates a signal cascade, inducing the activation of protein kinase G which, in turn, phosphorylates serine and threonine residues within other proteins, modulating their activity (Münzel et al. 2003).

The numerous biological targets with which NO and its related species react, coupled with its rapid diffusion coefficient, makes NO an ideal signalling molecule. Unsurprisingly, NO is an integral part of a vast number of physiological pathways, a small number of which are detailed below.

1.2.2 Roles of NO in animals

The identification of NO as a signalling molecule by Palmer 1987 and Ignarro 1987 represented the first example of NO in a physiological process (Ignarro et al. 1987; Palmer et al. 1987). Palmer et al. (1987) demonstrated the precursor for NO formation to be L-arginine, rather than D-arginine or urea, and that endothelium-dependent relaxation could be disrupted following the addition of L-NMMA (a nitric oxide synthase inhibitor) but not by its stereo-isomer D-NMMA (Palmer et al. 1988). NO signals endothelium-derived relaxation by stimulating cyclic GMP. This in turn inhibits RhoA kinase (Sauzeau et al 2003). RhoA kinase is activated by noradrenaline and intracellular calcium levels, and induces vasoconstriction by inhibiting the myosin light chain phosphatase (MLCP) and by activating the myosin light chain kinase (MLCK). Inhibition of RhoA kinase by cyclic GMP reduces phosphorylation of the myosin light chain and contraction of the smooth muscle, resulting in vasodilatation (Bruckdorfer 2005). Since its

discovery as the endothelium-derived relaxing factor, NO has been detailed as a signal in multiple physiological processes associated with the central nervous system. For example gastrointestinal function, neuronal differentiation and development, neuroprotection and memory (Friebe et al. 2007; Oh et al. 2010; Calabrese et al. 2007; Bouladakis et al. 2010).

NO however, is not only limited to cell signalling, but also plays a part in mammalian immunity, firstly, acting as an antimicrobial defence mechanism. Detection of pathogens leads to a rapid increase in NO synthesis from multiple immune specific cells (Bogdan 2001). NO, released by immune cells, kills or inhibits pathogen replication. Multiple studies have demonstrated the importance of NO in immunity, for example, Mastroeni et al. (2000) reported increased bacterial loads in *iNOS*^{-/-} mice after they were inoculated with *Salmonella typhimurium* (Mastroni et al. 2000). The readiness of NO to react with other free radicals and oxygen means its defensive activity is not limited to a single pathogen receptor or epitope, making NO an ideal antimicrobial defence molecule. Secondly, NO suppresses tumour growth. NO synthesised by macrophages and eosinophils inhibits enzymes required for tumour growth, including ornithine decarboxylase, ribonucleotide reductase and arginase, NO also induces tumour cell apoptosis through p53 accumulation and inhibits the tumour cell cycle (Pervin et al. 2001; Bauer et al. 2001).

Intriguingly, regarding tumours, NO functions as a double-edged sword, as it both inhibits and promotes tumour growth. All three *NOS* isoforms (see 1.3.1.1 for detailed information) have been detected within tumours isolated from breast, colon, cervix, head and neck tissue (Thomsen et al. 1995; Prazma et al. 1995). In each case *iNOS* expression was detected, with the highest expression levels recorded in the most invasive tumours. It is thought that NO promotes tumour growth through the regulation of angiogenesis (blood vessel growth); increased angiogenesis leads to accelerated primary tumour development and tumour metastasis (Folkman 1997).

1.2.3 Role of nitric oxide in plants

NO has not been as extensively studied in plants but has been identified in multiple physiological processes, including germination, leaf development and lateral root development (Wilson et al. 2008). NO has also been extensively studied with regard to i) abiotic stress and ii) plant innate immunity (Neill et al. 2008; Delledonne et al. 1998).

i) Multiple studies have confirmed the presence of NO during plant responses to abiotic stress, specifically during water deficit. Under water-limiting conditions cellular turgor pressure decreases, stimulating abscisic acid (ABA) generation (Neill et al. 2002). ABA triggers hydrogen peroxide (H_2O_2) synthesis by NADPH oxidase through a signal transduction process involving ABA receptors, calcium and calmodulin (Neill et al. 2002). H_2O_2 activates NO synthesis and induces stomatal closure through a combination of interactions with mitogen active protein kinases (MAPKs), Ca^{2+} and cGMP (Neill et al. 2008). NO is also involved as a key signalling intermediate in combating oxidative stress. Garcia-Mata & Lamattina (2001) observed that oxidative stress in wheat (*Triticum aestivum*) could be, in part, ameliorated through the addition of NO (Garcia-Mata & Lamattina 2001). Just how NO reduces the impact of oxidative stress is not understood. However current hypotheses suggest that NO interacts directly with free radicals such as superoxide, and converts them into less damaging molecules such as peroxynitrite, which are metabolised by separate physiological pathways (Neill et al. 2008). Alternatively NO elevates the expression of superoxide dismutase and catalase, which in turn convert superoxide to H_2O_2 , before being de-toxified to water and oxygen (Zhang et al. 2007). Evidence that NO ameliorates oxidative stress was reported by Zhao et al. (2007) who demonstrated increased sensitivity to oxidative stress in the *Arabidopsis thaliana* mutant *Atnos1*, which is impaired in NO synthesis (Zhao et al. 2007).

ii) NO plays an important role as a signal intermediate in plant innate immunity, in systemic acquired resistance (SAR). Following pathogen attack an increase in cellular salicylic acid (SA)

occurs which induces SAR in non-infected tissues (Moua Z 2003). SAR provides long-term protection from pathogens by inducing the expression of multiple pathogenesis related (PR) genes (Moua et al. 2003; Spoel et al. 2009). In *Arabidopsis thaliana* this pathway has been shown to be controlled by non-expressor of PR genes (Npr1), which translocates into the cell nucleus and activates PR gene expression (Delaney et al. 1995; Spoel et al. 2009). Tada et al. (2008) observed NO to be critical for translocation of Npr1 into the nucleus. In plants unchallenged by pathogens, Npr1 is localised to the cytoplasm as an oligomer, where it is conformationally constrained by a redox sensitive intermolecular disulfide bond (Tada et al. 2008). An increase in cellular SA causes nitrylation by S-nitrosoglutathione of the Npr1 disulfide bond, resulting in the breakdown of the Npr1 oligomer. The released monomer translocates into the nucleus and activates PR gene expression (Kinkema et al. 2000; Spoel et al. 2009). NO is also involved in the hypersensitive response (HR). HR is defined as *'an area of cell death that forms at the point of attempted pathogen ingress and which correlates with the exhibition of resistance a method by which plants limit the spread of infection by inducing rapid cell death in the cells surrounding the site of infection'* (Mur et al. 2008). Delledonne et al. (1998) showed that a combination of both H₂O₂ and NO were required for successful induction of cell death (Delledonne et al. 1998). The addition of the NO scavenger cPTIO to soybean cells previously induced for reactive oxygen species generation (ROS) inhibited the hypersensitive response. More recently, Boccara (2005) confirmed the link between NO and the HR through their work on the plant pathogenic bacteria *Erwinia chrysanthemi* and *Pseudomonas syringae* (Boccara et al. 2005). *Disruption of an NO scavenging flavohaemoglobin gene (HMPX) in E. chrysanthemi resulted in increased NO levels within the plant tissue, which induced the HR and suppressed pathogen invasion. Furthermore, the introduction of the HMPX gene into a non-host compatible P. syringae strain dramatically reduced the levels of HR within soy bean cells (Boccara et al. 2005). Therefore, NO is required to elicit the HR and prevent pathogen invasion.*

NO has yet to be extensively studied within rice, however the stress tolerance and SAR pathways are present within rice, meaning that NO generation is predicted to occur. As the importance of rice increase it is likely that more studies will be undertaken to fully explore the role NO plays.

1.2.4 The role of NO in fungi and slime moulds

In contrast to both animals and plants, the study of NO in fungi and slime moulds has been limited. A detailed search of the literature revealed that, from 1996 to 2011, fewer than 20 papers have been published detailing NO and its function(s) in fungi. These studies rely on pharmacological approaches to understand NO and its synthesis, and no fungal *NOS* genes have been identified, nor corresponding deletion mutants generated. Therefore, our current understanding of NO in fungi (and oomycetes) is very limited.

Three separate studies have reported a link between NO and conidiation. i) Ninnemann & Maier (1996) first identified a potential role for NO in the photoconidiation of *Neurospora crassa* (Ninnemann & Maier 1996). They showed that exogenous NO supplied via the NO donor SNP dramatically inhibited dark and light stimulated conidiation. The addition of mammalian NOS inhibitors, L-NA and L-NAME, but not their stereo isomers or L-arginine, resulted in increased *N. crassa* conidiation in the dark. They concluded that NO is synthesised via animal NOS-like enzymes and that it negatively regulates photoconidiation. ii) Gong et al. (2007) disrupted *Cmcps1* in *Coniothyrium minitans*, an L-arginine specific carbamoyl phosphate synthase, and found that the resulting mutant ($\Delta cmcps1$) displays reduced conidiation which could be restored to wildtype conidiation levels following treatment with L-arginine or SNP (Gong et al. 2007). The addition of L-NAME to the wildtype strain significantly suppressed conidiation, but had no effect on $\Delta cmcps1$. Staining of the wildtype strain with the NO sensitive dye DAF-FM DA revealed strong fluorescence in the undifferentiated pycnidia primordia, suggesting the involvement of NO in conidiation. The Griess assay was used to quantify NO generation and revealed significantly reduced NO levels in $\Delta cmcps1$ relative to the wildtype strain. iii) Most

recently, Li et al. (2010) detected NOS-like activity in *C. minitans* wildtype strain and demonstrated that cGMP is required for conidiation through the reversible inhibition of guanylate cyclase (GC) with ODQ (1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one) (Li et al. 2010). The addition of the NOS inhibitor L-NNA to ZS-1 reduced NO generation and inhibited conidiation; interestingly L-NNA also reduced the level of cGMP. These data represents the first evidence of a potential fungal NO signalling pathway using cGMP, mirroring the well-documented examples in animals. The addition of exogenous NO from SNP instantly recovered cellular cGMP levels, but exogenous cGMP could not restore conidiation in $\Delta cmcps1$ suggesting that cGMP is only one of the signalling molecules NO induces for successful conidiation (Li et al. 2010). These data indicate that NO is a positive regulator of conidiation in *C. minitans*.

Two studies have reported NO to be involved with fungal development. i) Wang & Higgins (2005) investigated the role of NO in the germination and development of *Colletotrichum coccodes* (Wang & Higgins 2005). Using the NO sensitive dye, DAF-FM DA, they 'observed' NO in germinating conidia and developing germings, with the strongest fluorescence observed in mature appressoria. Addition of the NO scavenger cPTIO reduced the observed fluorescence by 50%, indicating NO as the molecule responsible for eliciting fluorescence. A combination of treatments of NOS inhibitors, L-NMMA and L-NNA, and the NO donor SNP revealed a negative regulatory role for NO in germling development. Conidia treated with SNP exhibited significantly suppressed germination and development, but conversely the addition of either L-NAME or L-NNA accelerated germination. The D-stereo isomers of L-NAME and L-NNA had no impact on germination. From these data, Wang & Higgins (2005) concluded that NO, generated through animal-like NOS, positively regulates *C. coccodes* germination and development. ii) Prats et al. (2008) reported NO and its involvement in appressorium formation in *Blumeria graminis*. Utilising the NO sensitive dye DAF-2 DA, they observed fluorescence generation within appressorium germ-tubes during appressorium maturation (Prats et al. 2008). To confirm that DAF-2 DA correctly reports NO they applied cPTIO and L-NAME to DAF-2 DA loaded

germlings, and, in both cases, the treatment suppressed fluorescence. Germlings treated with L-NAME, but not D-NAME, also significantly reduced the number of appressorial lobes. Prats et al. (2008) concluded that NO is required for appressorium maturation in *B. graminis*.

NO may play both a cytostatic and a cytoprotective role in *Saccharomyces cerevisiae*. Domitrovic et al. (2003) treated cell cultures, in their exponential growth phase, with the NO donors SNP and SNAP (Domitrovic et al. 2003). At low concentrations, <1mM, neither treatment affected the cell budding rate, whilst at >1mM concentrations, NO increased the budding time suggesting NO has cytostatic activity. At low levels, NO was found to elicit a cytoprotective response in yeast cells. Significantly, Domitrovic et al. (2003) detected all three animal NOS isoforms in *S. cerevisiae* via Western blotting analyses using rabbit anti-NOS2/NOS3 and mouse anti-NOS1 antibodies. To date this remains the only example of animal NOS isoforms being demonstrated in fungi.

Xu (2011) suggested a role for NO and ABA in regulating trap formation by the nematode trapping fungus *Drechslerella stenobrocha* (Xu et al. 2010). The *in vitro* addition of 1-100µM ABA to *D. stenobrocha* greatly increased both the number of traps and percentage of trapped nematodes. The addition of 100µM SNP abolished trap formation and simultaneous addition of either L-NNA or cPTIO did not restore trap formation. The addition of L-NNA slightly increased trap formation, however, coupled with ABA a dramatic increase in trap numbers was observed. These data suggest that the trap formation is upregulated by ABA and down regulated by NO.

Golderer et al. (2001) identified and cloned an animal like NOS from the Mycetozoa slime mould *Physarum polycephalum*, the first and only example of animal-like NOS to be identified in a multicellular organism outside of the Animalia (Golderer et al. 2001). The cloned *P. polycephalum* NOS shares 39% amino acid identity with animal NOS and carries all the required NOS co-factor binding sites (see 1.3.1.1 for further information), with the exception of the calcium binding site. As in *C. minitans*, *P. polycephalum* NOS was significantly upregulated

during sporulation, suggesting a role for NO in this process. Sporulation was repressed following the addition of the NOS inhibitor NIL (L-N6-(1-iminoethyl)-lysine) and the guanylate cyclase (GC) inhibitor ODQ. These data suggest that NO and GC, synthesised by NOS and cGMP respectively, act as regulatory signals for sporulation. This remains the closest example to animal NO/cGMP signalling in lower eukaryotes.

1.2.5 The role of NO in Prokaryotes

Despite the well-documented presence of NOS within Prokaryotes it has been only relatively recently that the biological roles of prokaryote NO have been described.

Investigations into a pathogenicity island common to certain *Streptomyces* strains provided the first information as to the role of NO in prokaryotes (Kers et al. 2004). *Streptomyces turgidiscabies* NOS (*stNOS*) lies in close proximity to two nonribosomal peptide synthases which are essential for the thaxtomin (toxin which inhibits plant cell wall synthesis) biosynthesis pathway. Kers et al. (2004) suggested that NO generated by *stNOS* may nitrate thaxtomins (Kers et al. 2004). The addition of animal NOS inhibitors greatly reduced thaxtomin synthesis and the disruption of *stNOS* virtually abolished thaxtomin synthesis, which suggests that *stNOS* is closely related to animal-like NOS and is the source of NO synthesis (Wach et al. 2005). Thaxtomin synthesis was recovered following *stNOS* complementation. Johnson et al. (2008) postulated that *Streptomyces* may also influence plant growth via NO production, by increasing root tip extension, resulting in increased potential for bacterial invasion (Johnson et al. 2008)

In *Deinococcus radiodurans* NO has been shown to play a cytoprotective role. The disruption of *D. radiodurans* NOS (*drNOS*) increases its susceptibility to UV radiation, suppressing its growth (Patel et al. 2009). $\Delta drNOS$ displays moderately reduced growth relative to the wildtype in a non-stressed environment; however exposure to UV radiation greatly exaggerated this impaired growth phenotype. $\Delta drNOS$ phenotype was recovered following its transformation with a plasmid carrying a functional NOS (Patel et al. 2009).

NO also has a cytoprotective role in *Bacillus* species, protecting against oxidative stress. Following disruption of *Bacillus subtilis* NOS (*bsNOS*), the mutant displays elevated susceptibility to oxidative stress from reduced thiols (Gusarov et al. 2005). The presence of reduced thiols induces the Fenton reaction, which recycles ferrous iron, leading to the generation of the toxic hydroxyl radical (OH[•]) and hydroxyl anion (OH⁻). S-nitrosylation of thiols by NO is thought to inhibit ferrous iron recycling, disrupting the thioredoxin system, suppressing oxidative damage (Aruoma et al. 1989).

1.3 Nitric oxide generation

NO is involved in myriad physiological processes and is synthesised via a number of separate biochemical pathways depending on the organism. This section details the more important NO synthesising biochemical pathways.

1.3.1 Nitric oxide synthase

1.3.1.1 Animal NOS

NO in animals is generated by a family of nitric oxide synthase (NOS) enzymes. Three separate NOS isoforms exist, each encoded by a separate gene: neuronal NOS (*nNOS*, *NOS1* and type 1), inducible NOS (*iNOS*, *NOS2* and type 2) and endothelial NOS (*eNOS*, *NOS3* and type 3) (Alderton et al. 2001). nNOS is localised to nerve cells, skeletal muscle and heart muscle; iNOS is located in

multiple cell types, most of which are associated with the immune system; and eNOS is localised in vascular endothelial cells (Hall et al. 1994; Geller et al. 1993; Janssens et al. 1992). *nNOS* and *eNOS* are constitutively expressed within their respective tissues. Activation of *nNOS* and *eNOS* requires the binding of both calmodulin (CaM) and Ca^{2+} . The activity of *nNOS* and *eNOS* is strictly regulated, reflecting the role of NO as a signalling molecule (Forstermann et al. 1998). *iNOS* exhibits a strong affinity for CaM at physiological concentrations and is considered to be calcium insensitive (Bredt & Snyder 1990).

NOS catalyse the two step oxidation of L-arginine to citrulline and NO. During this reaction NOS first catalyse the N^{ω} -hydroxylation of arginine, forming N^{ω} -hydroxy-L-arginine (NOHA), Figure 1.4. Secondly, NOS cleave the $\text{C}=\text{N}(\text{OH})$ bond of NOHA, generating citrulline and NO. Step one involves a first monooxygenation reaction requiring one molecule of oxygen and NADPH, the second monooxygenase reaction consumes one molecule of oxygen and half a molecule of NADPH during the three-electron oxidation of NOHA (Bruckdorfer 2005).

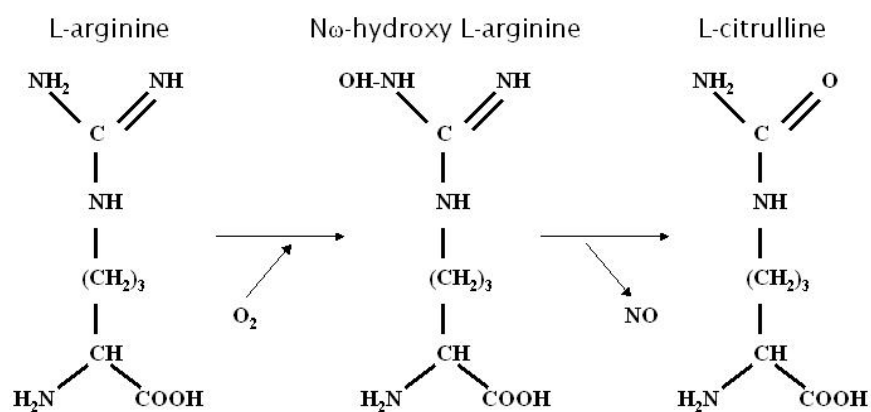


Figure 1.4. NOS catalysed NO synthesis from L-arginine, NADPH and O_2 adapted from (Bruckdorfer 2005).

Functional animal NOS isoforms (*nNOS*, *iNOS* and *eNOS*) are arranged as tetramers, carrying two NOS monomers and two calmodulins, Figure 1.5. Each NOS monomer consists of a C-terminal oxygenase and an N-terminal reductase domain, linked by a 32 amino acid calmodulin binding site (Knowles & Moncada 1994). The reductase domain carries flavine adenine

dinucleotide (FAD) and flavin mononucleotide (FMN) cofactors and NADPH binding sites, whilst the reductase domain carries the L-arginine, heme and tetrahydrobiopterin (BH₄) binding sites. These cofactors are deemed essential for NOS-derived NO synthesis (Marletta 1994). Calmodulin was the first protein identified to bind to NOS and increases the electron flow from NADPH to the reductase domain. BH₄ binding is also essential for NOS function as it stabilises the NOS tetramer, acts as an electron donor during oxygen activation and reclaims an electron from the ferrous nitrosyl complex to allow NO release (Matsuda & Iyanagi 1999). FAD and FMN cofactors are required for electron donation from NADPH to haem, via the formation of semiquinone radical intermediates. The transfer of electrons between FAD and FMN domains is thought to be under the control of CaM, and, in its absence, electron flow is reduced significantly (Matsuda & Iyanagi 1999).

NOS isoforms have several important differences. eNOS and nNOS both carry auto-inhibitory loops, represented by a 40-50 amino acid insert in their FMN sub-domains. These loops negative regulatory NO synthesis, destabilising CaM binding at low calcium concentrations and preventing electron flow from FMN to heme, thus inhibiting NO synthesis (Nishida & Ortiz de Montellano 1999). nNOS carries an N terminal 220 amino acid PDZ domain (PSD-95 discs large/ZO-1 homology domain), targets nNOS to the plasma membrane in neurons and skeletal muscle, Figure 1.5 (Brenman et al. 1996). eNOS is acylated by both myristate and palmitate, required for its localisation within endothelial cells caveolae (Michel 1999).

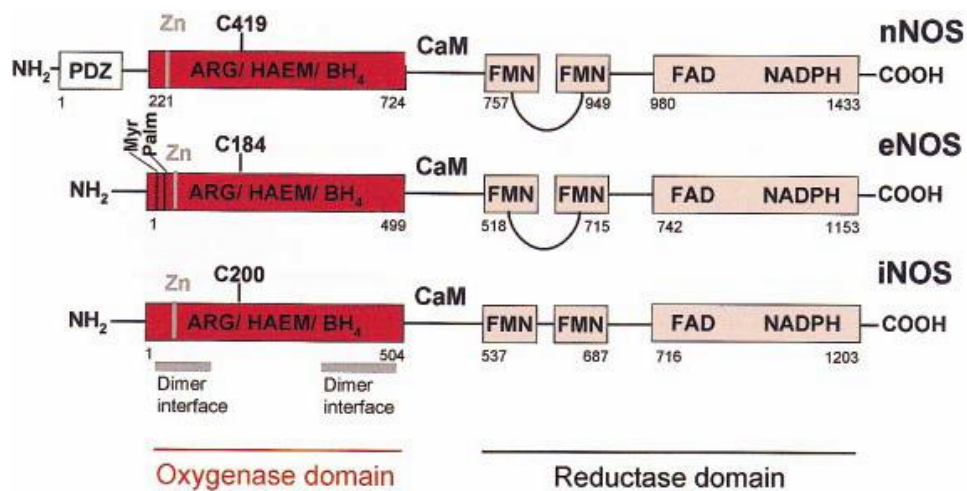


Figure 1.5. Animal nNOS, eNOS and iNOS protein structures. Adapted from Alderton et al. (2001).

Regulation of NOS gene expression and protein activity is achieved through a variety of mechanisms. Mammalian iNOS differs from eNOS and nNOS in that, following its expression, the resulting protein has little regulation. This widely reflects its role in the immune system (Kleinert et al. 2004). *iNOS* is therefore predominantly controlled at the transcript level (Ghosh et al. 1998). Lipopolysaccharides (LPS), Interleukin-1 beta (IL-1 β), Tumour necrosis factor-alpha (TNF- α) and oxidative stress all induce *iNOS* expression by interacting with the NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) protein complex (Upreti et al. 2004; Meraz et al. 1996). By contrast, glucocorticoids, Transforming growth factor beta 1 (TGF- β 1) and antioxidants suppress *iNOS* expression (Upreti et al. 2004; Meraz et al. 1996). eNOS and nNOS activities are regulated by intracellular calcium levels which, at high concentrations, induce CaM binding and protein kinase phosphorylation. In eNOS such phosphorylation leads to an increase in electron flow through the reductase domain, increasing NO synthesis. By contrast, phosphorylation of nNOS suppresses NO synthesis (Nakane et al. 1991; Komeima et al. 2000). nNOS is subject to alternative mRNA splicing, resulting in the generation of splice variants. Four nNOS splice variants (nNOS β , nNOS γ , nNOS μ and nNOS-2) have been isolated from specific

tissues, however their generation and biological significance remain poorly understood (Eliasson et al. 1997; Silvagno et al. 1996).

Aside from the well-documented nNOS, eNOS and iNOS isoforms it has been suggested that there are other forms of NOS; mitochondrial NOS (mtNOS) being the most cited example. Immunohistochemical, biochemical and electrochemical studies have provided evidence for the existence of mtNOS. However, technical difficulties associated with protein extraction combined with conflicting published data means that the existence of an mtNOS catalysing the two step conversion of arginine and oxygen to citrulline and NO remains a contentious issue (Dedkova & Blatter 2009).

1.3.1.2 Plant NOS

The enzymatic synthesis of NO in plants by animal-like NOS is one of the most controversial areas of plant biochemistry. Over the past 20 years multiple publications have reported the existence of NOS-like enzymes capable of arginine dependent NO synthesis (Moreau et al. 2010). These studies have mainly utilised pharmacological approaches, NOS inhibitors, NO donors and scavengers. Delledonne et al. (1998) observed a decrease in *A. thaliana* pathogen resistance, following treatment with the NOS inhibitor L-NNA (Delledonne et al. 1998). Subsequently multiple studies have reported the existence of an arginine dependent NO synthesis pathway (Besson-Bard et al. 2009). Indeed, several papers have reported the conversion of arginine to citrulline via proteins considered to be NOS-like enzymes (Corpas et al. 2006). However, these conclusions have been challenged for the following reasons: i) exogenous application of L-arginine derived NOS inhibitors and NO scavengers may impact on other physiological pathways, alter plant phenotypes and bias findings; ii), the L-citrulline assay, used to measure NOS activity by incubating extracted putative NOS and cofactors with radioactive L-arginine and recording the synthesis of radioactive citrulline, is problematic. Tischer et al. (2007) demonstrated that radioactive citrulline, thought originally to be

synthesised by *A. thaliana* NOS, was, in fact, synthesised by argininosuccinate lyase, a urea cycle enzyme which generates argininosuccinate from L-arginine and fumarate (Tischner et al. 2007).

Guo et al. (2003) identified *AtNOS1* as a putative NOS gene; its disruption generated a mutant with reduced NO levels (Guo et al. 2003). Atnos1 shares sequence homology with a protein linked to NO synthesis in the snail *Helix pomatia*, however neither protein is structurally similar to animal NOS. Guo et al. (2003) reported that purified Atnos1 used arginine and NADPH as substrates and was activated by calcium (Guo et al. 2003). More recently, the ability of Atnos1 to synthesise NO has been questioned and several independent researchers have confirmed that it is not a 'true' NOS. Indeed, Atnos1 was renamed Atnoa1 (*A. thaliana* nitric oxide-associated protein 1) and is thought to be part of the GTPase family involved in mitochondrial ribosome biogenesis (Zemojtel et al. 2006; Moreau et al. 2008). Our current understanding of the role that Atnoa1 plays in NO generation and accumulation is limited: under stressed conditions Δ *Atnoa1* exhibits reduced NO accumulation. However, when non-stressed Δ *Atnoa1* can accumulate wildtype levels of NO (Bright et al. 2006). Interestingly, Flores-Perez (2008) identified At3g47450, the *AtNOS1/AtNOA1* gene, as being part of the plastid localised methylerythritol phosphate pathway. Treatment with the NO donor sodium nitroprusside (SNP) failed to restore fully the wildtype phenotype in the *rif1/Atnoa1* mutant suggesting that the link between *AtNOA1* and NO synthesis is indirect (Flores-Perez et al. 2008). In short, the hunt for an animal-like plant NOS enzyme continues.

1.3.1.3 Bacterial NOS

NOS genes have been well studied in bacteria. Initial investigations into bacterial NOS began with pharmacological experiments designed to detect NOS activity. The first report of a bacterium with NOS-like activity was recorded for a 52 kDa enzyme purified from a *Nocardia* species, which catalyses the conversion of radio-labelled arginine to citrulline (Chen 1994). The addition of animal NOS inhibitors suppressed the formation of citrulline. Similarly, Sari (1998)

observed NOS-like activity in *Rhodococcus* sp. R312, after it successfully converted L-arginine and N-hydroxy-L-arginine (NOHA) to citrulline. This activity was inhibited by the addition of animal NOS inhibitors and increased following H₄B addition (Sari 1998). Choi et al. (2000) purified a 93 kDa protein from *Salmonella typhimurium* which generates citrulline and NO (Choi et al. 2000). However, in each example, the corresponding bacterial genomes carry no obvious NOS homologues. Therefore, the conclusion that NOS-like activity suppressed by animal NOS inhibitors is evidence for a NOS-like enzyme, must be viewed sceptically.

More recently, conclusive evidence for bacterial NOS arrived following genome sequencing; multiple genes were identified with strong sequence similarity to animal NOS genes, particularly from Gram positive bacteria, *Bacillales*, Actinobacteria and *Deinococcus* (Crane et al. 2010). With a single exception bacterial NOS do not carry contiguous reductase domains. This raises the question, what do they use as a reductase domain? Phylogenetic analyses report NOS gene clustering between species, which indicates evidence for horizontal gene transfer (HGT) (Sudhamsu & Crane 2009). The first bacterial NOS to be cloned was from *Deinococcus radiodurans* and when expressed in *E. coli*, Adaka et al. (2002) reported the protein forms a dimer complex, containing heme and generates nitrate and nitrite from arginine when fused with a mammalian reductase domain protein (Adak et al. 2002). To date, including *D. radiodurans* multiple NOS genes have been identified and cloned including *Staphylococcus aureus*, *Bacillus anthracis*, *Streptomyces turgidiscabies* and *Geobacillus stearothermophilis* (Crane et al. 2010).

The cofactor H₄B is essential for animal NOS function and is required by some bacterial NOS such as *S. aureus* where it increases the binding affinity of arginine and NOS; intriguingly, H₄B has no impact on the arginine binding affinity to NOS in *B. anthracis*, suggesting significant variation between bacterial NOS (Midha et al. 2005).

So, what associates with bacterial NOS? Adak et al. (2002) observed that *D. radiodurans* NOS synthesises NO when fused with a 'surrogate' mammalian reductase domain, implying that bacteria NOS may bind to a separate protein which exhibits reductase activity in order to complete NO synthesis (Adak et al. 2002). Wang et al. (2007) tested the ability of flavin-containing reductases from *Bacillus subtilis* to donate their electrons to its putative NOS. They identified the flavin-containing reductases YkuN and showed that when coupled with *B. subtilis* NOS the protein complex supports NO synthesis at comparable rates to animal NOS (Wang et al. 2007). Gusarov et al. (2008) reported that NOS from both *B. subtilis* and *B. anthracis* are able to 'hijack' cellular reductases, following their expression in *E. coli*. This remarkable finding suggests multiple reductase proteins may be able to support NO synthesis in bacteria (Gusarov et al. 2008). Currently only one NOS gene, identified in *Sorangium cellulosum*, contains both oxygenase and reductase domains (Schneiker et al. 2007).

1.3.1.4 Fungal and slime mold NOS

Fungi and slime molds are poorly studied with regards to NO synthesis and signalling. Of the few studies published on the role of NO in fungi, eight indicate NOS-like activities through the exogenous application of animal NOS inhibitors (Almeida et al. 2007; Prats et al. 2008; Wang & Higgins 2005; Ninnemann & Maier 1996; Gong et al. 2007; Song et al. 2000; Vieira et al. 2009; Maier et al. 2001).

However, these data provide little in the way of evidence for animal-like NOS enzymes in fungi. It is possible that the application of animal NOS inhibitors, as in plants, interfere with unrelated biochemical pathways, resulting in the observed fungal phenotypes. To date only two publications report credible findings of NOS in fungi and slime moulds. Domitrovic et al. (2003) identified animal NOS isoforms in *S. cerevisiae* during different growth phases (Domitrovic et al. 2003). Using Western blot analyses these researchers identified three animal NOS isoforms in yeast cells, during first and second exponential growth phases. The molecular masses of

identified yeast proteins were 160, 130, and 132 kDa, similar molecular weights to mouse NOS isoforms (Domitrovic et al. 2003). However, no animal-like NOS homologues have been identified in the *S. cerevisiae* genome, which casts doubt on this Western blot data.

Golderer et al. (2001) identified and cloned two *NOS* genes in the slime mould *Physarum polycephalum*. This remains the only published finding of animal NOS-like proteins outside of mammals (Golderer et al. 2001). Through screening of *Physarum* cDNA libraries, two NOS cDNAs called *phynosa* and *phynosb* were identified. Analysis of their protein sequences reveals they carry all the required mammalian NOS cofactor binding sites: FMN, FAD, NADPH, calmodulin and H₄B. Phynosa and Phynosb also show structural similarity to mammalian iNOS, as they do not carry the spacer sequence required for calcium activation. However, Golderer et al. (2001) did not generate *phynosa* and *phynosb* disruption mutants, limiting our understanding of the role NO plays in *Physarum polycephalum*.

In 2007 Mary Illes identified a series of putative *NOS* genes in the *M. oryzae* genome and, using targeted gene replacement, generated a disruption mutant termed $\Delta nos3$ (Mary Illes, DPhil Thesis, Oxford 2007). At the time of completion, this study was the first to have disrupted a putative *NOS* gene in a fungus.

1.3.2 Nitrate reductase

The role of nitrate reductase (NR) in nitrogen assimilation has been well studied, where it catalyses the reduction of nitrate to nitrite. Less well-studied is its ability to also catalyse the production of NO (Dean & Harper 1986). Unlike NOS, NR has been identified across all the Domains of Life.

1.3.2.1 Plant nitrate reductase

In plants, NR catalyses the first step of the nitrogen assimilation pathway, the NAD(P)H dependent reduction of nitrate to nitrite.



This pathway has been well-studied in plants and begins with NADH reducing the flavin adenine dinucleotide (FAD) domain at the first active site; the electrons are passed via cytochrome b to the molybdenum-molybdopterin (Mo-MPT) domain where nitrate is reduced to nitrite in the second active site, Figure 1.6 (Campbell 1999). NR is also thought to catalyse the ferric citrate reduction too (Redinbaugh & Campbell 1991).

Prior to the discovery of NOS in mammals, Dean & Harper (1986) reported the synthesis of NO by NR in soybean. They observed that a mutant deficient in NR activity was unable to generate NO, subsequently NR derived NO synthesis has been confirmed both *in vitro* and *in vivo* (Dean & Harper 1986; Yamasaki & Sakihama 2000; Rockel et al. 2002).



Figure 1.6. Nitrate reductase protein structure, detailing its five domains, Mo-MPT, cytochrome b (heme binding), FAD, NADH and dimer interface. Adapted from (Campbell 1999).

Functional NR is a homodimer (or in some examples a tetramer) containing two 100 kDa subunits. 3D structural analyses of NR reveal that each subunit carries five distinct domains: i) Mo-molybdopterin (Mo-MPT), ii) cytochrome b (heme binding), iii) FAD, iv) NADH and v) dimer interface (Campbell 1999). Protein sequence analysis highlights clear domain conservation between NRs across the Eukaryotes, and reveals three sequence locations which differ dramatically between species NR: i), the N-terminal region, which frequently contains additional acidic residues; ii), the first hinge, which contains a phosphorylation site; iii), hinge two, which contains a proteinase site (Campbell 1999). An interesting aspect of NR structure is that individual domains appear to have evolved independently before fusing to form the multi-

domain NR present in plants. For example, cytochrome b domain found in NR is closely related to Eukaryote cytochrome b_5 and its Mo-MPT domain is closely related to sulphite oxidase (SOX), one of only three other enzymes to use molybdenum as a cofactor (Nishida et al. 1995; Kisker C et al. 1997). SOX reduces sulphite to sulphate in mitochondria (Mendel 1997). Hyde & Campbell (1990) expressed a fragment of maize NR in *E. coli* and noted its similarities to mammalian cytochrome B_5 reductase and suggest a modular evolution for NR (Hyde & Campbell 1990).

An unusual feature of NR is the existence of NADH, NADPH and NAD(P)H bi-specific forms: why are there different forms of NR? Several hypotheses have been suggested including, i) multiple functions within separate physiological pathways. NAD(P)H NR in soybean is constitutively expressed without requirement for nitrate, it is thought to be involved in separate biochemical pathways aside from nitrate assimilation (Campbell 1989). NAD(P)H NR has been demonstrated to generate NO. ii) NR protein structure: relatively small changes in the pyridine nucleotide binding domain of NR are able to change the NR from NADPH to NADH specific. It is thought bi-specific forms arise following a partial conversion from one specific form to another (Scrutton et al. 1990; Shiraishi et al. 1998).

NR activity is controlled at two levels: transcriptional regulation and protein activation. Vincentz & Caboche (1991) reported that nitrate regulates the expression of NR genes, wildtype *Nicotiana plumbaginifolia* grown on nitrate shows high levels of NR mRNA expression, however, when grown on ammonium no NR mRNA was detected (Vincentz & Caboche 1991; Jolma et al. 2010; Krouk et al. 2010). Vincentz & Caboche (1991) also reported NR mRNA expression to be light-regulated: wildtype plants placed in darkness had no detectable NR mRNA, however, within four hours following transition from dark to light, NR mRNA levels increase. Interestingly, NR protein activity does not correspond with its transcript abundance. NR activity is suppressed following covalent modification by phosphorylation, in association with the transition from light dark and the presence of Ca^{2+} and Mg^{2+} cations (Kaiser & Brendle-Behnisch 1991; MacKintosh 1992).

1.3.2.2 NR in bacteria

Unlike Eukaryotes, bacteria carry three separate classes of NR: cytoplasmic assimilatory (Nas), membrane bound respiratory (Nar) and periplasmic dissimilatory (Nap) (Stolz & Basu 2002). Bacterial *NR* genes carry the molybdenum (Mo) cofactor binding domain, however, unlike Eukaryotes bacterial NRs contain molybdopterin-guanine dinucleotide rather than molybdopterin-guanine mononucleotide (Morozkina & Zvyagilskaya 2007).

Bacterial cytoplasmic assimilatory NR (Nas) are split into two separate groups, ferredoxin/ flavodoxin-dependent Nas and NADH-dependent Nas. Ferredoxin/ flavodoxin-dependent Nas carry Mo and [Fe-S] clusters in their active centres, but do not contain FAD or cytochrome domains (Rubio et al. 1999). NADH dependent Nas contain two separate subunits, a 45 kDa FAD containing subunit and a 95 kDa Mo and N-terminal containing subunit (Moreno-Vivián et al. 1999). *Nas* genes are frequently located in the same operon as other nitrate assimilation and nitrate transporter genes, as for example in *Klebsiella oxytoca (pneumoniae)* (Wu & Stewart 1998).

Bacterial membrane bound respiratory NR (Nar) are associated with denitrification and anaerobic nitrate respiration and are widespread among enterobacteria (Morozkina & Zvyagilskaya 2007). NarGHI has been extensively researched in enterobacteria, particularly in *E. coli* (Zumft 1997). NarGHI carries three separate subunits, α , β and γ , each of which is encoded by a separate gene: i) *NARG* which encodes the α -subunit, carries the [4Fe-S] and Mo cluster domains and is located in the cytoplasm; ii) *NARH* which encodes the β -subunit carries the [Fe-S] clusters and is also localised to the cytoplasm; iii) *NARI* which encodes the γ -subunit carries a five membrane spanning domain and two b-type hemes, and is localised to the membrane (Rothery et al. 1998; Jepson et al. 2004; Gregory et al. 2003). NarGHI functions by generating a transmembrane proton gradient, which allows ATP synthesis by utilising nitrate as an electron

acceptor under anaerobic conditions (Morozkina & Zvyagilskaya 2007). Because the NarGHI active site is localised to the cytoplasm, nitrate must be translocated first into the cell before reduction can occur (Moreno-Vivián et al. 1999). NarGHI is regulated by oxygen availability and is expressed in anaerobic conditions or in the presence of nitrate or nitrite (Härtig et al. 1999).

Bacterial periplasmic dissimilatory NR (Nap) is heterodimeric consisting of two subunits: a 90 kDa unit containing an Mo and [4Fe-4S] centre, and a 19 kDa subunit containing two heme cytochrome c (Stewart et al. 2002). Despite being periplasmically localised, Nap do not generate transmembrane potentials, but similar to Nar are encoded by multiple genes (Stewart et al. 2002). Nap are expressed under anaerobic environmental conditions, their physiological role is thought to vary between bacteria and may even alter in the same bacteria under differing metabolic conditions (Moreno-Vivián et al. 1999). Stewart (2002) reported that Nap may function in anaerobic respiration, supporting the hypothesis that Nap activity increases when low nitrate concentrations reduce the efficiency of Nar nitrate respiration (Stewart et al. 2002).

1.3.2.3 NR in fungi

Fungal NRs have received considerably more attention than fungal NOS, with a large number of studies published that give particular regard to fungal nitrogen metabolism. Intriguingly, not all fungi contain NR, for example the model yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* cannot assimilate nitrate (Böer et al. 2009). Fungal NRs are also separated into three separate groups based on their function: nitrate assimilation, denitrification and ammonium fermentation.

Fungal nitrate assimilation follows the same pathway as detailed in plants: nitrate is transported into the cell, via a dedicated nitrate transporter (Böer et al. 2009). Nitrate is reduced to nitrite by via NAD(P)H dependent NR, nitrite is further reduced to ammonium by nitrite reductase (NIR) before being incorporated into glutamate (Krappmann & Braus 2005). Nitrate assimilation genes in fungi are often clustered, such as in *Aspergillus nidulans*, however, many fungi carry

non-clustered nitrate assimilation genes, such as *M. oryzae* (Johnstone et al. 1990). Boer et al. (2009) characterised the nitrate assimilation gene cluster in the yeast *Arxula adeninivorans*, and identified a 9 Kb gene cluster on chromosome 2 which encode three genes *AYNT1*, *AYNR1* and *AYNI1*, nitrate transporter, NR and NIR, respectively (Böer et al. 2009). Sequence analysis of *AYNR1* revealed 50% homology to NR from *Hansenula polymorpha*, *Aspergillus oryzae*, *Aspergillus terreus* and *Aspergillus nidulans*. *AYNR1* also contains the required domains for a functional NR including Mo-co, cytochrome b₅, FAD and NADH binding domains. Promoter and transcript analyses revealed that *AYNR1* expression (along with *AYNT1* and *AYNI1*) is upregulated in the presence of nitrate and suppressed in the presence of ammonium. NR nitrate regulation is common in both yeasts and filamentous fungi such as *A. nidulans* (Cove & Pateman 1969). As well as being nitrate regulated, NR in the yeast *H. polymorpha* is regulated by two transcription factors, YNA1 and YNA2, which are essential for NR expression (Avila et al. 1998). Interestingly, yeasts (*Candida nitratophila* and *Candida utilis*) simultaneously metabolise ammonium and nitrate, indicating that ammonium neither suppresses NR expression nor down regulates NR activity, as is the case in *A. adeninivorans* (Navarro et al. 2003). Christensen et al. (2004) reported that *N. crassa* NR activity oscillates on a circadian time scale when nitrate is the sole nitrogen source (Christensen et al. 2004). These studies found that NR activity oscillated in both dark and continuous light environments. However, disruption of *FRQ* (a gene essential for circadian rhythms in *N. crassa*) had no impact on the oscillating activity of NR, which indicates that the rhythm is not circadian regulated. However, the rhythm was abolished following the addition of ammonium as a separate nitrogen source. This represents the only example of a nutritionally induced rhythm in fungi (Jolma et al. 2010).

Fungi NR denitrification is an anaerobic process through which nitrate is reduced to nitrite, and then further reduced to nitrous oxide (N₂O). This process requires three enzymes: NR, NIR and nitric oxide reductase (NOR) (Fujii & Takaya 2008). Denitrification, originally thought to only occur in Prokaryotes, was identified in *Fusarium oxysporum* by Shoun & Tanimoto (1991). It has

since been confirmed in ascomycetes, zygomycetes and basidiomycete fungi (Shoun & Tanimoto 1991; Tsuruta et al. 1998). In *F. oxysporum* and *Cylindrocarpon tonkinense* denitrification is linked with ATP synthesis, resembling the nitrate respiration system in *E. coli* (Kim et al. 2010). *F. oxysporum* NR is localised within the mitochondria, and thought to be found in the inter-membrane space, similar to bacterial Nar (Kobayashi et al. 1996). Analysis of *F. oxysporum* NR reveals homology with bacterial Nar, including its structural subunits and membrane bound location (Stouthamer 1992). Kobayashi et al. (1996) demonstrated that NR was required for ATP synthesis; the addition of deoxychlorate abolished malate/pyruvate dependent mitochondrial NR activity (Kobayashi et al. 1996). Fujii & Takaya (2008) confirmed that the expression of *F. oxysporum* NR was upregulated during denitrifying conditions (Fujii & Takaya 2008). The denitrifying nitrate respiration pathway allows *F. oxysporum* to survive conditions of hypoxia, endowing it with a key selective advantage over other fungi.

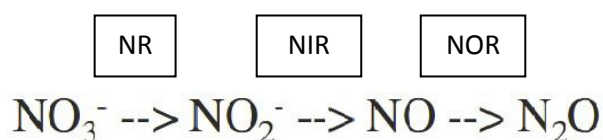


Figure 1.7. The fungal denitrification pathway, which requires nitrate reductase (NR), nitrite reductase (NIR) and nitric oxide reductase (NOR) enzymes. Not all fungi are capable of reducing nitrate, and therefore require only NIR and NOR (Fujii & Takaya 2008).

Fungi ammonia fermentation is a process similar to denitrification but differs in its end product, which is NH_4^+ rather than N_2O . Ammonia fermentation occurs under anoxic as opposed to hypoxic conditions and was first identified by Zhou et al. (2002) in *F. oxysporum* (Zhou et al. 2002). These researchers noted that NH_4^+ was not utilised for assimilatory purposes as it remained within the growth medium, and that the addition of nitrate resulted in significant growth, suggesting that nitrate metabolism generates energy. *F. oxysporum* reduces nitrate to ammonium via NR and NIR catalysis starting with ethanol as the main carbon source. Zhou et al.

(2002) identified the subcellular location of ammonia fermentation NR in the cytosol (Zhou et al. 2002), whilst Takasaki et al. (2004) identified ammonium fermentation in *A. nidulans* which requires cytosolic NR and NIR (Takasaki et al. 2004).

In summary, fungi NR are more closely-related to bacterial NR in both their function and structure than they are to plant NR. Indeed, plant mitochondria may have lost their ability to denitrify since diversifying from fungal NR.

1.3.2.4 NR in animals

Until recently NR activity was thought to be absent from animals; nitrate was considered to be an inert anion, generated through the oxidation of NO and absorbed from certain food sources. Any nitrate to nitrite reduction observed in mammals was considered solely to be catalysed by bacteria (Duncan et al. 1995). However, Jansson et al. (2008) reported NR activity, independent of bacteria, in liver homogenates from mice, rats and humans (Jansson et al. 2008). These researchers confirmed that NR activity was independent of bacteria by orally inoculating germ-free mice with sodium nitrate, and, after inoculation, observing nitrite formation. NR activity was found to be heat labile in liver homogenates, indicating the observed NR activity to be enzyme catalysed. The enzyme responsible for the observed NR activity is xanthine oxidoreductase (XOR), that is, following the addition of the XOR inhibitor allopurinol. The addition of this inhibitor results in a 40-80% inhibition of NR activity. However, as allopurinol does not completely abolish NR activity this suggests that there is an alternative (unknown) pathway which also reduces nitrate to nitrite. Intriguingly, Jansson et al. (2008) observed the reduction of nitrate to NO by XOR. NO levels were monitored using the NO sensitive dye DAF-2 DA, and strong fluorescence was observed in the rat liver homogenate. This was inhibited by allopurinol. This alternative NO synthesis pathway occurs at a physiologically relevant rate, under normoxic conditions and at nitrate concentrations found in tissues (Jansson et al. 2008).

Whilst XOR is not a true NR, it is clear that the process of nitrate reduction is physiologically important in animals.

1.3.3 Nitrite reductase

Nitrite reductase (NIR) is an essential enzyme for both nitrate assimilation and denitrification (nitrate respiration), and is closely-associated with NR. Like NR, NIR has been well-studied in plants, bacteria and fungi and only recently have reports been published demonstrating NIR activity in mammals (Jansson et al. 2008). Nitrite reductases encompass a wide spectrum of different enzymes which show considerable differences in amino acid sequence, cofactors specificity and conformations (Swamy et al. 2005).

1.3.3.1 NIR in plants

Nitrate is the main source of nitrogen for plants, and its assimilation starts with its uptake into plant root cells via specific nitrate transporters (Wang & Tsay 2011). Following uptake, nitrate is reduced in the cytosol by soluble NR to nitrite, before being translocated to the plastid (plant roots) or chloroplasts (plant leaves) (Crawford 1995). Once inside the plastid/chloroplast, nitrite is reduced by NIR to ammonium via a six electron reduction, ammonium is then incorporated into carbon skeletons via the glutamine synthetase and glutamate synthase cycle, Figure 1.7 (Fernandez & Galvan 2008).

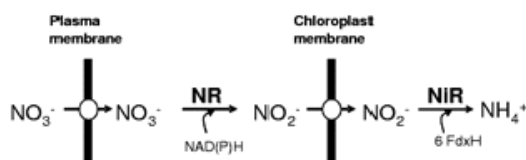


Figure 1.8. The nitrogen assimilation pathway in plants. Taken from Fernandez & Galvan (2008).

NIRs have been extensively studied in higher plants and also in the green alga *Chlamydomonas reinhardtii* (Fernandez & Galvan 2008). NIR is a soluble monomeric enzyme with a molecular mass of approximately of 65 kDa; it carries two prosthetic groups: an iron sulphur cluster and a siroheme (a reduced porphyrin containing two pairs of carbons located in two pyrrole rings) (Swamy et al. 2005). NIR has a single ferredoxin binding site, which in conjunction with its iron sulphur cluster can only donate a single electron at a time; therefore NIR must be able to maintain six electrons via its prosthetic groups in order for catalysis to proceed. In plant chloroplasts ferredoxin is reduced through electron transfer chain light dependent reactions and in the roots, it is reduced by NADPH (Hirasawa et al. 1984). Nitrite binds to the siroheme opposite the iron sulphur cluster and is thought to remain bound until fully reduced to ammonium as no reduced intermediates have been detected during catalysis (Kuznetsova et al. 2004). Plant NIRs share significant homology with sulphite reductase, which also contains a single siroheme domain. Sulphite reductases catalyse the conversion of sulphite to sulphide and are required for sulphur containing amino acid synthesis. Interestingly, NIR and sulphite reductase can each reduce sulphite and nitrite respectively, albeit less effectively than their preferred substrates (Crane & Getzoff 1996).

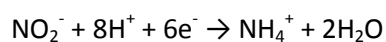
NIR expression is regulated by multiple environmental cues, such as cellular nitrate levels which also co-regulate NR expression (Lewis et al. 1981). Co-regulation of NR and NIR is important to prevent cellular nitrite levels from reaching toxic concentrations and upon nitrate uptake NR and NIR are both expressed. NIR must be imported into the chloroplast requiring additional time compared to NR before it is functional (Cr  t   et al. 1997). Jean-Denis et al. (1991) hypothesised that simultaneous expression of NIR with NR helps negate the increased time between expression and chloroplast localisation, preventing build-up of excessive cellular nitrite concentrations (Jean-Denis et al. 1991). Sharma & Sopory (1984) demonstrated that NIR activity, along with NR in maize, is regulated by phytochrome, and that after treatment with far red light their activities were increased significantly (Sharma & Sopory 1984). Plaut & Federman

(1979) observed the negative regulation of NIR activity by water stress in wheat (*Triticum vulgare* L. cv. *Mivhor*), whereby regulation is thought to occur via direct suppression of NIR activity, rather than its expression, due to the fast reversal of NIR inhibition following the addition of water (Plaut & Federman 1979).

1.3.3.2 NIR in bacteria

NIR in bacteria have been well-studied and like plants are closely associated with their corresponding NR. However, unlike plants, NIR in bacteria are involved in three distinct biochemical pathways: nitrate assimilation, denitrification and ammonia oxidation. Correspondingly, there is considerable diversity between bacterial NIRs.

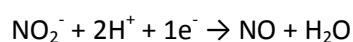
Nitrate assimilation in bacteria follows a similar pathway to plant nitrate assimilation. Uptake of nitrate occurs via a specific nitrate transporter, and, upon entering the cell, is reduced to nitrite by NR and then to ammonium by NIR (Amon et al. 2010). Finally ammonium is combined with carbon skeletons via the glutamine synthetase - glutamate synthase cycle (Frías et al. 1997). NIR have been well studied in cyanobacteria, catalysing the six electron reduction of nitrite to ammonium (Frías et al. 2005).



Cyanobacteria NIR are monomers with approximate 52-56 kDa molecular weights and contain two prosthetic groups: a siroheme and an iron sulphur cluster [4Fe-4S]. The iron sulphur cluster and siroheme are located side-by-side at the interface of the three protein domains, where the two prosthetic groups are linked by a cysteine residue, representing a common cystein thiolate ligand (Kuznetsova et al. 2004). Ferredoxin or flavodoxin, reduced by the photosynthesis electron transfer chain, donates electrons to NIR. Ferredoxin binding is stabilised by the electrostatic forces from positively charged amino acids (Curd et al. 2000). Electrons move from the bound ferredoxin to the iron sulphur centre and to the siroheme, where nitrite binds.

NIR and NR are also co-regulated; specifically their activities are suppressed by ammonium and de-repressed in its absence (Cai & Wolk 1997). This response is nitrate-independent and is regulated by NtcA (Herrero et al. 2001). NtcA is considered a global regulator of nitrate assimilation genes in cyanobacteria and is activated by the cellular carbon-nitrogen ratio (Ohashi et al. 2011). Frias et al. (2003) identified *cnaT* which, encodes a protein with activity similar to glycosyltransferase and is required for NIR and NR activity (Frías et al. 2003). However, the *cnaT* regulatory mechanism has not been identified. NIR mRNA stability has been shown to increase in the presence of nitrate, which suggests an alternative post-transcription regulation mechanism (Frías et al. 1997). Genome sequencing has revealed that NIR genes from cyanobacteria are similar to other photosynthesising organisms, plants and algae and also share homology with sulphite reductase (Luque et al. 1993). Assimilatory NIR genes have also been studied in *Mycobacterium tuberculosis* and *B. subtilis* (Malm et al. 2009; Ogawa et al. 1995).

Bacterial NIR are involved in denitrification, with two separate denitrifying NIRs identified. NIR containing cytochrome *cd₁* and NIR containing copper both catalyse the one electron reduction of nitrite to NO and provide an alternative pathway to generate a protonmotive force providing energy and promoting growth.



Cytochrome *cd₁* containing NIR have been identified in multiple denitrifying bacteria: *Pseudomonas denitrificans*, *Pseudomonas stutzeri*, *Alcaligenes fualis*, *Puracoccus halodenitrificans*, *Thiosphaera pantotropha* and *Ralstonia metallidurans* (Berks et al. 1995). These NIRs are dimers, with each 60 kDa subunit containing two heme domains; an N terminal located c-heme and a *d₁*-heme located in an eight-bladed β-propeller structure (Richardson & Watmough 1999). The *d₁*-heme is unique to denitrifying bacteria. Nitrite binds to the fully reduced enzyme at the *d₁*-heme where it is reduced by electrons donated from cytochrome c-

551, which are passed via *c*-heme to the d_1 -heme; finally the reduced and dehydrated nitrite is released as NO (Cutruzzolà et al. 2009; Zumft 1997).

Copper containing NIRs are trimeric proteins with each subunit containing a single type I and type II copper centre (Suzuki et al. 1999). The type I copper centre, bound by cysteine, methionine and two histidines, is the site of electron donation. The type II copper centre is bound by three histidines and an H₂O molecule, and is the site of nitrite reduction to NO. Electrons are passed from the type I to type II copper centre. Multiple type I copper centre electron donors have been identified as differing between species, and it has been suggested NIR from a single species can utilise multiple electron donors (Berks et al. 1995). Disruption of either the type I or II copper centres results in almost complete abolition of NIR activity (Kukimoto et al. 1994). NIR encoded by *aniA* in *Neisseria gonorrhoeae* has been demonstrated to be induced by anaerobic conditions and by nitrite (Hoehn & Clark 1992).

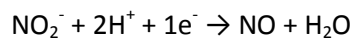
A third type of bacterial NIR is involved in ammonia oxidation. Ammonia oxidising NIR are similar to copper containing NIR and have been studied in the model ammonia oxidising bacterium *Nitrosomonas europaea* (Cantera & Stein et al. 2007).

1.3.3.3 NIR in fungi

NIR in fungi, akin to plants and bacteria, are closely-associated with NR. During nitrate assimilation NIR catalyses the six electron reduction of nitrite to ammonium. Electrons are donated from NAD(P)H to the bound FAD domain and through the iron sulphur centre to the siroheme where nitrite is bound (Prodouz & Garrett 1981). The resulting ammonium is converted into glutamate and glutamine, precursors for many nitrogen containing compounds. Characterisation of NIR in *N. crassa*, encoded by *nit-6*, revealed that NIR is a α_2 -type homodimer composed of two 127 kDa subunits which exhibit bi-specificity for NADPH and NADH (Exley et al. 1993). Each subunit contains a bound FAD, a siroheme and an iron sulphur centre (Exley et al. 1993). Catalysis of nitrite to ammonium requires three NAD(P)H molecules to transfer six

electrons to the FAD (Lafferty & Garrett 1974). Genomic analysis revealed the domains in NIR to be organised in a linear fashion, the FAD domain at the N terminus and the siroheme at the C terminus (Campbell & Kinghorn 1990). Both NIR and NR in *N. crassa* are regulated by transcriptional control and active protein degradation (Exley et al. 1993). Unlike certain *Aspergillus* species, NIR and NR in *N. crassa* are not contiguously located within their genomes; however their expression is co-regulated, demonstrating that the genes share the same regulatory sequences (Fu & Marzluf 1987). Iida et al. (2008) reported the up-regulation of NIR (*FoNIIA*) in *F. oxysporum* during conidiation (Iida et al. 2008). Disruption of *FoNIIA* resulted in mutants which generated significantly reduced numbers of macroconidia relative to the wildtype strain. Iida et al. (2008) also demonstrated *FoNIIA* to be upregulated by Ren1, a transcription factor required for micro and macroconidia development.

Fungal NIR have been identified in the denitrifying systems of both *F. oxysporum* and *C. tonkinense*. Fungal denitrification is an anaerobic respiration pathway in which ATP synthesis is coupled with reduction of nitrate to nitric oxide.



Kobayashi & Shoun (1995) demonstrated that NIR isolated from *F. oxysporum* were similar to bacterial copper containing NIR. Purification of *F. oxysporum* NIR revealed it to be a homodimer of two 41 kDa subunits, each subunit containing both type I and type II copper centres (Kobayashi & Shoun 1995). Kubota et al. (1999) reported *C. tonkinense* NIR activity to be induced following the addition of nitrate and under oxygen limiting conditions (Kubota et al. 1999). Partitioning of NIR activity between membrane and soluble fractions has been reported in *Bacillus halodenitrificans*, however it was later proved to be the same protein (Kubota et al. 1999; Payne & LeGall 1991).

Fungal NIRs are also involved in a second form of dissimilatory nitrate metabolism, ammonia fermentation. This pathway couples the reduction of nitrate to ammonium, catalysed by NIR

and NR, with the oxidation of ethanol to acetate and substrate phosphorylation (Takaya 2009). Ethanol oxidation to acetic acid requires three separate enzymes (alcohol dehydrogenase, acetaldehyde dehydrogenase and acetate kinase), which synthesise ATP. NIR associated with ammonia fermentation are distinct from the denitrification dissimilatory NIR described above and more closely resemble assimilatory NIR (Kobayashi et al. 1996). Fungal ammonia fermentation was first observed in *F. oxysporum* by Zhou et al. 2002, who found it to occur under anoxic conditions rather than the hypoxic conditions associated with denitrification (Zhou et al. 2002).

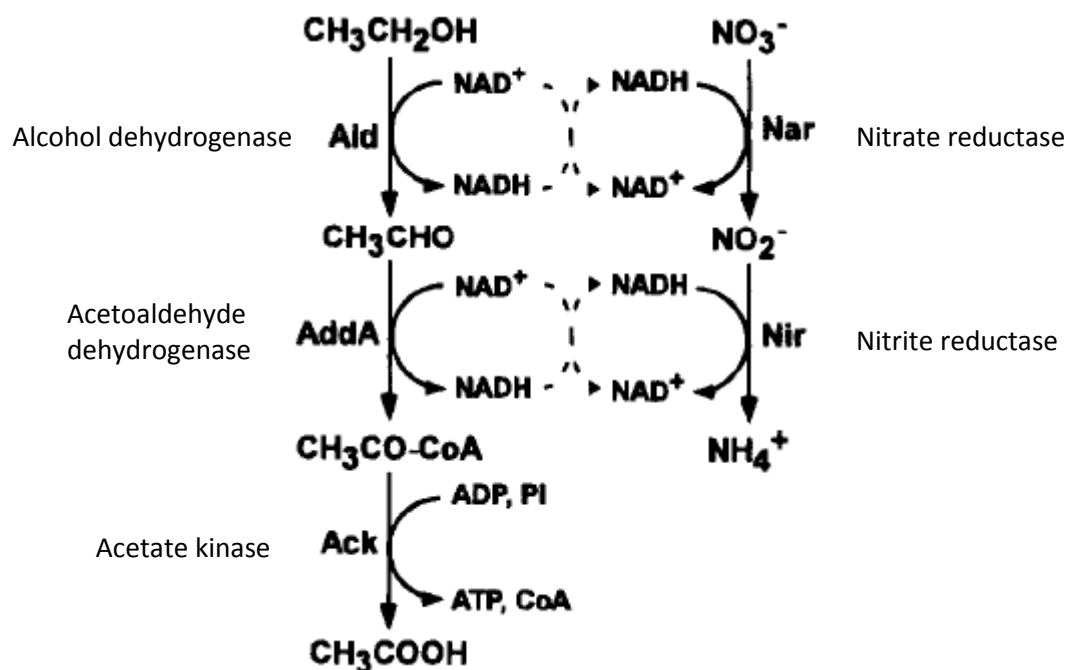


Figure 1.9. The ammonia fermentation pathway in *F. oxysporum*. Taken from Takaya (2002).

Shimizu et al. (2009) recently reported the finding of ammonia fermentation in *A. nidulans*. Using proteomic analysis they identified the up-regulation of genes required for ammonia fermentation and also genes required for the pentose phosphate pathway and thiamine synthesis in *A. nidulans* during aerobic and anaerobic conditions (Shimizu et al. 2009).

1.3.3.3 NIR in animals

Although animal genomes do not carry NIR genes, NIR activity has been studied in mammals, specifically its role in NO synthesis. Oral facultative anaerobic bacteria and intestinal anaerobic bacteria denitrify nitrite as part of their dissimilatory nitrate respiration pathways, and represent the only examples of true NIR present in animals (Lundberg et al. 2004). Deoxyhemoglobin has been known for over 70 years to exhibit NIR activity, synthesising NO (Brooks 1937). Nitrite reacts with ferrous deoxyhemoglobin forming methaemoglobin and NO, and this reaction pathway is considered analogous to bacterial dissimilatory NIR (Doyle et al. 1981). Studies suggest that haemoglobin may function as a regulator of hypoxic vasodilation (Nagababu et al. 2003; Cosby et al. 2003). Like haemoglobin, myoglobin also exhibits NIR activity, converting nitrite to NO. Due to its low haem redox potential and high oxygen affinity it is capable of reducing nitrite to NO thirty times faster than haemoglobin (Shiva et al. 2007). Myoglobin has been demonstrated to regulate cardiac energies and oxygen use during hypoxia; myoglobin deletion mice were unable to regulate nitrite dependent cardiac consumption (Shiva et al. 2007). Several other enzymes including XOR, cytochrome P450s and the mitochondrial electron transport chain have been reported to use nitrite instead of oxygen as an electron acceptor resulting in NO synthesis (Lundberg et al. 2008).

1.4 Thesis objectives

NO is involved in a diverse array of physiological processes in animals, plants and bacteria. It is synthesised by several enzymes, each of which have been well-documented within specific domains. However, NO has been poorly-studied in fungi, with regard to both its synthesis and its physiological roles. This study seeks to further our understanding of NO in fungi, placing specific emphasis on understanding the role of NO in the rice blast fungus *M. oryzae*, by seeking to:

- 1) Identify genes within the *M. oryzae* genome considered capable of NO synthesis.
- 2) Disrupt these genes through targeted gene replacement, generating mutant strains.
- 3) Assess mutant phenotypes, specifically their development of infection-related structures and pathogenicity as compared with the wildtype strains.
- 4) Confirm the presence of NO and monitor its real-time generation in *M. oryzae* germlings.

Chapter 2: Materials and Methods

2.1 Chemicals, solutions and oligonucleotide primers

Chemicals were purchased from Sigma-Aldrich Co. (3035 Spruce St., St. Louis, Missouri 63103) or VWR International Ltd (Hunter Boulevard, Magna Park, Lutterworth, Leicestershire LE17 4XN, UK), unless otherwise stated.

Oligonucleotide primers were purchased from MWG Biotech (90 Long Acre, Covent Garden, London WC2E 9RZ, UK) and Sigma- Aldrich Co. (3035 Spruce St., St. Louis, Missouri 63103).

Solutions listed in Table 2.1 were made using demineralised water and, where indicated, autoclaved at 121°C & 15lb.sq.in⁻¹ for 20-30 minutes.

Complete Medium (CM)	Liquid	10g D-glucose, 2g Peptone, 1g Yeast extract, 1g Casamino acids, 50ml 20X Nitrate salts, 1ml 1000X Vitamin solution & 1ml 1000X Trace elements. pH 6.5 with 1M NaOH, final volume 1L, autoclaved.		
	Solid	As above with 1.5% agar (w/v), autoclaved.		
Water Agar (WA)	Solid	1.5% agar (w/v), final volume 1L, autoclaved		
Minimal Medium (MN)	Nitrate salts	Liquid	10g D-glucose, 50mL 20X nitrate salts, 5µl 0.5% Biotin solution, 1mM 1% Thiamine solution & 1ml 1000X Trace elements. pH 6.5 with 1M NaOH, final volume 1L, autoclaved.	
		Solid	As above with 1.5/1% agar (w/v), autoclaved.	
		1mM Sodium Tungstate	Liquid	10g D-glucose, 50mL 20X nitrate salts, 5µl 0.5% Biotin solution, 1mM 1% Thiamine solution, 1ml 1000X Trace elements & 0.329g Na ₂ WO ₄ . pH 6.5 with 1M NaOH, final volume 1L, autoclaved.
			Solid	As above with 1.5% agar (w/v), autoclaved.
	Non-Nitrate salts	10mM Sodium Nitrate	Liquid	10g D-glucose, 50mL 20X non-nitrate salts, 5µl 0.5% Biotin solution, 1mM 1% Thiamine solution, 1ml 1000X Trace elements & 0.85g NaNO ₃ . pH 6.5 with 1M NaOH, final volume 1L,

				autoclaved.
			Solid	As above with 1.5% agar (w/v), autoclaved.
		10mM Sodium Nitrite	Liquid	10g D-glucose, 50mL 20X non-nitrate salts, 5µl 0.5% Biotin solution, 1mM 1% Thiamine solution, 1ml 1000X Trace elements & 0.69g NaNO ₂ . pH 6.5 with 1M NaOH, final volume 1L, autoclaved.
			Solid	As above with 1.5% agar (w/v), autoclaved.
		300mM Potassium Chlorate	Liquid	10g D-glucose, 50mL 20X non-nitrate salts, 5µl 0.5% Biotin solution, 1mM 1% Thiamine solution, 1ml 1000X Trace elements & 36.76g KClO ₃ . pH 6.5 with 1M NaOH, final volume 1L, autoclaved.
			Solid	As above with 1.5% agar (w/v), autoclaved.
		3.3mM Ammonium Sulphate	Liquid	10g D-glucose, 50mL 20X non-nitrate salts, 5µl 0.5% Biotin solution, 1mM 1% Thiamine solution, 1ml 1000X Trace elements & 0.434g (NH ₄) ₂ SO ₄ . pH 6.5 with 1M NaOH, final volume 1L, autoclaved.
			Solid	As above with 1.5% agar (w/v), autoclaved.
		3.3mM Ammonium Sulphate & 300mM potassium Chlorate	Liquid	10g D-glucose, 50mL 20X non-nitrate salts, 5µl 0.5% Biotin solution, 1mM 1% Thiamine solution, 1ml 1000X Trace elements, 0.434g (NH ₄) ₂ SO ₄ & 36.76g KClO ₃ . pH 6.5 with 1M NaOH, final volume 1L, autoclaved.
			Solid	As above and 1.5% agar (w/v), autoclaved.
20X Nitrate Salts	120 g NaNO ₃ , 10.4 g KCl, 10.4 g MgSO ₄ .7H ₂ O, 30.4 g KH ₂ PO ₄ . Final volume 1L, autoclaved.			
20X Non-Nitrate Salts	10.4 g KCl, 10.4 g MgSO ₄ .7H ₂ O, 30.4 g KH ₂ PO ₄ . Final volume 1L, autoclaved.			
1000X Trace elements	80 ml dH ₂ O, 2.2 g ZnSO ₄ .7H ₂ O, 1.1 g H ₃ BO ₃ , 0.5 g MnCl ₂ .4H ₂ O, 0.5 g FeSO ₄ .7H ₂ O, 0.17 g CoCl ₂ .6H ₂ O, 0.16 g CuSO ₄ .5H ₂ O, 0.15 g Na ₂ MoO ₄ .2H ₂ O & 5 g NaEDTA. Components added in order, boiled and the pH adjusted to 6.5 (with 1M KOH) at 60 °C. Final volume 100 ml & stored at 4 °C.			

1000X Vitamin solution	0.01g of: biotin, pyridoxine, thiamine, riboflavin, <i>p</i> -aminobenzoic acid & nicotinic acid. Final volume 100ml and stored at 4°C.	
Luria-Bertani (LB)	Liquid	10g NaCl, 10g Bacto-tryptone & 5g Yeast-extract. pH7 with 1M NaOH, final volume 1L, autoclaved.
	Solid	As above and 1.5% agar (w/v), autoclaved.
	Blue-White colony screening	As above and 100µg/ml Ampicillin, 80µg/ml X-Gal in Dimethyl sulfoxide (DMSO) & 0.5mM Isopropyl β-D-thiogalactopyranoside (IPTG)
SOC Broth	20g Tryptone, 5g Yeast-extract, 0.5g NaCl & 10ml 250mM KCl. pH 7 with 1M NaOH, make to 975ml, autoclave. Prior to use add filter-sterilized 5ml 2M MgCl ₂ & 20ml 1M Glucose, final volume 1L	
X-Broth	0.5g Yeast-extract, 2g Tryptone, 0.4g MgSO ₄ & 10ml 100mM KCl. pH 7.6 with 1M KOH, final volume 100ml & autoclaved.	
Osmotic Medium buffer (with lysing enzymes) OM	44.37 g MgSO ₄ , 1 ml 1M NaH ₂ PO ₄ .H ₂ O & 2.1 g lysing enzymes from <i>Trichoderma harzianum</i> ; pH 5.8 with dibasic Na ₂ HPO ₄ , final volume 150ml. Stir for 15 minutes, filter-sterilised and used immediately.	
ST Buffer	54.66 g sorbitol & 50 ml 1 M Tris-HCl pH 7.0. Final volume 500 ml & autoclaved.	
STC Buffer	109.32 g sorbitol, 5 ml 1 M Tris-HCl pH 7.5 & 5 ml 1 M CaCl ₂ . Final volume 500 ml & autoclaved.	
PTC Buffer	40 g PEG 4000, 18.22 g sorbitol, 1 ml 1 M Tris-HCl pH 7.5 & 1 ml 1 M CaCl ₂ . Final Volume 100 ml filter-sterilized.	
OCM	273.84g D-sucrose, 10g D-glucose, 2g Peptone, 1g Yeast extract, 1g Casamino acids, 50ml 20X Nitrate salts, 1ml 1000X Trace elements & 1ml 1000X vitamin solution. pH to 6.5 with 1M NaOH, add 1.5% agar (w/v), final volume 1L & autoclaved.	
Defined Complex Medium (DCM)	1.7g Yeast nitrogen base without amino acids, 2g L- Asparagine, 1g Ammonium nitrate & 10g D- Glucose. pH 6 with dibasic Na ₂ HPO ₄ , add 1.5% agar (w/v), final volume 1L & autoclaved.	
Penicillin & Streptomycin stock solution	500mg Penicillin G Sodium & 500mg Streptomycin Sulphate. Final volume 10ml, filter-sterilized, aliquoted & stored at -20°C	
DNA loading buffer	0.21 % bromophenol blue (w/v), 0.21 % xylene cyanolFF (w/v), 0.2 M EDTA pH8, 50 % glycerol (v/v) & 0.1 % SDS. Autoclaved.	
50X Tris acetate EDTA buffer (TAE)	242g Trizma base (Tris), 57.1ml glacial acetic acid & 100ml 0.5M EDTA pH8. Final volume 1L	
DNA Extraction Buffer	20ml 1M Tris HCl pH 7.5, 5ml 5M NaCl, 5ml 500mM EDTA & 5ml 10% SDS. Final volume 100ml.	
TE buffer	0.5ml 1M Tris pH 7.6 & 100µl 0.5M EDTA. Final volume 50ml, Autoclaved.	
Depurination Buffer	11ml Conc. HCl; Final volume 1L	
Denaturation Buffer	87.66g NaCl & 20g NaOH; pH 7.5 with conc. HCl, final volume 1L	
Neutralization Buffer	87.66g NaCl & 60.5g Trizma base (Tris); pH 7.5 with conc. HCl, final volume 1L	
Nucleic Acid Transfer Buffer (20X SSC)	88.23g Tri-sodium Citrate & 175.32g NaCl. pH 7-8 with either 1M NaOH, final volume 1L	
Southern blot washing solutions	2X SSC & 0.1% SDS	50ml 20X SSC & 5ml 10% SDS. Final volume 500ml
	1X SSC & 0.1% SDS	25ml 20X SSC & 5ml 10% SDS. Final volume 500ml
	0.1X SSC & 0.1% SDS	2.5ml 20X SSC & 5ml 10% SDS. Final volume 500ml

Table 2.1. Media and solutions used in this thesis.

Name of primer	Sequence direction 5' to 3'
MgTub-F	CTGCCATCTTCCGTGGAA
MgTub-R	GTGAACTCCATCTCGTCC A
NOS3-RTPCR-F	GTGCTTTGCCACCTACTACTACCTTC
NOS3-RTPCR-R	GCTTGATCTTGAGCTTGTAAATCG
MS12-BAR-F	AAAACCTCGAGGTCGACAGAAGATGATATTGAAGGAGC
MS13-BAR-R	AAAACCTCGAGGTCGACCTAAATCTCGGTGACGGGCAG
NEW-BAR-F	TCGTTTGACAAGATGGTTTCATTTAGGC
NEW-BAR-R	ACTTCAGCAGGTGGGTGTAGAGCG
NOS2-CHECK-F	CAATCTACTACGGTTCCAACCTCGG
NOS2-CHECK-R	GTCTCAAAGTCGGAGAACATGTCTG
NOS2-FL-F	CGGGCGTAAACCGTCGGATGTC
NOS2-FL-R	CCACAGGGCGCCATATCTTTTCG
NEWNOS2-FL-F	CTCTGCTTTTACTATGTACAGAGGTTCCG
NEWNOS2-FL-R	TTATTCAGCTCTTAATAGACTACCAAGCCG
NOS2-BAR-F	GTGGTGGTTTATCGTCTGTTGGGTC
NOS2-BAR-R	CGTGCTTGTCTCGATGTAGTGTTG
PacI-NOS2-F	AACCTTAATTAAGGGCGTAAACCGTCGGATGTC
PacI-NOS2-R	AAGGTTAATTAACCACAGGGCGCCATACTTTTCG
NOS2SEQ1-F	CAGATCGTCTGCTTCTGACCAAATCC
NOS2SEQ1-R	CCCCGCGAGAGCGTGGCGCGCATCAT
NOS2SEQ2-F	GCCGGCGACTACCTGGCCGTCCTGC
NOS2SEQ2-R	CCCGGACGCGGACGAGTTCAAGCC
NOS2SEQ3-F	CTGCCCCGACCTCGATGTCAAGTTTCCGC
HYG-F	GTCGACGTAACTGATATTGAAGGAG
HYG-R	GTCGACGTAACTGGTTCCCGGTCGGC
NIR5-HYG-R	AGCCCCAAAATGCTCCTTCAATATCATCTTCTCTCGACGTAAACGTCGACG GTGACGTTGTTGTGCGCGTCCACCTC
NIR3-HYG-F	CAAAGGAATAGAGTAGATGCCGACCGGGAACCAGTTAACGTCGACCAAA CTCGACAACATGGAAGTGGAGGTG
NIR5-F	TTATCGGGAACCATCTGAAGCAAGGCG
NIR3-R	CCTTTTCTGTATCTAGTTGAGTAATTTATTGTTAAAGC
Nitrite-integration-F	CATCAATTGGACAGTAAAGTTGCACCC
HYG-NEW-F	CCGGTCATACCTTCTTAAGTTC
HYG-NEW-R	GCTCCAGTCAATGACCGCTGTTATGC
HYG-SEQ1-R	CCGCAGGACATATCCACGCCC
HYG-SEQ2-F	CAGTGCGTCCGTCGCGCAGGC
NIR5-HYG-NIR3-SEQ2-R	CCTCATCTTAGCGACTGCTTCACC
NIR5-HYG-NIR3-SEQ2-R	CCAGTTGACCCGCTGCGGCCGCTCCC

Table 2.2. List of oligonucleotide primers used, including primers used to sequence gene replacement constructs.

2.2 Fungal material

2.2.1 Maintenance

Magnaporthe oryzae wildtype strains and mutant strains were cultured on Complete Medium (CM) (Table 2.1) and incubated in a Sanyo growth cabinet at 24°C with a 14/10 hour light/dark photoperiod. Wildtype strains Guy11 and $\Delta ku70$ were acquired from N. J. Talbot, Exeter University. $\Delta nos3$ was generated by Mary Illes (DPhil student), $\Delta nia1$ and $\Delta nos3\Delta nia1$ were generated by Marketa Samalova (postdoctoral).

For long-term storage of wildtype and mutant genotypes, strains were grown on solid CM overlaid with sterile filter papers (No. 2, Whatman Plc, 27 Great West Rd, Brentford, Middlesex, TW8 9BW, UK) and after 10 days were desiccated over silica gel for 72 hours and stored at -20°C.

2.2.2 Harvesting conidia

Conidia were harvested from 10 day old fungal cultures growing on CM, 5ml of demineralised (demin.) water was added to each culture and conidia were released by scraping the surface of the mycelium with a sterile glass microscope slide. The resulting suspension was passed through sterile Miracloth (Merck Chemicals Ltd, Padge Rd, Nottingham, NG9 2JR, UK) to remove damaged mycelium and centrifuged for 3 minutes at 4750rpm (5251g) to pellet the conidia. The supernatant was removed and the conidia pellet re-suspended in 1ml demin water. The conidia concentration was adjusted to 1×10^4 per ml (unless otherwise stated) using a haemocytometer.

2.3 Plant material

2.3.1 Maintenance

Susceptible Rice (*Oryza sativa*) cv. CO-39 and susceptible barley (*Hordeum vulgare* L.) cv. Golden Promise were grown in a 50:50 (w/w) mixture of Erin Multipurpose Compost and John Innes No. 3 Soil-based compost (21 and 7 days respectively). Rice and Barley were incubated in a Gallenkamp Fi-totron PG660 controlled environment chamber under a 14/10 hour light-dark photo period ($700\mu\text{Mol.m}^{-2}.\text{s}^{-1}$); rice at 25°C and 90% humidity, barley at 20°C and 84% humidity.

2.3.2 Barley epidermal peels

Seven day old barley leaves were harvested and placed adaxial side up. A sterile scalpel was used to cut through each leaf leaving the lower epidermis intact. The upper leaf material was peeled off and discarded leaving the intact epidermis, which was then placed with its abaxial side facing upwards on 1% water agar (WA) Petri dishes. The WA prevented epidermal peel desiccation.

2.4 Scoring assays

2.4.1 Preparation of plastic surfaces

TAAB plastic cover-slips (TAAB Laboratories Equipment Ltd, 3 Minerva House, Calleva Park, Aldermaston, Berkshire, RG7 8NA, UK) were washed individually for 30 seconds in 70% ethanol and rinsed three times in sterile demin. water. Rinsed cover-slips were air-dried lying on labelled microscope slides in a laminar flow hood for 30 minutes before inoculation with conidia suspensions.

2.4.2 Preparation of glass surfaces

Glass microscope slides (VWR International Ltd, Hunter Boulevard, Magna Park, Lutterworth, Leicestershire LE17 4XN, UK) and glass cover-slips (Gerhard Menzel, Glasbearbeitungswerk GmbH & Co. KG - Saarbrückener Str. 248 - D-38116 Braunschweig) were both rinsed three times in sterile demin. water before being air-dried for 30 minutes in a laminar flow hood.

2.4.3 Inoculation of infection-related surfaces

Conidia were harvested, as detailed above, and the resulting suspension diluted to 1×10^4 conidia per ml, before 50 μ l of conidia suspension was inoculated onto various infection-related surfaces: glass microscope slides, TAAB plastic cover slips, Menzel glass cover slips or Barley epidermal peels.

2.4.4 Germling scoring

Conidia were inoculated onto various previously detailed surfaces. After 12 hours incubation in a high humidity chamber at 24°C under a 14/10 hour light/dark photoperiod, the morphology of 120 germlings were assessed per genotype per treatment, via light microscopy. Conidia/germlings were classified into four separate categories: 1) Failed to germinate; no appressoria germ tube emerged; 2) Germ-tube; conidia produced elongate appressorium germ-tubes, at which point development arrested; 3) Appressoria; conidia germinated and developed non-melanised appressoria; 4) Melanized Appressoria; conidia germinated and developed melanised appressoria. In this latter category (4), the germlings were considered to have completed infection-related development. For each biological replicate 120 conidia were scored. A minimum of three biological replicates were undertaken per independent experiment, and each experiment was

repeated three times. Pairwise Two-tailed Students T-test was used to assess statistical differences between fungal strains. The error was calculated as the standard error of mean (SEM), $SE = \frac{\sigma}{\sqrt{n}}$, where σ equals the sample standard deviation and n represents the sample size.

For chemical genetics experiments, harvested conidia were diluted to 2×10^4 per ml and 25 μ l conidia suspension was added to 25 μ l chemical treatment solution, i.e. solution was added at two fold the final concentration. The conidia suspension was inoculated on the infection-related surface, (as detailed in 2.4.3). The various surfaces were placed in a high-humidity chamber and incubated at 24°C under a 14/10 hour light/dark photoperiod. After 12 hours inoculation germling morphologies were scored, (as detailed in 2.4.4).

2.5 Pathogenicity assays

2.5.1 Leaf preparation

Seven day old barley (cv. Golden Promise) leaves and 21 day old rice (cv. CO-39) plants were harvested and were cut into 6cm (barley) or 10 cm (rice) lengths. The leaves were washed for 30 seconds in a 2% bleach solution, before being rinsed three times in sterile demin. water. Sterilised leaves were left for 30 minutes to dry in a sterile flow hood. Following drying, 10 leaves were positioned per 120x120mm water agar (WA) Petri dish. Sterile microscope slides were placed over the top and bottom 5mm of the leaves to maintain their position and prevent leaf curl. A cut leaf assay as supposed to a whole plant assay was chosen for the following reasons: 1) Even spraying of conidia; 2) Even incubation conditions, e.g. light, temperature and humidity; 3) Conidia inoculated onto whole plants are readily washed off by excess solution and contact with the container; 4) cut leaf assays gave the most reliable results.

2.5.2 Preparation and inoculation of conidia

Conidia were harvested, (as detailed in 2.2.2), and diluted in a 0.2% gelatine solution to 1×10^4 per ml. Each WA Petri dish, containing sterilized leaves, was aerosol inoculated with 2ml of 1×10^4 conidia/0.2% gelatine suspension using a sterile artist's air brush. For each *M. oryzae* genotype a separate WA plate was inoculated, and a control plate was inoculated with 0.2% gelatine. Conidia were prepared in a weak (0.2%) gelatine solution to enhance conidia attachment to the plant cuticle. Care was taken to ensure the leaves had an even covering of conidia suspension. The air brush was sterilised between use with separate genotypes by submerging it in 70% ethanol for 30 seconds, and rinsing it three times in sterile demin. water. Sterile demin water was removed from the air brush prior to its use for inoculating dried rice and barley leaves.

Inoculated Rice and Barley leaves were incubated in a Gallenkamp Fi-totron PG660 controlled environment chamber under a 14/10 hour light/dark photoperiod ($700 \mu\text{Mol.m}^{-2}.\text{s}^{-1}$), for five days: Rice at 25°C and 90% humidity, and Barley at 20°C and 84% humidity.

2.5.3 Pathogenicity and virulence assays

Following five days incubation, images of infected Rice and Barley leaves were captured and the pathogenicity of each fungal genotype recorded via image analysis software, written in MatLab by Dr. Mark Fricker (University of Oxford). A minimum of three biological replicates was used in each independent experiment, and each experiment was repeated three times. Pairwise Two-tailed Students T-test was used to assess statistical differences between fungal strains. The error was calculated as the standard error of mean (SEM) $SE = \frac{\sigma}{\sqrt{n}}$ where σ equals the sample standard deviation and n represents the sample size.

2.6 Fungal growth assays

Petri dishes were filled with 15ml of molten agar and inoculated with a 20 μ l droplet of 1×10^4 conidia suspension before being incubated under a 14/10 hour light/dark photoperiod

($700\mu\text{Mol.m}^{-2}.\text{s}^{-1}$) for two weeks at 24°C . The diameter of the Total Mycelia (TM) growth, the maximum distance non-melanised mycelia extended, and the diameter of the Melanized Mycelia (MM) growth, the maximum distance melanised mycelia extended, were recorded. A minimum of six biological replicates were used for each experiment and two-tailed Pairwise Students T-test was used to assess statistical differences in growth between fungal strains. Wildtype and mutant strains growth were assessed on the following media (Table 2.1):

CM

MN containing nitrate-salts,

MN containing non-nitrate salts,

MN containing non-nitrate salts 11.7mM Sodium Nitrate,

MN containing non-nitrate salts 10mM Sodium Nitrite,

MN containing nitrate salts 300mM Potassium Chlorate,

MN containing non-nitrate salts 3.3mM Ammonium Sulphate,

MN containing non-nitrate salts 300mM Potassium Chlorate & 3.3mM Ammonium Sulphate,

MN containing nitrate salts & 1mM Sodium Tungstate.

2.7 Fungal biomass assays

Conidia were harvested, (as detailed in 2.2.2), and $20\mu\text{l}$ of conidia suspension (1×10^4 per ml) was inoculated into 20ml of each liquid media and was incubated at 24°C in darkness, shaken at 150rpm for 14 days. The resulting fungal culture was then passaged through pre-dried and weighed glass microfiber filters (Whatman Plc, 27 Great West Rd, Brentford, Middlesex, TW8 9BW, UK), 16hrs at 80°C . The collected filtrate was heat dried (16hrs at 80°C), weighed and the fungal biomass calculated. A minimum of six biological replicates were used for each experiment and two-tailed Pairwise Students T-test was used to assess statistical differences in growth between fungal strains. Wildtype and mutant strains growth were assessed in the following liquid media (Table 2.1):

CM

MN containing nitrate-salts,

MN containing non-nitrate salts,

MN containing non-nitrate salts 11.7mM Sodium Nitrate,

MN containing non-nitrate salts 10mM Sodium Nitrite,

MN containing nitrate salts 300mM Potassium Chlorate,

MN containing non-nitrate salts 3.3mM Ammonium Sulphate,

MN containing non nitrate salts 300mM Potassium Chlorate & 3.3mM Ammonium Sulphate,

MN containing nitrate salts & 1mM Sodium Tungstate.

2.8 Conidiation assessment assay

The number of conidia generated per unit surface area of mycelium was compared between the strains. A sterile core borer was used to cut out three 9mm mycelium plugs from 10 day old fungal cultures, growing on complete media. Mycelia plugs were vortexed for 30 seconds releasing conidia into 4ml demin water. Three 10 μ l samples were removed from the vortexed mycelia suspension, and their concentrations were calculated using a haemocytometer, each conidiation experiment was repeated three times.

2.9 Collection of fungal material for DNA and RNA extraction

Mycelium from 10 day old cultures was harvested for genomic DNA extraction by carefully removing the surface of the fungal culture with a sterile scalpel; the amount of agar collected was kept to a minimum. Collected mycelium was flash frozen in liquid nitrogen and stored at -80°C.

For fungal RNA extraction, barley epidermal peels were placed abaxial side up on water agar Petri dishes. Peels were saturated with conidia and incubated at 24°C in a high humidity chamber

under a 14/10 hour light/dark photoperiod. At the chosen time point inoculated peels were flash frozen in liquid nitrogen and stored at -80°C.

2.10 DNA methods

2.10.1 Genomic DNA extraction

Two methods were used to extract fungal gDNA. gDNA used in Southern blots and transformations, was extracted using the Qiagen DNEasy Plant Minikit (Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ, UK) as per the manufacturer's instructions. gDNA was eluted in 50µl Milli-Q (ultra-pure water) for PCR methods and 100µl Milli-Q for Southern blots.

gDNA used in the initial assessment of putative transformants was extracted using a method adapted from Edwards et al. (1991). Mycelium, from 10 day old cultures, was collected using a sterile scalpel and macerated using a homogeniser in an Eppendorf tube for 15 seconds, before 400µl of DNA extraction buffer (Table 2.1) was added. The mixture was vortexed for five seconds, and incubated for 15 minutes at room temperature. The sample was centrifuged for 60 seconds at 13,000 rpm (17,900g) and the supernatant removed into a separate Eppendorf tube, where upon 300µl isopropanol was added. The solution was centrifuged for a further five minutes at 13,000rpm (17,900g), and the supernatant discarded, leaving the pelleted gDNA. Finally, the dried DNA pellet was re-suspended in 100µl TE buffer (Table 2.1) and stored at 4°C.

2.10.2 DNA phenol-chloroform precipitation

DNA was dissolved in 200µl in Milli-Q water to which 200µl Phenol:Chloroform:Isoamyl Alcohol 25:24:1 saturated with 10 mM Tris (pH 8.0) was added. The solution was vortexed for 30 seconds and centrifuged for 60 seconds at 13,000rpm (17,900g), to separate the solution phases. The

upper aqueous phase was removed into a new Eppendorf tube and 5M NaCl was added (3µl per 100µl DNA solution), before 100% ethanol was added (3X the total solution volume). The solution was incubated at -20°C, for 30 minutes, and centrifuged for 30 minutes at 13,000rpm (17,900g). The supernatant was removed and the pelleted DNA was washed with 1ml 70% ethanol (pre-chilled to -20°C), inverted and left to air dry in a flow hood. The DNA pellet was re-suspended in 10-20µl Milli-Q water.

2.10.3 Ligation of vectors and DNA insertion fragments

Ligation of pGEM®-T Easy vector and DNA fragments were carried out in accordance with pGEM®-T Easy vector system (Promega Delta House, Enterprise Road, Southampton Science Park, Southampton SO16 7NS, UK) and the manufacturer's instructions.

2.10.4 Preparation of plasmid DNA

DH5α *Escherichia coli* and JM109 High Efficiency Competent Cells (Promega) were inoculated in 5ml liquid LB containing 100µg/ml Ampicillin, for 12 hours, shaking at 200 rpm. Bacterial cells were collected by centrifugation 8000 rpm (6000g) and plasmid DNA extracted using Qiagen's QIAprep Miniprep Kit, following the manufacturer's instructions. The eluted plasmid DNA was stored at -20°C.

2.10.5 DNA restriction endonuclease digests

Restriction enzymes were purchased from either New England Biolabs (73 Knowl Piece, Wilbury Way, Hitchin, Hertfordshire, SG4 0TY) or Fermentas (Thermo Fisher, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG). Enzymes were used in accordance to their manufacturer's instructions, typically using one unit of enzyme per µg of DNA per hour.

2.10.6 Agarose gel electrophoresis of DNA fragments

Unless otherwise stated, 1% (w/v) agarose gels contained $0.2\mu\text{g}/\text{ml}^{-1}$ ethidium bromide, prepared with 1X Tris acetate EDTA buffer (TAE) pH8. 1X TAE was also used as the running buffer during electrophoresis. Prior to loading the DNA samples into the gel wells, 10X loading buffer ($1/10^{\text{th}}$ the total solution volume) was added to the samples. $0.5\mu\text{g}$ 1 kb DNA Ladder (New England Biolabs, 73 Knowl Piece, Wilbury Way, Hitchin, Hertfordshire, SG4 0TY) was used. DNA was visualised via a UV transilluminator ($\lambda = 245\text{ nm}$).

2.10.7 Gel extraction of PCR products

DNA bands, identified using a UV transilluminator, of interest were excised with a scalpel, taking care to minimise the amount of excess agarose collected. DNA was extracted from the agarose gel using the Qiagen QIAquick Gel Extraction Kit as per the manufacturer's instructions.

2.11 PCR methods

2.11.1 Proof-reading enzyme PCR

The proofreading enzyme Herculase II Fusion DNA polymerase (Agilent Technologies, 5301 Stevens Creek Boulevard, Santa Clara, CA 95051-7201, USA) was used to amplify high quality DNA for fungal transformations. PCR mixtures were made up in a total volume of $50\mu\text{l}$, in the following order:

$34.5\mu\text{l}$ Milli-Q water

$1.25\mu\text{l}$ 200pM forward and reverse primers (Table, 2.2)

$10\mu\text{l}$ 5X Herculase buffer

$1\mu\text{l}$ dNTPS (dATP, dTTP, dCTP & dGTP, 25mM)

$1\mu\text{l}$ ~5ng DNA template

$1\mu\text{l}$ ~1 rxn unit Herculase II Fusion DNA polymerase

PCR reactions were conducted in both Biometra® T-gradient and Bio-Rad PTC-100 Peltier Thermal Cycler. The following cycling reaction was used:

Step 1	DNA denaturation	94°C	4 minutes
Step 2	DNA denaturation	94°C	20 seconds
Step 3	Primer annealing	58-60°C	20 seconds
Step 4	Elongation	72°C	3 minutes
Step 5	Repeat steps 2-4, 35 times		
Step 6	Final elongation step	72°C	3 minutes
Step 7	Cooling	4°C	infinity

2.11.2 Non-proof reading PCR

Taq DNA polymerase (recombinant) was purchased from Fermentas. PCR mixtures were made up in a total volume of 50µl, in the following order:

37µl Milli-Q water
 1µl 200pM forward and reverse primers (Table, 2.2)
 5µl 1.25mM Taq buffer + (NH₄)₂SO₄ – MgCl₂
 5µl 25mM MgCl₂
 0.5µl dNTPS 2.5mM (dATP, dTTP, dCTP & dGTP)
 1µl ~5ng DNA template
 0.5µl 2.5U Taq DNA polymerase (recombinant)

PCR reactions were conducted in both Biometra® T-gradient and Bio-Rad PTC-100 Peltier Thermal Cycler. The following cycling reaction was used:

Step 1	DNA denaturation	94°C	5 minutes
Step 2	DNA denaturation	94°C	30 seconds
Step 3	Primer annealing	58-60°C	30 seconds
Step 4	Elongation	72°C	2 minutes 10 seconds

Step 5	Repeat steps 2-4, 35 times		
Step 6	Final elongation step	72°C	10 minutes
Step 7	Cooled	4°C	infinity

2.11.3 PCR product cleaning

Promega Wizard® SV Gel and PCR clean-up system kit, and Qiagen QIAquick PCR Purification kit were used to purify PCR products following the manufacturer's instructions.

2.11.4 DNA sequencing

Cleaned plasmid, genomic and PCR DNA samples were sent, with their respective sequencing primers (Table 2.2), to Geneservice Sequencing, Department of Biochemistry, University of Oxford, for sequencing via Illumina GAIIx and HiSeq 2000 sequencing systems.

2.12 RNA methods

2.12.1 Maintaining a ribonuclease-free environment

Work surfaces and pipettes were cleaned with RNase-ZAP spray and wipes (Ambion, Applied Biosystems, Lingley House, 120 Birchwood Boulevard, Warrington, WA3 7QH, UK) and disposable RNase/DNase plastic was used where appropriate. Other plasticware was treated with 0.2% (v/v) diethylpyrocarbonate (DEPC) overnight, before being autoclaved. Pestles and mortars were washed with ethanol, wrapped in foil and baked overnight at 200 °C.

2.12.2 Total plant and fungal RNA extraction from frozen material

Fungal RNA was extracted from frozen Barley epidermal peels previously inoculated with conidia. Frozen tissue was ground under liquid nitrogen with a pre-cooled pestle and mortar, ground material was transferred to an RNase free Eppendorf tube and the total RNA extracted using the Qiagen RNeasy Minikit, following the manufacturer's instructions.

2.12.3 Conversion of extracted RNA to cDNA

Contaminating DNA was removed from the extracted RNA via the following reaction, incubated at room temperature for 15 minutes:

2.1µl RNase free water

0.9µl DNase I buffer (**Invitrogen Ltd**, 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, UK)

0.5µl (1/10 diluted) DNase I

0.5µl RNasin[®] Plus RNase Inhibitor

5µl template RNA (2.5µg)

cDNA synthesis was achieved by adding 1µl Oligo (dt)18 (Bioline Pty Ltd. 16 The Edge Business Centre, 122 Humber Road, London NW2 6EW) and 1µl dNTPs (10mM each) to the above detailed reaction on ice. 2µl 100mM dithiothreitol (DTT) (Promega), 4µl 5X SuperScript[®] III Reverse Transcriptase buffer (Invitrogen), 0.5µl RNasin[®] Plus RNase Inhibitor (Promega) and 1µl SuperScript[®] III Reverse Transcriptase were added, before being incubated at 50°C for two minutes, 42°C for 60 minutes and finally at 70°C for 15 minutes. Synthesised cDNA was stored at -20°C.

2.13 Transformations

2.13.1 DH5α *E. coli* & JM109 High Efficiency Competent Cell transformation

2µl of ligation reaction was added to 100µl thawed DH5α *E. coli* cells and left on ice for 30 minutes. Cells were heat-shocked at 42°C, for exactly two minutes, and 1ml of X-broth added (Table 2.1). Cells were shaken at 150 rpm for 45 minutes, and centrifuged at 4000rpm (2150g) for four minutes. The supernatant was removed, except for 50µl into which the cell pellet was re-suspended. Finally, the cell suspension was inoculated onto LB agar Petri dishes containing ampicillin, IPTG and X-gal (Table 2.1), and incubated overnight at 37°C.

JM109 High Efficiency Competent Cells were transformed following to pGEM®-T Easy vector system and manufacturer's instructions.

2.13.2 *Magnaporthe oryzae* transformation

Conidia were harvested from 10 day old Guy11 or $\Delta ku70$ cultures (as described in 2.2.2), and inoculated in 300ml liquid CM (Table 2.1) containing 100µg/ml Penicillin & Streptomycin (Table 2.1). Liquid cultures were incubated in the dark for 72-96 hours, at 25°C and 150rpm.

Following the incubation stage the cultures were filtered through two layers of sterile Miracloth. The resulting mat of mycelium was pressed dry in sterile paper towelling and divided in two. Each piece was added to a 50ml Falcon tube with filter-sterilized Osmotic Medium buffer (containing *Trichoderma harzianum* lysing enzymes, Table 2.1). A sterile scalpel was used to homogenize the mycelium and the resulting suspension was digested at 30°C for three hours, shaking at 150rpm.

Following digestion, half the protoplast suspensions from each tube were poured into two new Falcon tubes, and 4°C ST buffer (Table 2.1) was added to a final volume of 50ml. The protoplast ST suspension was centrifuged in a Beckman-Coulter Allegra® X-15 centrifuge for 15 minutes at 4°C at 5000rpm (2744g). Protoplasts were harvested from the OM/ST buffer interface into two new Falcon tubes. 10ml 4°C STC buffer was used to overlay the suspension before being centrifuged for 10 minutes 3000rpm (2095g) at 4°C. Following centrifugation the supernatant was discarded and the protoplast pellet re-suspended in 10ml 4°C STC buffer, which was then transferred to the

second tube and used to re-suspend the second pellet. This step was repeated a further three times. Following the final centrifugation step the pellet was re-suspended in 1ml STC buffer and the protoplast concentration measured and diluted to $2-6 \times 10^4$. Into a sterile Eppendorf tube 150µl of diluted protoplast suspension was transferred and combined with 5-10µg of linear DNA construct, before being incubated at room temperature for 25 minutes. Into the mixture 1ml of freshly made filter-sterilized PTC buffer (Table 2.1) was added and the tube gently inverted, and incubated at room temperature for 20 minutes.

Transformed protoplasts were added to 200ml molten (48°C) OCM agar (Table 2.1), mixed gently and poured into 10 sterile petri dishes. The transformation plates were incubated for 24 hours in the dark at 24°C before being overlaid with either 1% (w/v) DCM containing 100µg/ml Bialaphos or Minimal Media (MN) containing 300µg/ml Hygromycin B. Plates were further incubated in the dark at 24°C for seven days.

2.14 Screening of putative transformant strains

2.14.1 Selection of putative transformants

After seven days incubation, fungal colonies which had grown through the top overlay of agar were picked off onto fresh 1.5% (W/V) DCM containing 100µg/ml Bialaphos or MN containing 300µg/ml Hygromycin B. After a further seven days mycelia plugs were transferred to Complete Media Petri dishes and incubated for 10 days at 24°C (2.2.1). From these cultures, freezer paper stocks and genomic DNA extractions were made.

Putative transformant stains were subsequently screened for the presence of the resistance cassette via PCR and endonuclease gDNA digestions. Southern blot analyses determined the number of locations of resistance cassette integration.

2.14.2 Southern blot analysis

Genomic DNA was extracted from 10 day old cultures of putative transformant strains (as detailed in 2.10.1) and digested for seven hours with the appropriate restriction enzyme, at 37°C. Transformants carrying the Bialaphos resistance cassette were digested with KpnI, whilst transformants carrying the Hygromycin B resistance cassette were digested with EcoRI. Digested DNA was loaded into an ethidium bromide free 0.8% agarose gel and separated by gel electrophoresis at 20 V for 16 hours.

Following electrophoresis, the gel was treated with depurination buffer (Table 2.1) whilst gently agitated for 20 minutes. The depurination buffer was replaced with denaturation buffer (Table 2.1) and the gel agitated for a further 30-60 minutes. Finally, the gel was treated with neutralization buffer (Table 2.1) and agitated for 30-60 minutes.

The capillary transfer tower was constructed as described in Sambrook & Russell (third edition), two layers of Whatman 3M filter paper were used as wicks, supported on a plastic island their ends were submerged in 10x SSC. The gel (with its wells removed) was placed upside down, on top of the wicks and surrounded with Parafilm. A nylon Hybond N+ membrane (Amersham plc, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, UK) was cut to the same dimensions as the gel and placed on top, three identically dimensioned pieces of filter paper (pre-wetted in 10x SSC) were placed over the N+ membrane. A tall stack of paper towels and 1kg weight were positioned on top of the filter paper. The tower was left for 16 hours to allow DNA transfer.

The transferred DNA was cross-linked to the membrane using a Stratagene UV Stratalinker 2400. The UV cross-linked membrane was prehybridised in 7-10ml Perfect HYB plus hybridization buffer 1X (Sigma-Aldrich) for one hour at 65°C in a hybridisation oven under gentle rotation.

DNA fragment probe-labelling was carried out using the Amersham Ready-To-Go DNA labelling beads (GE healthcare, Life Sciences, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA,

UK). Probe DNA (25-50ng) was mixed with 45µl TE buffer in the supplied tube, boiled for 10 minutes and then ice-cooled (ethanol treated). The mixture was centrifuged for 10 seconds before 5µl α ³²P dCTP was added and incubated at 37°C for ~1 hour.

Incorporated ³²P labelled probe was purified from unincorporated radiolabel nucleotides using Illustra NICK columns (GE healthcare) kits. The columns were prepared by removing the cap and pouring off the storage liquid, columns were then washed with 3ml TE buffer. The probe was mixed with 70µl dextran blue and applied to the centre of the column; additional TE buffer was applied. The blue dextran solution, containing only the radio labelled probe was collected and stored on ice for future use. The remaining solution, containing unincorporated radiolabel nucleotides was collected and used to determine the labelling efficiency.

The radio-labelled probe was boiled for 10 minutes and then ice cooled, before being added to <7ml hybridisation buffer. The cross-linked membrane was hybridised overnight at 65°C. Following hybridization the membrane was washed as follows: two 30 minutes washes in 2X SSC, 0.1% SDS at 65°C, one wash for 20 minutes in 1X SSC, 0.1% SDS at 65°C and 1-2 20 minutes washes in 0.1X SSC, 0.1% SDS at 65°C.

The washed membrane was air dried and exposed to Amersham Hyperfilm MP (Amersham) in a cassette with intensifier screens for 24 hours at -80°C. The film was developed using Kodak GBX fixer and developer (Sigma-Aldrich).

2.14 Light Microscopy

Light microscopy was conducted using an Olympus BX50 microscope (Olympus Microscopy, KeyMed House, Stock Road, Southend-on-Sea). Images were captured through a Q Image Retiga Exi digital camera (19535 56th Avenue, Suite 101 Surrey, BC, Canada) and Image-Pro software (Media Cybernetics, Inc. 4340 East-West Hwy, Suite 400, Bethesda, MD, 20814-4411 USA).

2.15 Nitric oxide detection

2.15.1 Fluorescent plate reader assay

NO production was measured using the NO sensitive fluorescent dyes DAR-4M (non-cell permeable) and DAR-4M AM (cell permeable) in a BMG Labtech FLUORostar Galaxy (BMG LABTECH GmbH, Hanns-Martin-Schleyer-Str. 10, D-77656 Offenburg/Germany) 96-well fluorescence plate reader.

M. oryzae conidia viability and optimisation of fluorescence detection in the 96 well plate format was assessed using necrotic cell dye propidium iodide (PI) the live cell dye fluorescein diacetate (FDA). PI readily binds to DNA within cells, however it is membrane impermeable to living cells, hence fluorescence is only observed within dead cells. FDA is cell permeable and readily diffuses into live cells, where its acetate groups are cleaved by intracellular esterases, yielding highly fluorescent membrane, impermeable fluorescein. Signals increased to saturation point over 20-30 minutes with 10-100 μ M FDA, (data not shown).

Different positioning of excitation and emission fiber optics were tested and, the positioning of both optics below the 96-well plate resulted in the strongest fluorescence from FDA loaded Guy11 conidia as they settled to the base of the well, (data not shown).

Different 96-well plates were also assessed for their ability to induce germination in *M. oryzae*. NUNC 96-well white optical bottom plates (VWR International, Hunter Boulevard, Magna Park, Lutterworth, Leicestershire LE17 4XN) were selected as the most suitable. Of the different concentrations of Guy11 conidia tested, 1x10⁴ conidia per ml generated strong fluorescence without compromising the conidia's ability to develop. At higher concentrations conidia development was compromised by the density of conidia settled on the base of each well, (data not shown).

To maintain temperature control the FLUORostar was run in a controlled temperature (18°C) environment.

The effect of both DAR-4M and DAR-4M AM on the germination and development of Guy11 conidia inoculated into a NUNC 96 well optical bottom plate over 16 hours was tested. No differences were observed between Guy11 conidia loaded with DAR-4M, DAR-4M AM or 10mM HEPES pH7.

2.15.2 Conidia loading with DAR-4M (AM)

DAR-4M AM (solution) was diluted to 1mM in dimethyl sulfoxide (DMSO) before being aliquoted and stored at -20°C. DAR-4M (powder) was dissolved in acetonitrile, aliquoted, vacuum dried under anoxic conditions, sealed with Parafilm and stored at -20°C. The preparation of DAR-4M (AM) in 50ml plastic Falcon tubes led to dye auto-activation, resulting in increased fluorescence. Preparation of DAR-4M (AM) within glass 50ml Duran centrifugation tubes prevented this auto-activation.

During conidia harvesting (Chapter 2, 2.2.2) damage to the fungal mycelia released cellular enzymes, including esterases, into the supernatant along with conidia. These cleaved the diacetate group of FDA and the acetoxymethyl ester of DAR-4M AM during dye loading, resulting in extracellular dye activation and reduced intracellular dye accumulation. To alleviate this problem, conidia were washed via centrifugation and re-suspension, in demin water three times to remove extracellular esterases.

Conidia were harvested from three 10 day old cultures, (detailed in Chapter 2, 2.2.2), and filtered through a double layer of Miracloth into a 50ml glass Duran centrifuge tube, before being centrifuged for three minutes at 4750rpm (5251g) at room temperature. The supernatant was discarded and the conidia pellet re-suspended in 5ml demin water before being centrifuged for a further three minutes at 4750rpm (5251g), at room temperature. The supernatant was again

discarded and the conidia pellet re-suspended in 6ml demin water. 3ml of the conidia suspension was transferred to a fresh 50ml Duran tube. The two tubes, each containing 3ml conidia suspension for the test and control samples, were centrifuged again for three minutes at 4750rpm (5251g) at room temperature and the supernatant removed. On ice 10 μ l 1mM DAR-4M (AM) in DMSO was diluted with 250 μ l 100mM HEPES pH7 and 2240 μ l demin water to a final concentration of 2 μ M DAR-4M (AM) 10mM HEPES pH7. Conidia were re-suspended in this solution, while control conidia were re-suspended in 2.5ml 10mM HEPES pH7. Conidia suspensions were incubated for 30 minutes at room temperature in the dark to allow dye loading. Following their incubation, conidia were centrifuged twice for three minutes at 4750rpm (5251g) at room temperature, after each spin the supernatant (containing excess DAR-4M (AM) was discarded). Both test and control conidia were re-suspended in 1ml 10mM HEPES pH7. Their conidia concentrations were measured using a haemocytometer and adjusted to 1×10^4 . 200 μ l 1×10^4 conidia suspension was inoculated into each well of the NUNC optical bottom plate. Fluorescence ($\lambda_{\text{excitation}} = 544 \text{ nm}$, $\lambda_{\text{emission}} = 590 \text{ nm}$) from loaded conidia was recorded for 16/12 hours at 20°C, unless otherwise stated. The viability of the conidia was tested using fluorescent microscopy and a combination of FDA and propidium iodide dyes. Each experiment contained a minimum of three biological replicates and was independently replicated on three separate occasions.

2.16 Bioinformatics methods

2.16.1 Identifying nitric oxide synthase, nitrate reductase and nitrite reductase genes in *M. oryzae*

Genes of interest were identified in the annotated *M. oryzae* genome, release six (http://www.broadinstitute.org/annotation/genome/magnaporthe_grisea/MultiHome.html)

using the gene index search function and *Basic Local Alignment Search Tool (BLAST)*: *blastp* (protein) and *blastn* (nucleotide). Mammalian nitric oxide synthase, nitrate reductase and nitrite reductase genes were downloaded from NCBI (<http://www.ncbi.nlm.nih.gov/>). All sequence data was stored in Biology Work Bench 3.2 (<http://workbench.sdsc.edu/>) allowing easy access to bioinformatics tools. Biology Work Bench 3.2 was also used for sequence alignments (CLUSTALW), restriction site analysis (TACG) and sequence reverse complementation (REVCMP).

2.16.2 Protein subcellular localisation

Five programs were used to predict protein subcellular localisation. SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) predicts the likelihood of protein secretion. TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) predicts protein subcellular localisation. WoLF PSORT (<http://wolfsort.org/>) predicts protein subcellular localization sites based on the proteins amino acid sequence. MITOPROT (<http://ihg.gsf.de/ihg/mitoprot.html>) predicts the likelihood of the protein to be mitochondrial targeted. NucPred (<http://www.sbc.su.se/~maccallr/nucpred/>) predicts the likelihood of a protein being localised to the nucleus.

2.16.3 Intron-exon architectures and domain locations

Gene intron-exon and domain architectures were identified using the Broad Institute fungi sequence database (<http://www.broadinstitute.org/scientific-community/data>).

2.16.4 Phylogenetic trees

Gene sequences for phylogenetic analysis were identified in the Broad Institute fungal sequence database (<http://www.broadinstitute.org/scientific-community/data>). Sequences were edited in BioEdit (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) before neighbour joining trees were constructed with bootstrap support using MEGA (<http://www.megasoftware.net>).

2.16.5 Promoter analysis

The 1kb nucleotide sequences upstream of the start of the genes of interest were assessed for motifs using MEME (<http://meme.sdsc.edu/meme/website/meme.html>).

Chapter 3: Putative nitric oxide synthase, nitrate reductase and nitrite reductase genes in *Magnaporthe oryzae*

3.1 Introduction

In Chapter 1, the different classes of enzymes capable of synthesising NO were discussed: nitric oxide synthase (*NOS*), nitrate reductase (*NR*) and nitrite reductase (*NIR*). Herein, putative *NOS*, *NR* and *NIR* genes were identified within the *M. oryzae* genome and examined *in silico*. Phylogenetic analyses were used to elucidate the relationships between putative *NOSs*, *NRs* and *NIRs* in *M. oryzae*, fungi, animal and plant species. Identified *M. oryzae* *NOSs*, *NR* and *NIR* sub-cellular localisations were predicted using multiple online recourses. The intron positions, domain motifs, and gene promoter motifs were also assessed.

3.1.1 *Magnaporthe oryzae* genome sequence

Following the sequencing of the first Eukaryotic organism, the fungus *Saccharomyces cerevisiae*, there has been a tremendous increase in the amount of available genome sequence data (Cherry et al. 1997). The area of comparative genomics is expanding at an ever-increasing rate as new technologies enable faster and more cost effective sequencing and advances in computational biology allow complex bioinformatics studies to be conducted. The Broad Institute web-site alone, currently displays over 109 complete and draft fungal genome sequences (<http://www.broadinstitute.org/>).

M. oryzae (strain 70-15), was the first phytopathogenic fungus to be sequenced, via a whole genome shotgun method, achieving seven fold genome coverage (Dean et al. 2005). Completion was aided by previously published genetic maps (Nitta et al. 1997). Within the *M. oryzae* genome, 12,842 putative genes were identified and genome comparisons with the

closely-related Ascomycote fungi *Neurospora crassa* and *Aspergillus nidulans* reveal several interesting differences (Galagan et al. 2003; Galagan et al. 2005). *M. oryzae* carries more predicted genes than either *N. crassa* or *A. nidulans* and has several expanded gene families associated with fungal pathogenicity. These include cutinases, isotrichodermin C-15 hydroxylases, cytochrome P450s and subtilisin-like serine protease families (Dean et al. 2005). *M. oryzae* also has an expanded family of G-protein coupled receptors (GPCRs), 74 of which have not previously been described. Within this family, a subset of twelve GPCRs carry conserved fungal-specific extracellular membrane spanning domains (CFEM). Microarray analysis reveals all CFEM GPCRs are upregulated during fungal pathogenesis, including *Pth11*, which is required for appressorium formation and pathogenesis (DeZwaan et al. 1999; Dean et al. 2005). By contrast, three GPCRs were identified in *N. crassa* and *A. nidulans* (Dean et al. 2005). Interestingly, the predicted secretome for *M. oryzae* is double that predicted for *N. crassa*. The large predicted secretome for *M. oryzae*, coupled with its expanded gene families and novel GPCRs provides insight into the genetic basis of its hemibiotrophic phytopathogenic life-style.

3.1.2 Phylogenetics

Phylogenetic analyses allow evolutionary relationships and diversity between organisms to be established. Analyses facilitate the identification of multigene families, gene clusters and highlight genes undergoing rapid evolution. There are several different phylogenetic analysis techniques. These can be split into i) traditional methods and ii) Bayesian methods. The traditional methods include: Neighbour-Joining (NJ), Maximum Likelihood, Minimum Evolution and Parsimony. Traditional methods calculate the most probable phylogenetic tree and assess the confidence of each branch of the tree.

Neighbour-Joining is rapid relative to other techniques and provides accurate results for non-divergent sequences (Saitou & Nei 1987). NJ analysis starts by converting DNA or protein

sequences into a distance matrix, in which the evolutionary distances between sequences are estimated. Holder & Lewis (2003) defined the evolutionary distance as “the number of changes that have occurred along the branches between sequences” (Holder & Lewis 2003). From the distance matrix, a single phylogeny is created following a star decomposition method, which uses an approximation to the minimum evolution optimality criteria.

The NJ approach suffers in that; sequence differences do not always reflect accurate evolutionary distances. Effectively, the more divergent the sequences are the greater the likelihood of encountering this problem (Studier & Keppler 1988). To counteract this, it is advantageous to generate distance matrices using protein rather than DNA sequences. Protein sequences are more conserved than their corresponding coding DNA sequences. They are unaffected by synonymous DNA substitutions, which can influence distance matrix generation.

An inherent weakness of all traditional methods is that they generate only a single estimate of the true relationship. It is therefore important to have an indication of the strength of fit between the data and the final phylogeny. This can be achieved using the statistical method of Bootstrapping, which calculates the support between the data and the estimated phylogeny, through randomly re-sampling the original data matrix generating pseudo data sets (Efron 1979). A typical bootstrap analysis contains 100 - 1000 replicates. For each pseudo-dataset a separate phylogeny is generated, the frequency of taxa positions within each pseudo phylogeny is evaluated, so generating the Bootstrap value.

The Bootstrap value represents the strength of support between the data and the phylogeny. A high Bootstrap value indicates that the addition of extra sequence data is unlikely to affect a specific clades position. A low bootstrap value suggests that the addition of extra sequence data is likely to alter the position of a specific clade (Felsenstein 1985). Bootstrap values of 70% or higher are considered strong support for a clades position (Hillis & Bull 1993).

NJ, combined with Bootstrap support represents a 'cost-effective' technique with regard to time and computational power, for building phylogenies. However, if highly divergent sequences are to be analysed then Bayesian methods are more appropriate. Bayesian methods generate a phylogenetic tree and an estimate for each branch within the phylogeny; however, they require prior distribution parameters to be specified and are more time and computationally demanding (Holder & Lewis 2003).

3.1.3 Subcellular protein localization

Further useful information regarding putative *NOSs*, *NR* and *NIR* can be determined by predicting their protein subcellular locations. There are multiple different, web-based tools, of which the following were used.

- NucPred (<http://www.sbc.su.se/~maccallr/nucpred/>) predicts the likelihood of a protein being localised to the nucleus. A candidate protein sequence is analysed by 100 sequence based predictors, which individually predict the protein localisation. The NucPred score reflects the percentage of predictors which suggest protein localisation in the nucleus (MacCallum 2007). The sequence-based predictors are continuously updated using an evolutionary machine-learning technique (MacCallum 2007).
- SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) predicts the presence and location of signal peptide cleavage sites in protein sequences. This method combines a prediction of cleavage sites and signal peptide/non T signal peptide estimation, based on the combination of multiple artificial neural networks and hidden Markov models (Emanuelsson et al. 2007).
- TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) predicts the subcellular location of the protein of interest. The location is based on the predicted presence of any of the N-terminal presequences (Emanuelsson et al. 2007).
- WoLF PSORT (<http://wolfpsort.org/>) predicts protein subcellular localization sites based on their amino acid sequences. WoLF PSORT is an extension of the PSORTII program and generates its

predictions using both known sorting signal motifs and correlative sequence features (Horton et al. 2007). The prediction accuracy WoLF PSORT has been calculated at over 80%.

- MITOPROT (<http://ihg.gsf.de/ihg/mitoprot.html>) evaluates the N-terminal protein region for mitochondrial targeting sequences and the cleavage sites, based on 47 separate parameters. MITOPROT has a 75-97% accuracy of correctly identifying mitochondrial targeted genes (*Claros & Vincens 1996*).

3.1.4 Intron-exon architectures

The relative abundance and position of introns within putative *NOSs*, *NR* and *NIR* were observed and compared to closely related genes identified from the phylogenetic analyses. Information regarding intron-exon architectures was obtained from the Broad Institute website (<http://www.broadinstitute.org/>).

3.1.5 Gene domain sequences

The location of protein domain sequences within putative *M. oryzae* *NOSs*, *NR* and *NIR*, and in proteins from related species, identified through the phylogenetic analyses, were assessed. Information regarding gene domain sequences was obtained from the Broad Institute website (<http://www.broadinstitute.org/>).

3.1.6 Gene promoter analysis

The 1kb sequence 5' to ATG sequences for *M. oryzae* *NOSs*, *NR* and *NIR* were examined for common sequence motifs using MEME (http://meme.sdsc.edu/meme4_6_0/cgi-bin/meme.cgi). MEME uses statistical modelling methods to assign the best width, number of occurrences, and description for each motif (Bailey & Elkan 1994.)

3.1.7 Expression profiles of putative *NOS* genes

Quantitative real time RT-PCR was used to profile the mRNA transcript levels of *NOS1*, *NOS2*, *NOS3* and *NOS4*, Figures 3.1 and 3.2 (Iles M, 2007, DPhil thesis, Oxford). mRNA was extracted from Guy11 germlings inoculated on barley leaves at 0.5, 1, 2, 5 and 12 hours post inoculation (hpi), mRNA was also extracted from un-germinated Guy11 conidia, 0 hpi (see Chapter 2, 2.12 for details).

Relative *NOS* mRNA transcript abundances were compared with two constitutively expressed genes: *β-TUB* and *EF-1α*. The transcript profile of each *NOS* gene was normalized to 1 at 0 hpi.

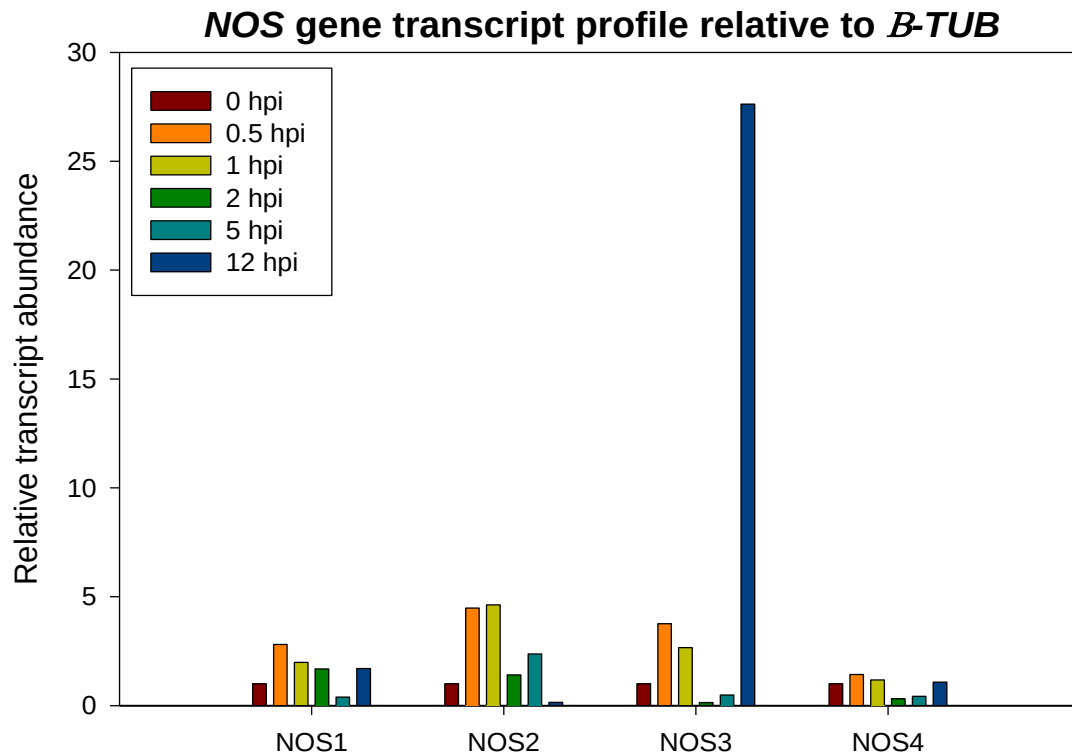


Figure 3.1. The expression levels of *NOS1*, *NOS2*, *NOS3* and *NOS4* genes in Guy11 germlings differentiating on barley leaves over a 12 hour time-course, relative to *β-TUB* expression (Iles M, 2007, DPhil thesis, Oxford). No amplification was observed from control reactions without either cDNA template or reverse transcriptase. Data represents three independent experimental replicates. Data was analysed with Pfaffl, comparing expression values with the control, 0 hpi.

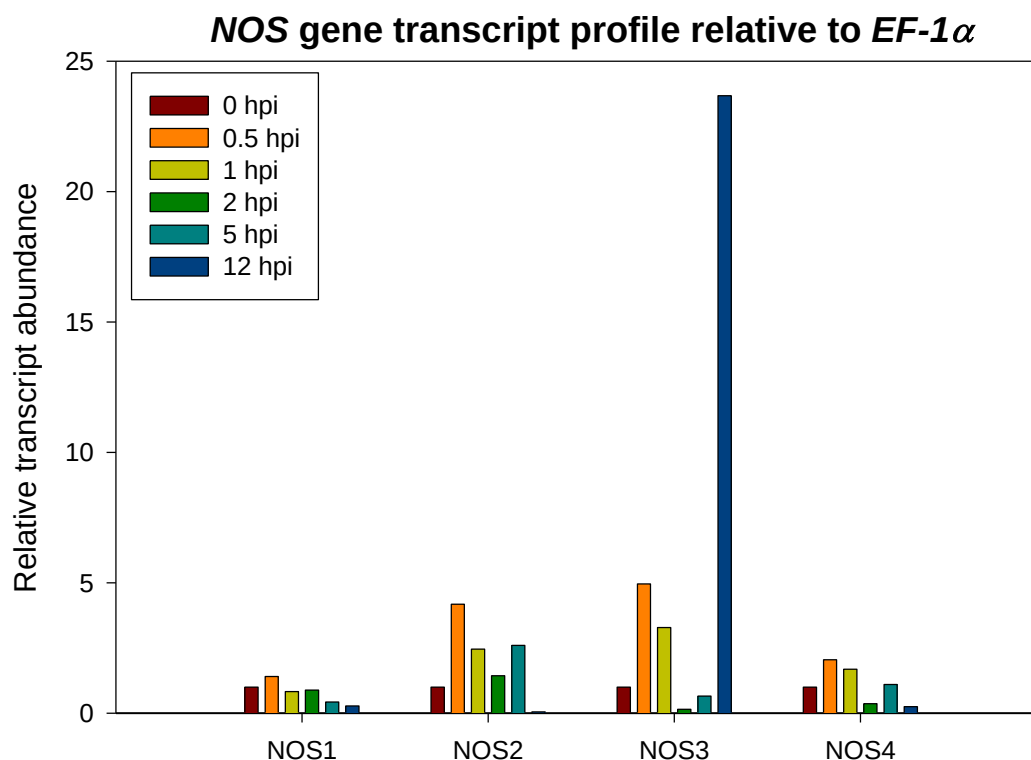


Figure 3.2. The expression levels of *NOS1*, *NOS2*, *NOS3* and *NOS4* genes in Guy11 germlings differentiating on barley leaves over a 12 hour time-course, relative to *EF-1 α* expression (Iles M, 2007, DPhil thesis, Oxford). No amplification was observed from control reactions without either cDNA template or reverse transcriptase. Data represents three independent experimental replicates. Data was analysed with Pfaffl, comparing expression values with the control, 0 hpi.

The expression level of *NOS3* was significantly increased relative to both constitutively expressed genes: *β -TUB* and *EF-1 α* at 12 hpi, approximately 25 fold higher than at 0 hpi. *NOS1*, *NOS2* and *NOS4* exhibited reduced expression levels during pathogenesis, with *NOS2* being the second most highly expressed gene at 0.5hpi, corresponding with germ tube formation.

3.2 Experimental objectives

1. To identify putative *NOS*s, nitrate reductase and nitrite reductase genes within the *M. oryzae* genome.
2. To build individual *NOS*, *NR* and *NIR* phylogenies using neighbour-joining.
3. To examine putative *NOS*, *NR* and *NIR* protein sub-cellular localisation.
4. To examine the DNA sequence of putative *M. oryzae* *NOS*, *NR* and *NIR* genes, and genes from related species, with regard to their intron-exon architectures, domains and upstream promoters.

3.3 Results

3.3.1 Identification of putative *NOS*, nitrate reductase and nitrite reductase genes

Mary Illes (DPhil, Oxford 2007) identified ten putative *NOS*s within the *M. oryzae* genome (fourth release) using the BLASTp analysis tool. Of these initial *NOS*s she discarded six as they lacked the required domains for a functional NOS enzyme. The four remaining *NOS*s (classified as bifunctional P-450:NADPH-P450 reductases by the Broad Institute) were named *NOS1*, *NOS2*, *NOS3* and *NOS4* and considered as putative *NOS*s, Table 3.1. The original BLASTp analysis was re-conducted using the sixth release of the *M. oryzae* genome (<http://www.broadinstitute.org/>), and confirmed the originally identified putative *NOS*s.

Gene name	Accession number	Super Contig	Genomic DNA length (bp)	Coding DNA length (bp)	Amino Acid residues
<i>NOS1</i>	MGG_01925	25	3645	3467	1093
<i>NOS2</i>	MGG_05401	15	3814	3198	1066
<i>NOS3</i>	MGG_07953	28	3447	3258	1086
<i>NOS4</i>	MGG_10879	25	3623	3277	1084

Table 3.1. Putative nitric oxide synthase genes identified in *M. oryzae* genome. Accession number, genomic DNA length, coding DNA length, amino acid residue numbers and Super Contig position are detailed.

Nitrate and nitrite reductase genes were located in the sixth *M. oryzae* genome release. To confirm the genes were correctly annotated within the *M. oryzae* genome, *Arabidopsis thaliana* nitrate reductase Nia1 and nitrite reductase Nir1 protein sequences were used in conjunction with BLASTp to identify nitrate and nitrite reductase genes within *M. oryzae*, Table 3.2.

The *M. oryzae* nitrate reductase gene carries a molybdopterin binding domain, cytochrome b5 reductase and heme/steroid binding domains, Figure 3.12 (Campbell 1999). The nitrite reductase gene carries a nitrite/sulfite reductase ferredoxin-like domain and ferredoxin-nitrite reductase domain (Campbell & Kinghorn 1990). The genes were named *NIA1* (nitrate reductase)

and *NII1* (nitrite reductase), in accordance with the current *M. oryzae* protocol (Lau & Hamer 1996).

Gene name	Accession number	Super Contig	Genomic DNA length (bp)	Coding DNA length (bp)	Amino Acid residues
<i>NIA1</i>	MGG_06062	24	2815	2736	912
<i>NII1</i>	MGG_00634	21	3601	3462	1154

Table 3.2. Nitrate reductase and nitrite reductase genes identified in *M. oryzae* genome. Accession number, genomic DNA length, coding DNA length, amino acid residue numbers and Super Contig position are detailed.

3.3.2 Phylogenetic analyses

Nitric oxide synthase, nitrate and nitrite reductase protein sequences were selected from Fungi, Animals, Plants and Bacteria using the pBLAST analysis tool. Sequences were edited in BioEdit (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) before NJ trees, with Bootstrap support, were constructed using MEGA (<http://www.megasoftware.net>).

3.3.2.1 NOS phylogenies

Three separate NOS phylogenies were constructed containing; i) the complete putative NOS amino acid sequences; ii) NOS oxygenase domain amino acid sequences and iii) NOS reductase domain amino acid sequences.

The complete putative NOS protein sequence phylogeny placed the fungal and animal-specific NOS sequences into two monophyletic groups, Figure 3.3. The putative *M. oryzae* NOS are not localised to a single clade, instead they are positioned next to putative *Colletotrichum graminicola* NOS. Intriguingly, *M. oryzae* and *C. graminicola* represent the only fungal species with no evidence of recent NOS duplication events, with *Aspergillus oryzae*, *Botrytis cinerea*, *Fusarium oxysporum*, *Rhizopus oryzae* and *Verticillium albo-atrum* all showing evidence of recent NOS duplication events.

Whilst searching for putative *NOSs*, multiple genes were identified with strong homologies to either NOS oxygenase or reductase domain sequences. To include these additional sequences in the phylogenetic analyses, two further phylogenies were constructed using only NOS oxygenase or reductase amino acid sequences, Figures 3.4 and 3.5. The resulting NOS oxygenase and reductase domain protein sequence phylogenies closely-resemble the complete NOS protein sequence phylogeny. The putative *M. oryzae NOSs* remain positioned alongside the putative *C. graminicola NOSs*. Putative *NOSs* from *A. oryzae*, *B. cinerea*, *F. oxysporum*, *R. oryzae* and *V. albo-atrum* retain their associations, potentially showing evidence for recent gene duplication.

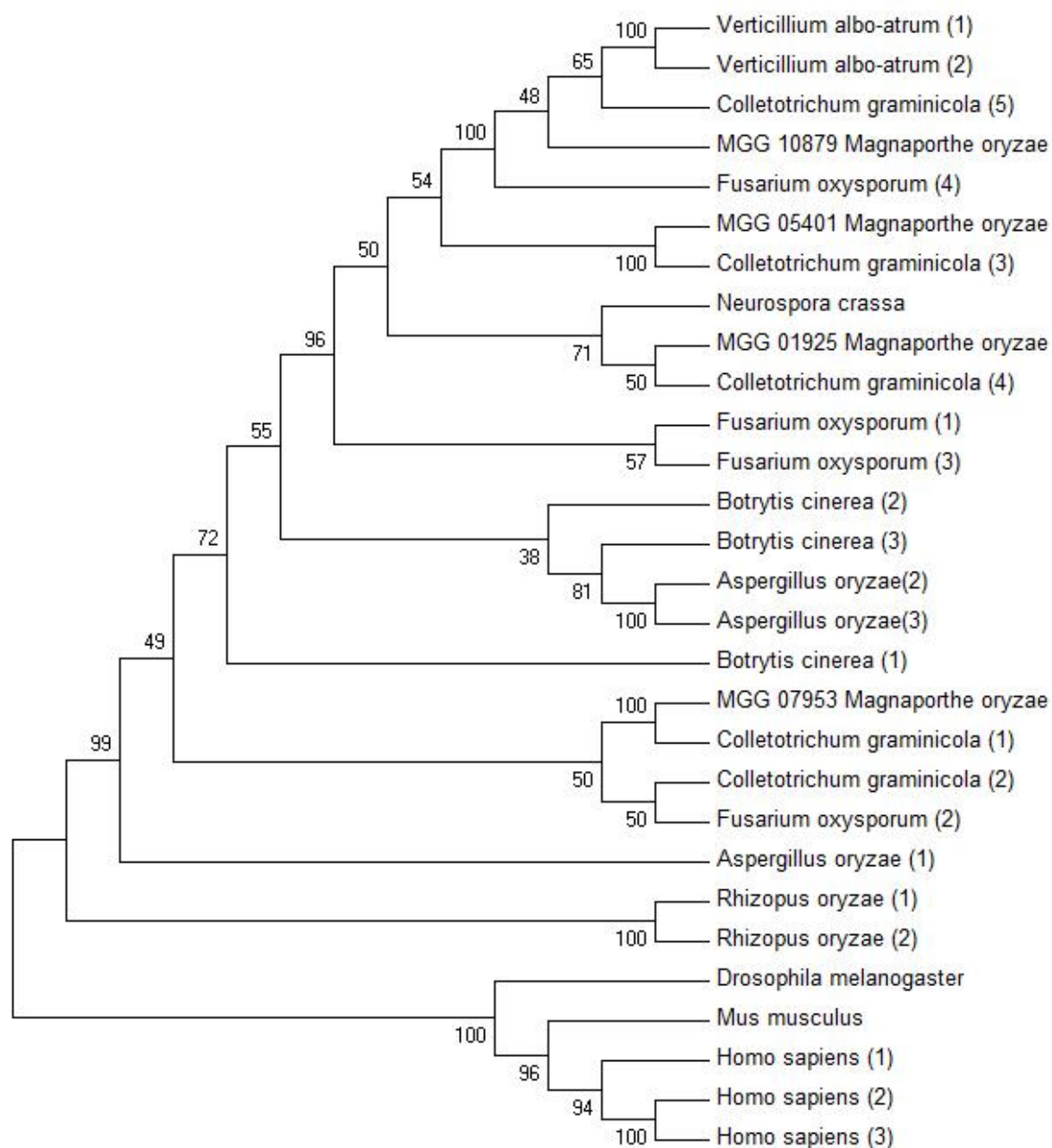


Figure 3.3. NJ analysis of putative fungi and animal complete NOS protein sequences. NOS sequences were edited in BioEdit before a single phylogeny was constructed in MEGA, rooted on the *H. sapiens* (1) sequence. Bootstrap values represent 1000 replicates. See page 93 for further details regarding gene information.

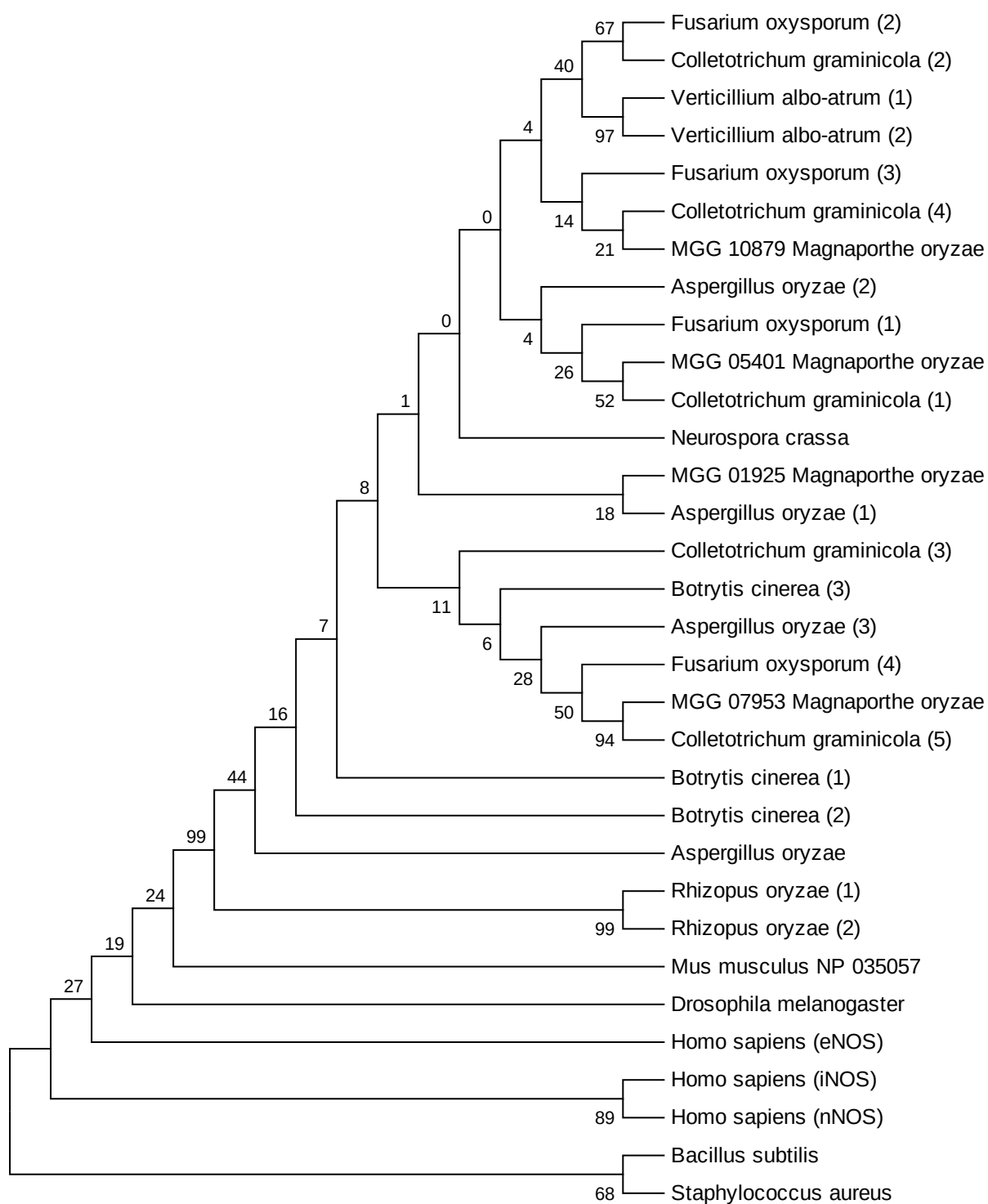


Figure 3.4. NJ analysis of NOS oxygenase domain protein sequences from fungi, animal and bacteria. NOS sequences were edited in BioEdit before a single phylogeny was constructed in MEGA, rooted on the *B. subtilis* sequence. Bootstrap values represent 1000 replicates. See page 93 for further details regarding gene information.

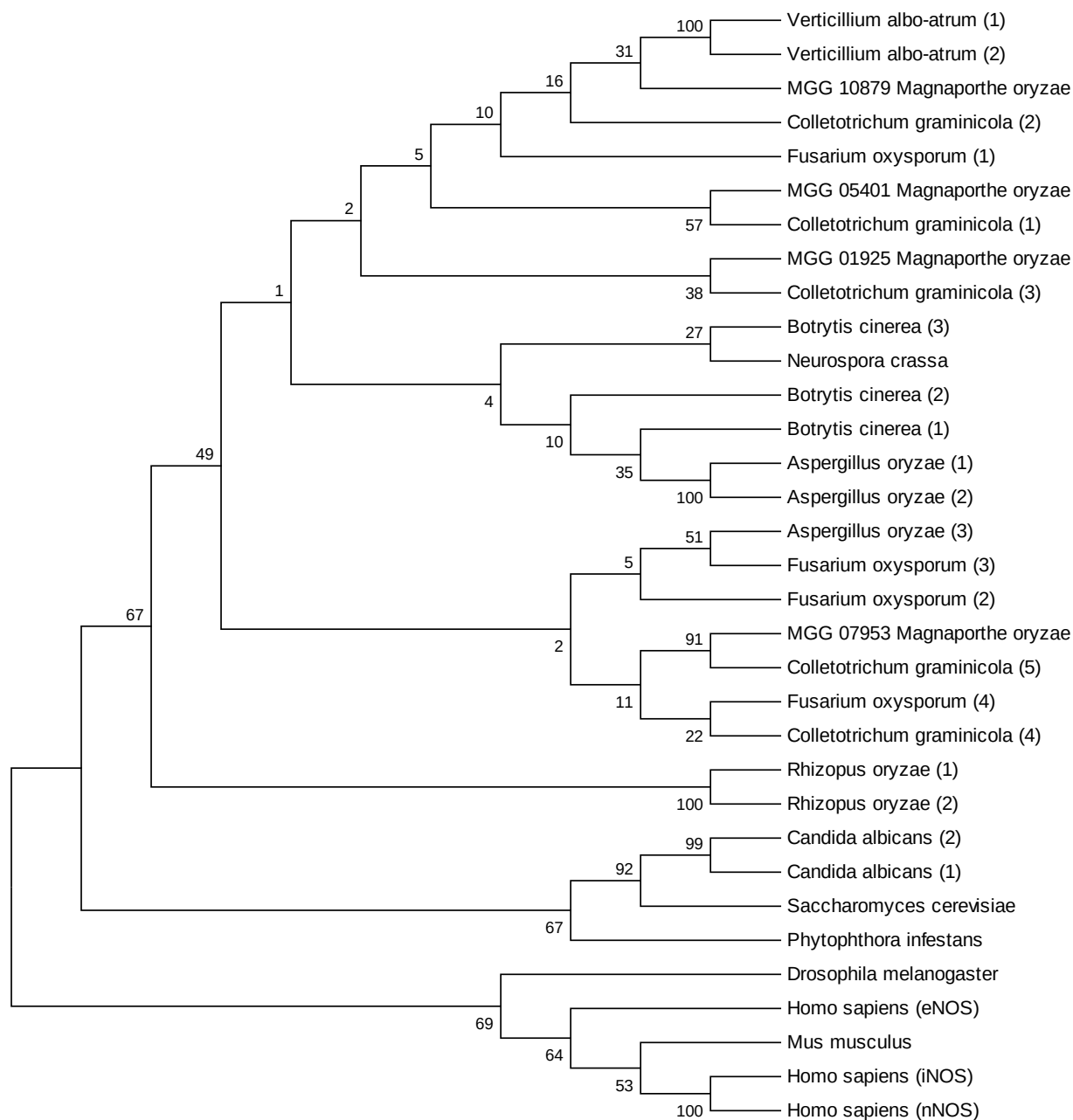


Figure 3.5. NJ analysis of NOS reductase domain protein sequences from fungi, oomycete and animal. NOS sequences were edited in BioEdit before a single phylogeny was constructed in MEGA, rooted on the *H. sapiens* (nNOS) sequence. Bootstrap values represent 1000 replicates. See page 93 for further details regarding gene information.

The complete NOS protein sequences aligned in Figure 3.3:

A. oryzae (1) AO090001000445, *A. oryzae* (2) AO090005001313, *A. oryzae* (3) AO090020000369, *B. cinerea* (1) BC1G_15490.1, *B. cinerea* (2) BC1G_05268.1, *B. cinerea* (3) BC1G_11409.1, *C. graminicola* (1) GLRG_06866.1, *C. graminicola* (2) GLRG_08937.1, *C. graminicola* (3) GLRG_07212.1, *C. graminicola* (4) GLRG_11431.1, *C. graminicola* (5) GLRG_11426.1, *D. melanogaster* NP_001027241, *F. oxysporum* (1) FOXG_04152.2, *F. oxysporum* (2) FOXG_17581.2, *F. oxysporum* (3) FOXG_17221.2, *F. oxysporum* (4) FOXG_15285.2, *H. Sapiens* (*iNOS*) AAB49040, *H. sapiens* (*nNOS*) EAW98109, *H sapiens* (*eNOS*) CAA53950, MGG_01925 *NOS1* *M. oryzae*, MGG_05401 *NOS2* *M. oryzae*, MGG_07953 *NOS3* *M. oryzae*, MGG_10879 *NOS4* *M. oryzae*, *M. musculus* NP_035057, *N. crassa* NCU05185, *R. oryzae* (1) RO3G_10498, *R. oryzae* (2) RO3G_17132, *V. albo-atrum* (1) VDAG_07177.1 and *V. albo-atrum* (2) VDBG_05527.1.

The NOS oxygenase protein sequences aligned in Figure 3.4:

A. oryzae (1) AO090001000445, *A. oryzae* (2) AO090005001313, *A. oryzae* (3) AO090020000369, *B. subtilis* YP_004206779, *B. cinerea* (1) BC1G_15490.1, *B. cinerea* (2) BC1G_05268.1, *B. cinerea* (3) BC1G_11409.1, *C. graminicola* (1) GLRG_06866.1, *C. graminicola* (2) GLRG_08937.1, *C. graminicola* (3) GLRG_07212.1, *C. graminicola* (4) GLRG_11431.1, *C. graminicola* (5) GLRG_11426.1, *D. melanogaster* NP_001027241, *F. oxysporum* (1) FOXG_04152.2, *F. oxysporum* (2) FOXG_17581.2, *F. oxysporum* (3) FOXG_17221.2, *F. oxysporum* (4) FOXG_15285.2, *H. Sapiens* (*iNOS*) AAB49040, *H. sapiens* (*nNOS*) EAW98109, *H sapiens* (*eNOS*) CAA53950, MGG_01925 *NOS1* *M. oryzae*, MGG_05401 *NOS2* *M. oryzae*, MGG_07953 *NOS3* *M. oryzae*, MGG_10879 *NOS4* *M. oryzae*, *M. musculus* NP_035057, *N. crassa* NCU05185, *R. oryzae* (1) RO3G_10498, *R. oryzae* (2) RO3G_17132, *S. aureus* ZP_05693526, *V. albo-atrum* (1) VDAG_07177.1 and *V. albo-atrum* (2) VDBG_05527.1.

The NOS reductase protein sequences aligned in Figure 3.5:

A. oryzae (1) AO090001000445, *A. oryzae* (2) AO090005001313, *A. oryzae* (3) AO090020000369, *B. cinerea* (1) BC1G_15490.1, *B. cinerea* (2) BC1G_05268.1, *B. cinerea* (3) BC1G_11409.1, *C. albicans* CAWG_03482 (1), *C. albicans* orf19.2672 (2), *C. graminicola* (1) GLRG_06866.1, *C. graminicola* (2) GLRG_08937.1, *C. graminicola* (3) GLRG_07212.1, *C. graminicola* (4) GLRG_11431.1, *C. graminicola* (5) GLRG_11426.1, *D. melanogaster* NP_001027241, *F. oxysporum* (1) FOXG_04152.2, *F. oxysporum* (2) FOXG_17581.2, *F. oxysporum* (3) FOXG_17221.2, *F. oxysporum* (4) FOXG_15285.2, *H. Sapiens* (*iNOS*) AAB49040, *H. sapiens* (*nNOS*) EAW98109, *H sapiens* (*eNOS*) CAA53950, MGG_01925 *NOS1* *M. oryzae*, MGG_05401 *NOS2* *M. oryzae*, MGG_07953 *NOS3* *M. oryzae*, MGG_10879 *NOS4* *M. oryzae*, *M. musculus* NP_035057, *N. crassa* NCU05185, *P. infestans* PITG_06954, *R. oryzae* (1) RO3G_10498, *R. oryzae* (2) RO3G_17132, *S. cerevisiae* SCRG_04751.1, *V. albo-atrum* (1) VDAG_07177.1 and *V. albo-atrum* (2) VDBG_05527.1.

3.3.2.2 NR phylogenies

Two NR phylogenies were constructed, the first, a fungal specific tree containing NR protein sequences from Fungi and the oomycete *P. infestans*, rooted on *Arabidopsis thaliana*, Figure 3.6. The second tree constructed contained NR protein sequences from Fungi, oomycetes, Animals, Plants and Bacteria, rooted on *Bacillus cereus*, Figure 3.7.

The fungal specific NR phylogeny placed the Ascomycetes together as a monophyletic group, with *M. oryzae* positioned alongside *V. fungicola*. The lower fungi *U. maydis*, *P. graminis* and *R. oryzae* form a paraphyletic group, but collectively fungi form a monophyletic group.

Addition of the plant, animal and bacterial NR protein sequences, in the second analysis did not influence the grouping of the fungal sequences. The Ascomycotes remain a monophyletic group, whilst the Basidiomycotes, Zygomycotes and oomycetes form a polyphyletic group. Plants and bacteria were both grouped as new monophyletic groups.

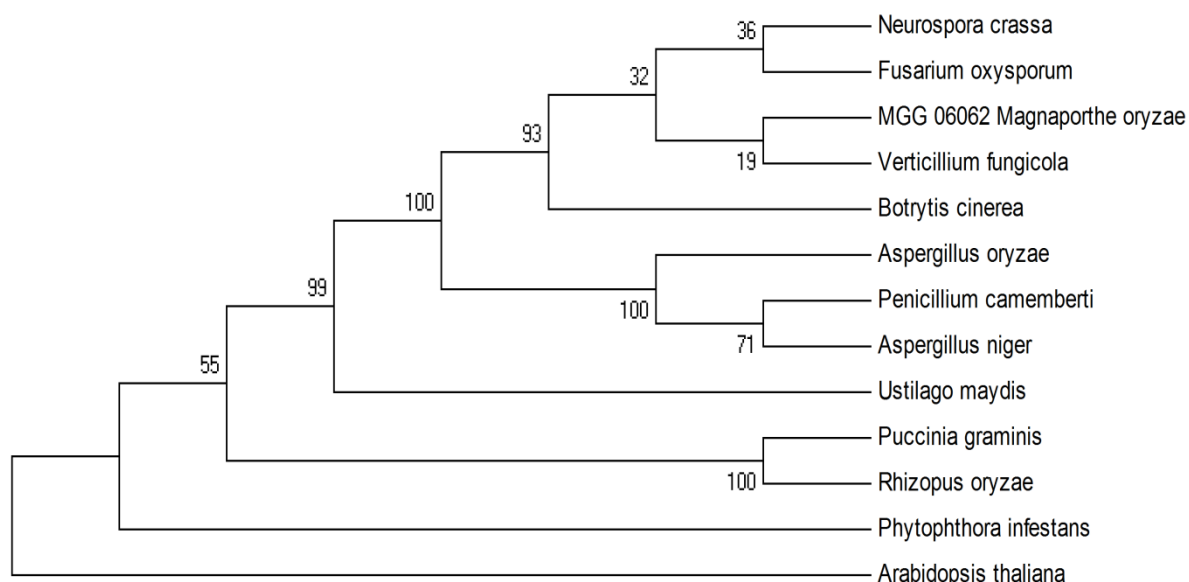


Figure 3.6. NJ analysis of fungi and the oomycete *P. infestans* NR protein sequences. NR sequences were edited in BioEdit before a single phylogeny was constructed in MEGA, rooted on the *A. thaliana* sequence. Bootstrap values represent 1000 replicates.

A. thaliana NP_174901.1, *A. niger* XP_001392678.1, *A. oryzae* XP_001727611.2, *B. cinerea* BC1G_09772.1, *F. oxysporum* FOXG_04181, *M. oryzae* MGG 06062, *N. crassa* NCU05298.4, *P. camemberti* ACI29313.1, *P. infestans* XP_002900553.1, *P. graminis* EFP77954.1, *R. oryzae* RO3G_03220.3, *U. maydis* CAA47918.1 and *V. fungicola* AAO63560.

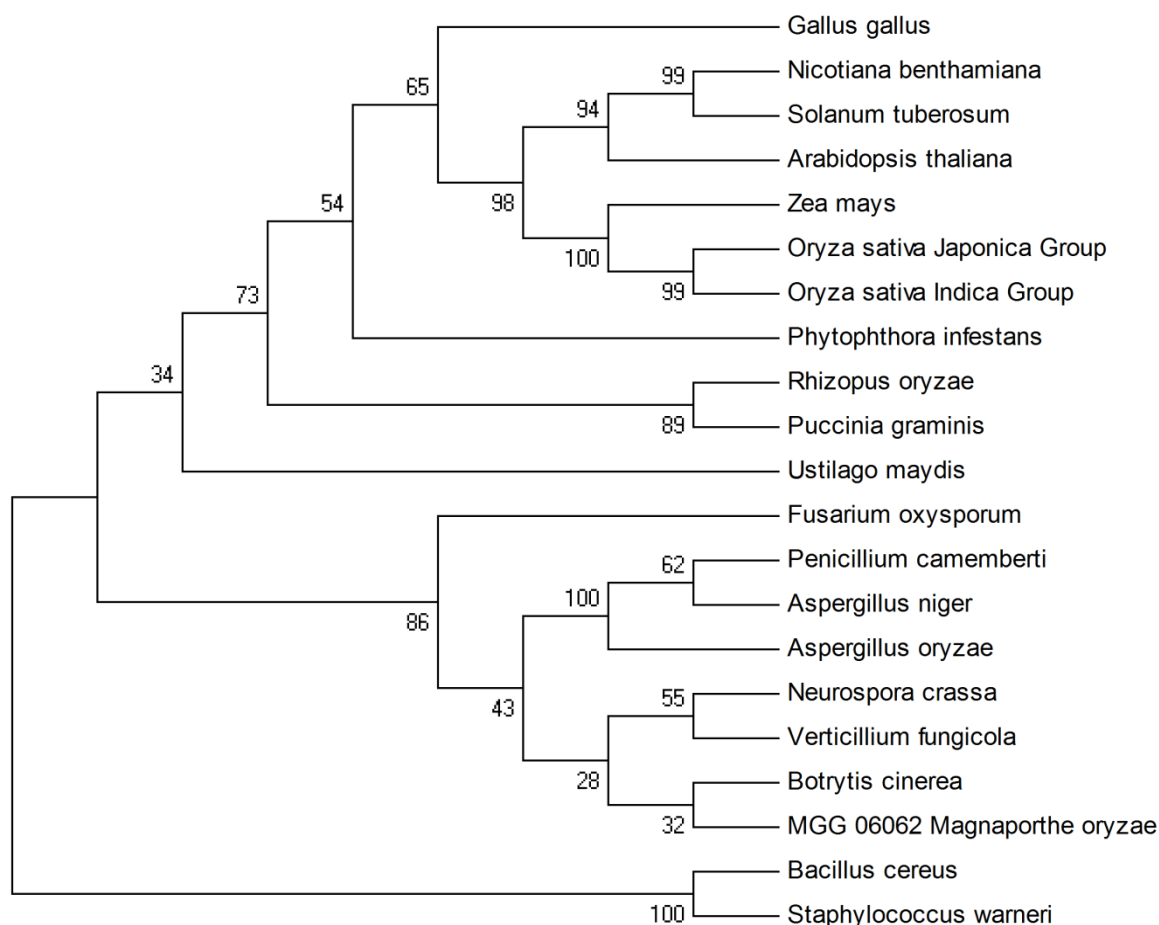


Figure 3.7. NJ analysis of fungi, oomycete, plant, animal and bacterial NR protein sequences. NR sequences were edited in BioEdit before a single phylogeny was constructed in MEGA, rooted on the *S. warneri* sequence. Bootstrap values represent 1000 replicates.

A. thaliana NP_174901.1, *A. niger* XP_001392678.1, *A. oryzae* XP_001727611.2, *B. cereus* ZP_04311574.1, *B. cinerea* BC1G_09772.1, *F. oxysporum* FOXG_04181, *G. gallus* XP_420957, *M. oryzae* MGG 06062, *N. crassa* NCU05298.4, *N. benthamiana* BAE46746.1, *P. camemberti* ACI29313.1, *P. infestans* XP_002900553.1, *P. graminis* EFP77954.1, *O. sativa Indica* EEC83712.1, *O. sativa Japonica* NP_001062006.1, *R. oryzae* RO3G_03220.3, *S. tuberosum* BAB93534.1, *S. warneri* ZP_04677041.1, *U. maydis* CAA47918.1, *V. fungicola* AAO63560 and *Z. mays* AAD38068.1

3.3.2.3 NIR phylogenies

Two NIR phylogenies were also constructed; the first contained only fungi and the oomycete *P. infestans*, Figure 3.8, the second contained additional plant and bacterial NIR protein sequences, Figure 3.9. The fungi specific NIR phylogeny is markedly different from the fungi specific NR phylogeny. Ascomycetes, Basidiomycetes, Zygomycetes and oomycetes were aligned alongside each other without any phyla specific monophyletic groups. *M. oryzae* is located next to the phytopathogen *B. cinerea* and is positioned next to other closely-related Ascomycete fungi, *N. crassa* and *C. higginsianum*. The addition of plant and bacterial protein sequences has no significant effect on the positioning of the fungi, which remain a polyphyletic group. The plants were positioned as a new monophyletic group. *M. oryzae* remains located alongside closely-related Ascomycete fungi.

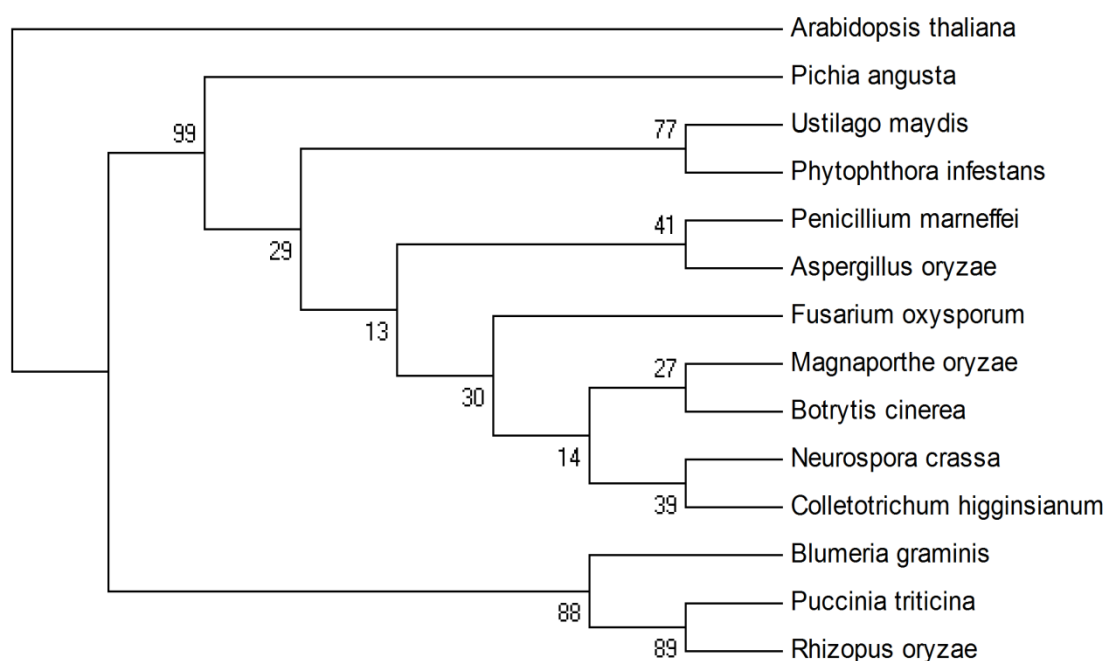


Figure 3.8. NJ analysis of fungi and the oomycete *P. infestans* NIR protein sequences. NIR sequences were edited in BioEdit before a single phylogeny was constructed in MEGA, rooted on the *A. thaliana* sequence. Bootstrap values represent 1000 replicates.

A. thaliana NP_174901.1, *A. oryzae* XP_001727611.2, *B. graminis* AAL05260.1, *B. cinerea* BC1G_00437.1, *C. higginsianum* CH063_01363.1, *F. oxysporum* BAF31848.1, *M. oryzae* MGG_00634, *N. crassa* XP_960355.2, *P. marneffeii* XP_002146261.1, *P. infestans* XP_002900554.1, *P. angusta* CAA92206.1, *P. tritici* EFP83381.1, *R. oryzae* RO3G_09852.3 and *U. maydis* CAC41649.1.

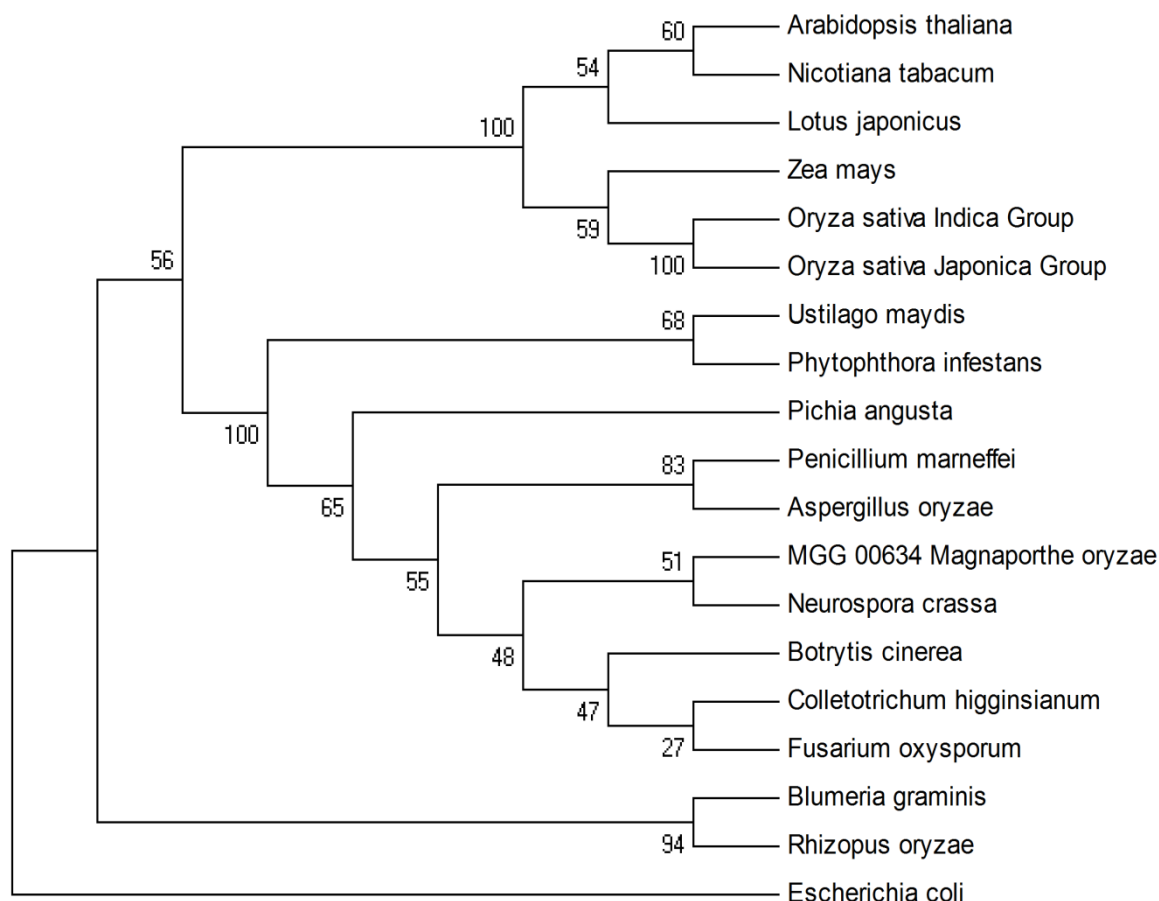


Figure 3.9. NJ analysis of fungi, the oomycete *P. infestans*, plant, animal and bacterium NIR protein sequences. NIR sequences were edited in BioEdit before a single phylogeny was constructed in MEGA, rooted on the *E. coli* sequence. Bootstrap values represent 1000 replicates.

A. thaliana NP_174901.1, *A. oryzae* XP_001727611.2, *B. graminis* AAL05260.1, *B. cinerea* BC1G_00437.1, *C. higginsianum* CH063_01363.1, *E. coli* ZP_07133646.1, *F. oxysporum* BAF31848.1, *L. japonicus* CAC06095.1, *M. oryzae* MGG_00634, *N. crassa* XP_960355.2, *N. tabacum* BAD15365.1, *O. sativa Indica* EEC70619.1, *O. sativa Japonica* NP_001043008.1, *P. marneffeii* XP_002146261.1, *P. infestans* XP_002900554.1, *P. angusta* CAA92206.1, *P. tritici* EFP83381.1, *R. oryzae* RO3G_09852.3 and *U. maydis* CAC41649.1 and *Z. mays* ACG29734.1.

3.3.3 Protein sub-cellular localisation

Five, previously described, web-based *in silico* analyses were used to predict the protein sub-cellular localization of Nos1, Nos2, Nos3, Nos4, Nia1 and Nii1, Table 3.3.

TargetP, WoLF PSORT and MITOPROT predict a mitochondrial localization for Nos1. NucPred predicts a slight probability of nuclear localisation and SignalP, suggests no evidence for Nos1 secretion.

Nos2 is strongly predicted for mitochondrial localization by WoLF PSORT, and to a lesser extent by TargetP and MITOPROT. Nos2 is not predicted to be localised to the nucleus (NucPred) and SignalP provides no support for it being secreted.

Nuclear localization was predicted for Nos3 by WoLF PSORT, TargetP, and to a lesser extent by MITOPROT. Nos3 is not predicted to be localised to the nucleus (NucPred) with no evidence to suggest it is secreted (SignalP).

Nos4 nuclear localisation was predicted by NucPred, in contrast, MITOPROT and TargetP predict its localisation outside of the mitochondrion. WoLF PSORT indicates cytoplasmic localisation, with SignalP not predicting its secretion.

Protein	Program	Predicted cellular location
Nos1 MGG_01925	NucPred	Nucleus localisation probability 0.2
	SignalP	Signal peptide probability: 0.000 Signal anchor probability: 0.000
	TargetP	Mitochondrion export probability 0.683
	WoLF PSORT	Probabilities: mito: 14.0, cyto: 8.0, pero: 2.0, cysk: 2.0
	MITOPROT	Mitochondrion export probability 0.8081
Nos2 MGG_05401	NucPred	Nucleus localisation probability 0.2
	SignalP	Signal peptide probability: 0.000 Signal anchor probability: 0.000
	TargetP	Mitochondrion export probability 0.41
	WoLF PSORT	Probabilities: mito: 16.0, cyto: 5.0, nucl: 3.0, pero: 2.0
	MITOPROT	Mitochondrion export probability 0.3242

Nos3 MGG_07953	NucPred	Nucleus localisation probability 0.11
	SignalP	Signal peptide probability: 0.003 Signal anchor probability: 0.000
	TargetP	Mitochondrion export probability 0.573
	WoLF PSORT	Probabilities: mito: 16.0, cyto: 8.0, nucl: 2.0
	MITOPROT	Mitochondrion export probability 0.192
Nos4 MGG_10879	NucPred	Nucleus localisation probability 0.32
	SignalP	Signal peptide probability: 0.003 Signal anchor probability: 0.000
	TargetP	Mitochondrion export probability 0.058
	WoLF PSORT	Probabilities: cyto: 9.0, cysk: 8.0, cyto_nucl: 7.0, mito: 6.0
	MITOPROT	Mitochondrion export probability 0.192

Table 3.3. The predicted sub-cellular localisation for *M. oryzae* Nos1, Nos2, Nos3 and Nos4. Protein locations calculated using NucPred, SignalP, TargetP, WoLF PSORT and MITOPROT.

Comparing putative *M. oryzae* NOS proteins with *H. sapiens* NOS isoforms nNOS, iNOS and eNOS, reveals little similarity between their predicted subcellular locations (Table 3.3 and 3.4). *H. sapiens* nNOS, iNOS and eNOS have high probabilities of nuclear localisation (Nucpred values >0.5) and low probabilities of mitochondrial export (MITOPROT values <0.18). WoLF PSORT predicted cytoplasmic localisation for nNOS and iNOS, and a nuclear positioning for eNOS. In summary, the putative *M. oryzae* NOS sub-cellular locations are separate from the *H. sapiens* NOS isoforms.

Protein	Program	Predicted cellular location
NOS1/nNOS <i>H. sapiens</i> NC_000012.10	NucPred	Nucleus localisation probability 0.57
	SignalP	Signal peptide probability: 0.000 Signal anchor probability: 0.000
	TargetP	Mitochondrion export probability 0.224
	WoLF PSORT	Probabilities: cyto: 10.5, cyto_nucl: 10.3, mito_nucl: 7.3, mito: 4.5
	MITOPROT	Mitochondrion export probability 0.172
NOS2/iNOS <i>H. sapiens</i> NC_000017.10	NucPred	Nucleus localisation probability 0.58
	SignalP	Signal peptide probability: 0.000 Signal anchor probability: 0.000
	TargetP	Mitochondrion export probability 0.141
	WoLF PSORT	Probabilities: cyto: 8.0, plas: 7.0, nucl: 6.0, mito: 4.0
	MITOPROT	Mitochondrion export probability 0.046

NOS3/ NOS <i>H. sapiens</i> NC_000007.13	NucPred	Nucleus localisation probability 0.86
	SignalP	Signal peptide probability: 0.269 Signal anchor probability: 0.070
	TargetP	Mitochondrion export probability 0.089
	WoLF PSORT	Probabilities: nucl: 7.0, mito: 5.0, cyto: 5.0, pero: 5.0
	MITOPROT	Mitochondrion export probability 0.029

Table 3.4. The predicted subcellular localisations for *H. sapiens* NOS1, NOS2 and NOS3. Protein locations calculated using NucPred, SignalP, TargetP, WoLF PSORT and MITOPROT.

TargetP, WoLF PSORT and MITOPROT predicted Nia1 localisation outside of the mitochondria, possibly in the cytoplasm, NucPred suggested a weak probability of nuclear localisation and SignalP suggested no likelihood for Nia1 secretion, Table 3.5.

TargetP, WoLF PSORT and MITOPROT predicted cytoplasmic localisation for Nii1. NucPred predicted only a weak probability of nuclear localisation, and SignalP suggested a low probability of secretion with a strong signal anchor, potentially indicating Nii1 to be membrane localised, Table 3.5.

Protein	Program	Predicted cellular location
Nia1 MGG_06062	NucPred	Nucleus localisation probability 0.22
	SignalP	Signal peptide probability: 0.004 Signal anchor probability: 0.000
	TargetP	Mitochondrion export probability 0.104
	WoLF PSORT	Probabilities: cyto: 12.5, cyto_nucl: 8.0, mito: 4.0, pero: 4.0
	MITOPROT	Mitochondrion export probability 0.011
Nii1 MGG_00634	NucPred	Nucleus localisation probability 0.21
	SignalP	Signal peptide probability: 0.0013 Signal anchor probability: 0.707
	TargetP	Mitochondrion export probability 0.272
	WoLF PSORT	Probabilities: Cyto: 19.0, extr: 3.0, E.R.: 3.0
	MITOPROT	Mitochondrion export probability 0.008

Table 3.5. The predicted sub-cellular localisation for *M. oryzae* Nia1 and Nii1. Protein locations calculated using NucPred, SignalP, TargetP, WoLF PSORT and MITOPROT.

In summary, NucPred, SignalP, TargetP, WoLF PSORT and MITOPROT suggest that Nos1, Nos2 and Nos3 are localised to the mitochondria, whilst Nos4, Nia1 and Nii1 are predicted to be localised in the cytoplasm. Caution must be taken when interpreting these data, as each prediction program has inherent drawbacks. However, using multiple programs to analyse each protein sequence reduces the probability of an incorrectly predicted sub-cellular localisation.

3.3.4 Gene intron-exon architectures

Comparison of the number, length and positions of introns within the four putative *NOS* genes reveals no conservation; with considerable variation between the four genes. All 13 introns (Figure 3.10) carry the GT/AG consensus splice sites and no intron identity was observed between genes.



Figure 3.10. Intron-exon architectures for putative *M. oryzae* *NOS1*, *NOS2*, *NOS3* and *NOS4*. Exons are shown as the blue bars with introns as single lines. Figures adapted from the Broad Institute website (<http://www.broadinstitute.org/scientific-community/data>).

Exon and intron sequence lengths *NOS1* (2453, 91, 681, 87 and 333bp), *NOS2* (351, 69, 36, 73, 690, 81, 817, 97, 575, 65, 44, 231 and 685bp), *NOS3* (3248, 189 and 10bp) and *NOS4* (704, 103, 179, 83, 2148, 95, 119, 65 and 127bp).

The intron-exon architectures of the nitrate reductase genes from *N. crassa*, *F. oxysporum*, *A. nidulans* and *B. cinerea* were compared with *M. oryzae* *NIA1*, Figure 3.11. The genes were

selected based on their close proximity to *NIA1* in the fungi specific NR phylogeny. *NIA1*, *N. crassa* and *F. oxysporum* carry conserved intron-exon architectures suggesting a close evolutionary relationship. *A. nidulans* and *B. cinerea* do not share intron-exon architectures with *NIA1*, and contain six and zero introns respectively. The nine introns identified across the five genes, contain the GT/AG consensus splice sites. The single intron in *NIA1*, *N. crassa*, *F. oxysporum* and *A. nidulans* (located in the cytochrome b5-like heme/steroid binding domain sequence) have similar lengths, but sequence alignment revealed no identity (data not shown).

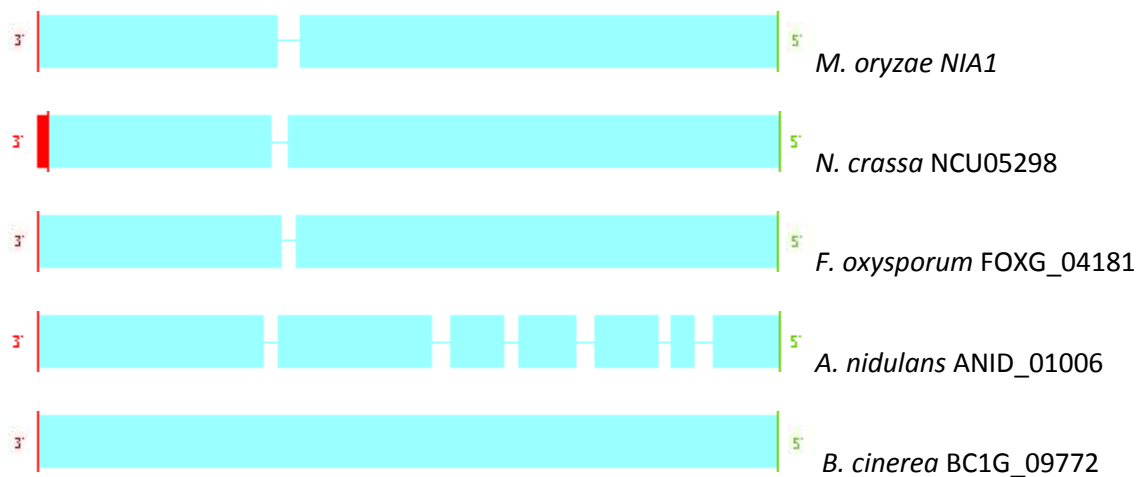


Figure 3.11. Intron-exon architectures for *M. oryzae NIA1*, *N. crassa* NCU05298, *F. oxysporum* FOXG_04181, *A. nidulans* ANID_01006 and *B. cinerea* BC1G_09772 nitrate reductase genes. Exons are shown as the blue bars with introns as single lines. Figures adapted from the Broad Institute website (<http://www.broadinstitute.org/scientific-community/data>).

Exon & intron sequence lengths *M. oryzae NIA1* (1825, 79 and 911bp), NCU05298.4 *N. crassa* (2029, 61 and 974bp), FOXG_04181.2 *F. oxysporum* (1807, 57 and 974bp), ANID_01006.1 *A. nidulans* (614, 52, 624, 67, 224, 53, 236, 68, 259, 50, 100, 66 and 265bp) and BC1G_09772.1 *B. cinerea* (2724bp).

The intron-exon architectures of the nitrite reductase genes from *N. crassa*, *F. oxysporum*, *A. nidulans* and *B. cinerea* were compared with *M. oryzae NII1*, Figure 3.12. Conserved intron-exon architectures were observed between *NII1*, *N. crassa*, *F. oxysporum* and *B. cinerea* with each

gene containing a single intron in their nitrite and sulphite reductase 4Fe-4S and pyridine nucleotide-disulphide oxidoreductase domain sequences. *N. crassa*, *F. oxysporum* and *B. cinerea* carry an additional intron in their pyridine nucleotide-disulphide oxidoreductase domain. There is little conservation in the intron-exon architectures between *A. nidulans* and *NII1*, *A. nidulans* contains an additional five introns. The 18 introns identified across the five genes contained the GT/AG consensus splice sites. Similar to the nitrate reductase intron-exon architectures, the conserved introns within the nitrite and sulphite reductase 4Fe-4S and pyridine nucleotide-disulphide oxidoreductase domains have comparable lengths but share no sequence identity.

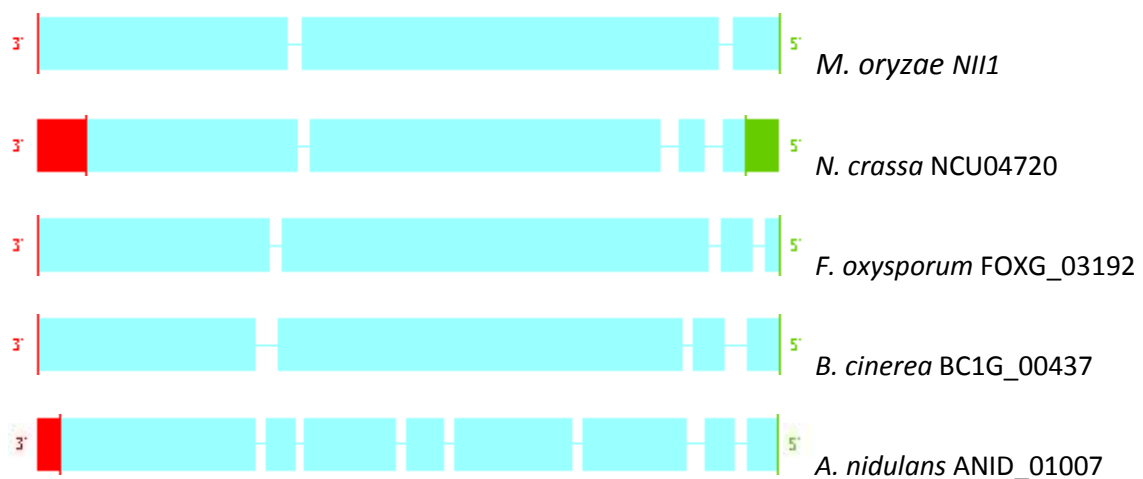


Figure 3.12. Intron and exon architectures for *M. oryzae* NII1, *N. crassa* NCU04720, *F. oxysporum* FOXG_03192, *B. cinerea* BC1G_00437 and *A. nidulans* ANID_01007 nitrate reductase genes. Exons are shown as the blue bars with introns as single lines. Figures adapted from the Broad Institute website (<http://www.broadinstitute.org/scientific-community/data>).

Exon & intron sequence lengths NII1 (1222, 66, 2024, 73 and 216bp), *N. crassa* NCU04720 (1511, 65, 2033, 106, 148, 107 and 315bp), *F. oxysporum* FOXG_03192 (1081, 53, 1973, 51, 151, 53 and 65bp), *B. cinerea* BC1G_00437 (1066, 113, 1967, 55, 151, 106 and 155bp) and *A. nidulans* ANID_01007 (176, 61, 151, 92, 540, 52, 611, 52, 203, 47, 473, 48, 158, 48 and 1124bp).

3.3.5 Protein domains

The position of domains within the four putative NOS proteins was assessed, Figure 3.13. The location of the domains are conserved across the four proteins, with the Cytochrome P450 domains located at the N-terminus, the oxidoreductase domains located at the C-terminus and the flavodoxin domains positioned in the centre of each protein. NOS2 does not carry a FAD binding domain. In summary, there is a high level of domain conservation between the putative NOS proteins.

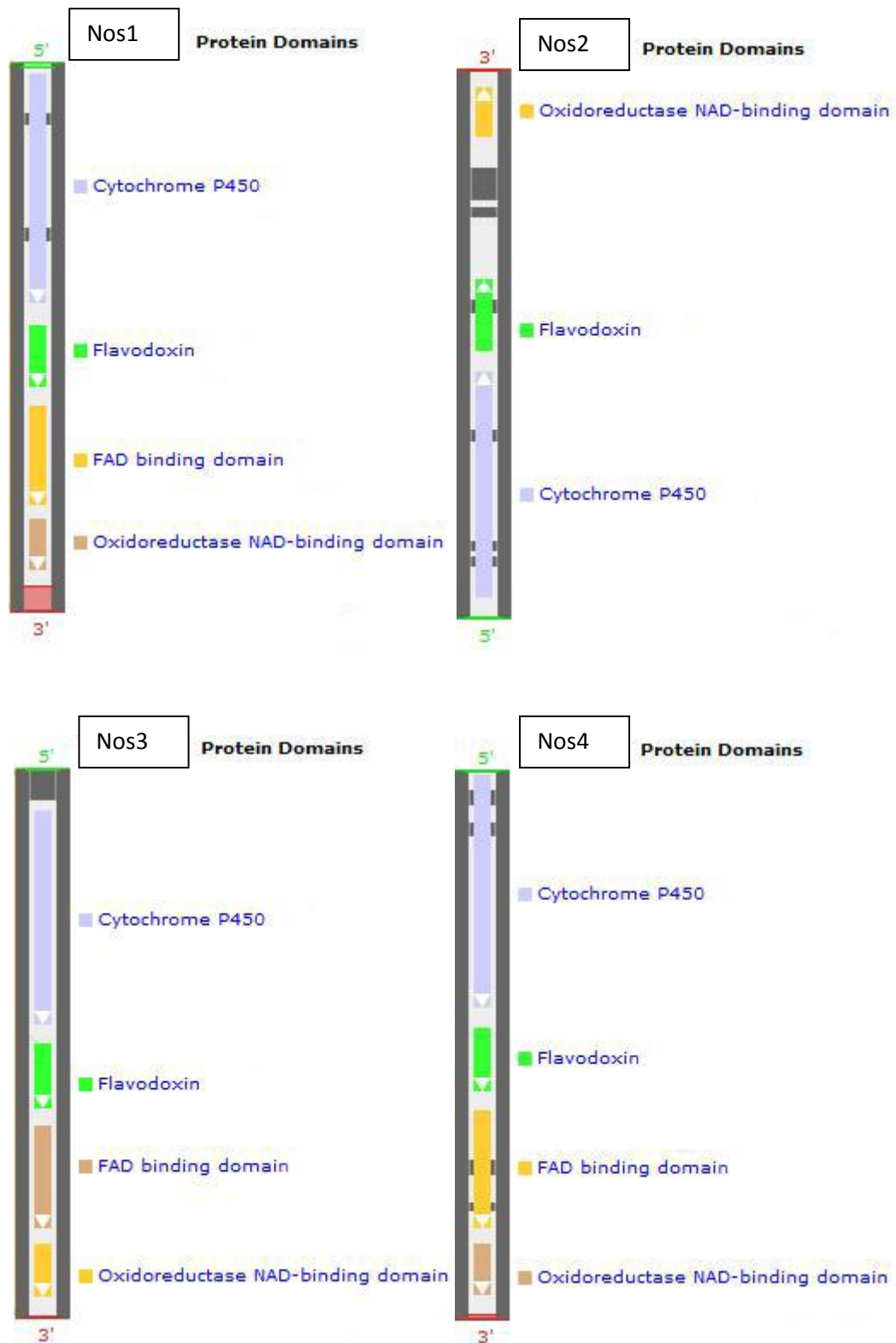


Figure 3.13. The domain architectures of the four putative *M. oryzae* NOS proteins. The intron-exon architectures are detailed beneath the domains, the exons depicted as the white vertical band and the introns represented as the grey bands. Figures adapted from the Broad Institute website

(http://www.broadinstitute.org/annotation/genome/magnaporthe_grisea/MultiHome.html).

The position of the domains within *M. oryzae* Nia1 and NR proteins from *N. crassa*, *F. oxysporum* and *A. nidulans* were analysed, Figure 3.14. Nia1, *N. crassa*, *F. oxysporum* and *A. nidulans* NRs carry the same five domains. *A. nidulans* has slightly altered domain locations with the two oxidoreductase domains located in reversed positions relative to Nia1, *N. crassa* and *F. oxysporum*. Overall, there is clear conservation of domain positions between the four NR proteins, suggesting a close evolutionary relationship.

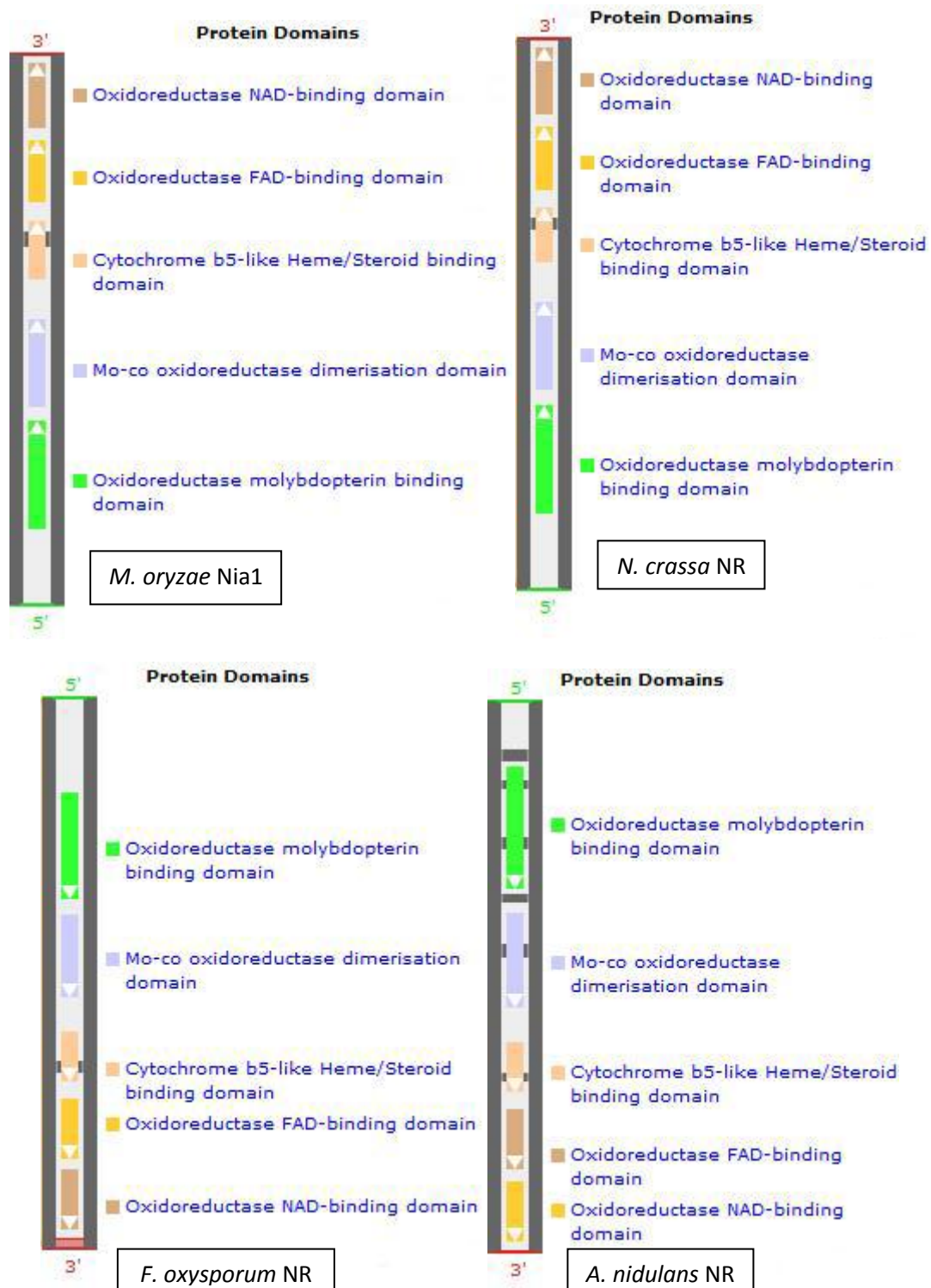


Figure 3.14. The domain architectures of *M. oryzae* Nia1 and NR proteins from *N. crassa* (NCU05298), *F. oxysporum* (FOXG_04181) and *A. nidulans* (ANID_01006). The intron-exon architectures are detailed beneath the domains, the exons depicted as the white vertical band and the introns represented as the grey bands. Figures adapted from the Broad Institute website

(http://www.broadinstitute.org/annotation/genome/magnaporthe_grisea/MultiHome.html).

The identity and position of the domains within *M. oryzae* Nii1 and nitrite reductase proteins from *N. crassa*, *F. oxysporum* and *A. nidulans* were analysed, Figure 3.15. The domains and their positions in Nii1, *N. crassa* and *F. oxysporum* demonstrate a strong degree of conservation. The domain architecture of *A. nidulans* is similar to Nii1, *N. crassa* and *F. oxysporum* but does not contain a BDF like [2Fe-2S] binding domain. The nitrite reductase [NAD(P)H] large and small subunits detailed in *N. crassa* and *F. oxysporum* do not represent additional domains, but rather, show the domains which are grouped together to form either the large or small subunits found in NIR enzymes. Overall, there is a high degree of conservation between Nii1, *N. crassa*, *F. oxysporum* and *A. nidulans* NIRs, inferring a close evolutionary relationship between these fungi.

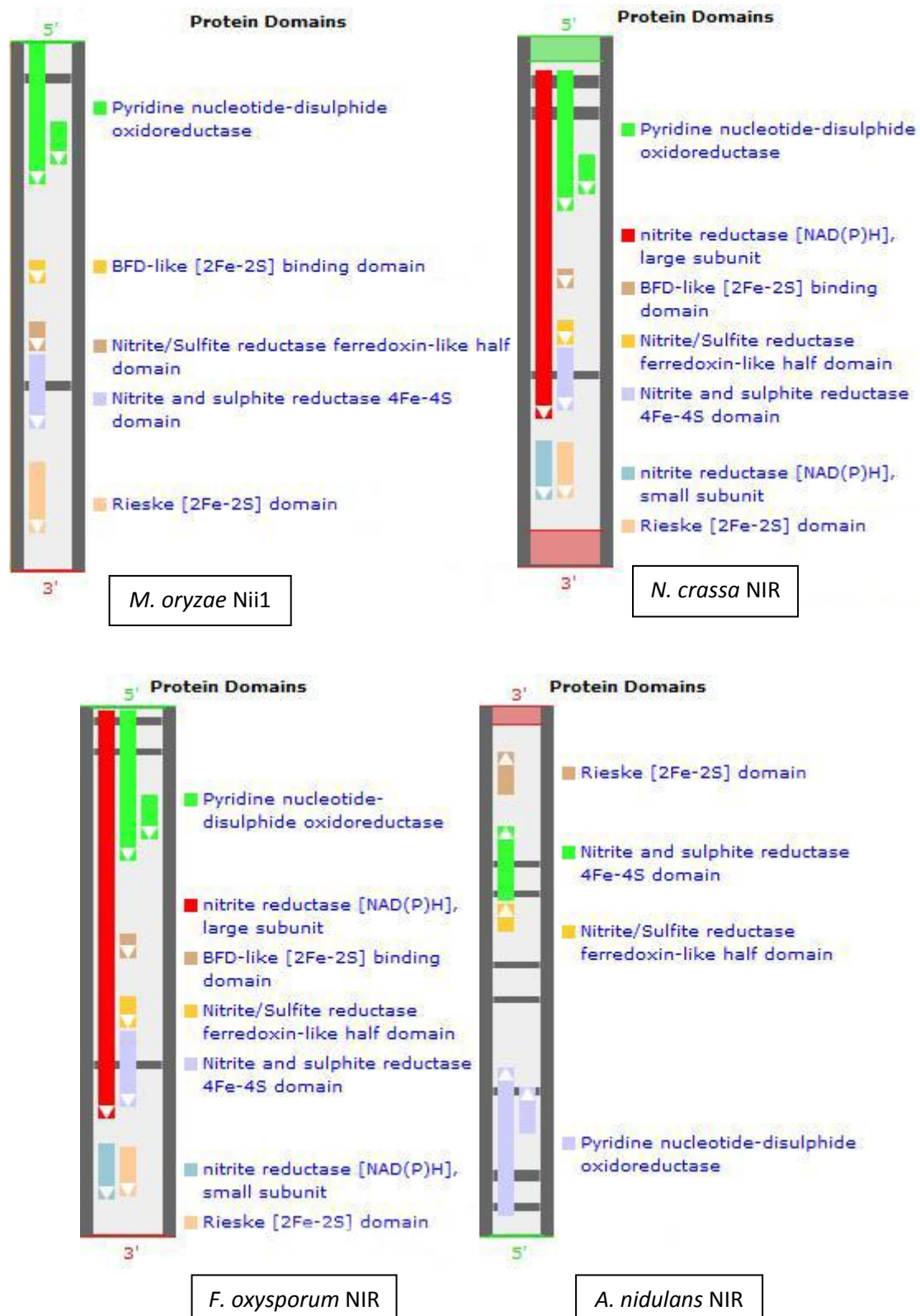


Figure 3.15. The domain architectures of *M. oryzae* Nii1 and NIR proteins from *N. crassa* (NCU04720), *F. oxysporum* (FOXG_03192) and *A. nidulans* (ANID_01007). The intron-exon architectures are detailed beneath the domains, the exons depicted as the white vertical band and the introns represented as the grey bands. Figures adapted from the Broad Institute website (http://www.broadinstitute.org/annotation/genome/magnaporthe_grisea/MultiHome.html).

3.3.6 Promoter analysis

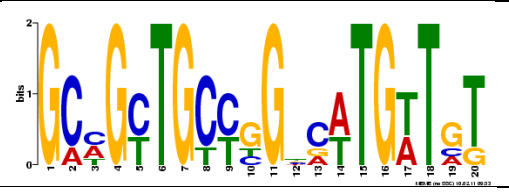
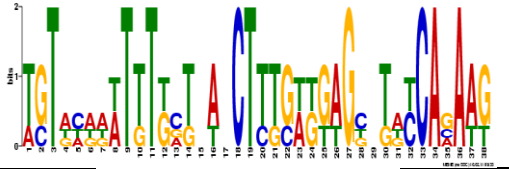
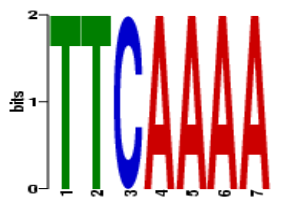
The 1kb sequence 5' to ATG of *NOS1*, *NOS2*, *NOS3*, *NOS4*, *NIA1* and *NII1* were assessed for common sequence motifs using MEME (<http://meme.sdsc.edu/meme/website/meme.html>).

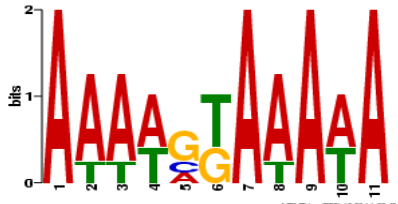
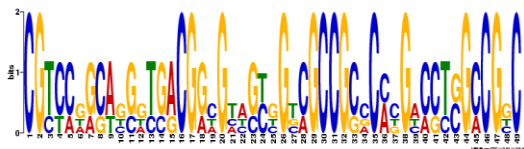
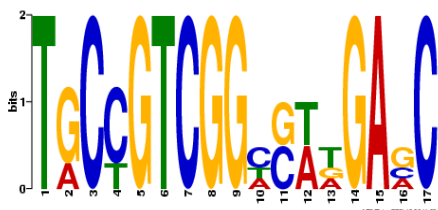
Gene promoter analyses provide insight into the regulation of the genes e.g. co-expression, potentially revealing shared transcription factors and promoter motifs. The three most commonly identified motifs per promoter sequence are outlined in Table 3.6.

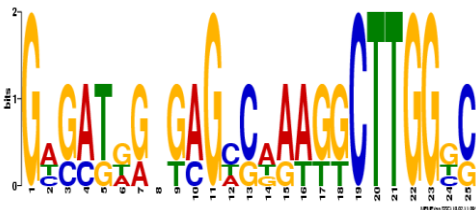
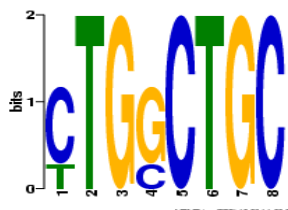
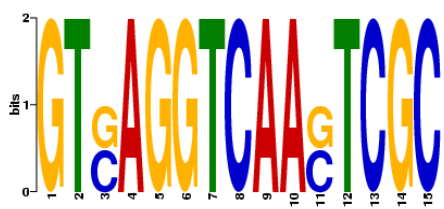
The TATA box is considered the predominant DNA promoter sequence and is the binding site of transcription factors or histones; controlling transcription (Tora & Timmers 2010). Multiple TATA boxes were identified upstream of *NOS1*, *NOS3* and *NOS4*. The motif CAAA is also present upstream of *NOS1*, *NOS2*, *NOS3*, *NOS4*, *NIA1* and *NII1*, but has no known function. Otherwise, there is little conservation of promoter motif sequences.

<i>NOS1</i>	Number of sites	Motif consensus	Base pairs upstream of start codon	Statistical significance
	5		23 240 480 920	$>3.36 \times 10^{-6}$
	5		1 318 385 395 496	$>1.29 \times 10^{-5}$
	3		208 911	4.59×10^{-5}

NOS2	Number of sites	Motif consensus	Base pairs upstream of start codon	Statistical significance
	5		348 472 547 694 974	$>1.14 \times 10^{-6}$
	4		406 840 961	$>1.36 \times 10^{-6}$
	3		259 597 621 713 871	$>1.13 \times 10^{-5}$

NOS3	Number of sites	Motif consensus	Base pairs upstream of start codon	Statistical significance
	5		224 266 348 372 636	$>1.15 \times 10^{-8}$
	4		65 730 824 909	$>2.43 \times 10^{-13}$
	2		110 411	4.37×10^{-5}

<i>NOS4</i>	Number of sites	Motif consensus	Base pairs upstream of start codon	Statistical significance
	5		122 147 218 237 249	$>1.32 \times 10^{-7}$
	4		387 516 666 894	$>1.41 \times 10^{-16}$
	2		450 624 726 840	2.03×10^{-8}

<i>NIA1</i>	Number of sites	Motif consensus	Base pairs upstream of start codon	Statistical significance
	4		189 272 441 591	$>1.52 \times 10^{-10}$
	4		110 162 170 528	$>1.31 \times 10^{-5}$
	2		409 479	3.31×10^{-9}

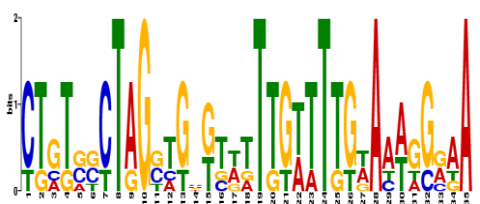
<i>NII1</i>	Number of sites	Motif consensus	Base pairs upstream of start codon	Statistical significance
	4		344 463 510 854	$>1.89 \times 10^{-5}$
	5		87 697 736 781 933	$>1.33 \times 10^{-11}$
	4		266 310 657 874	1.92×10^{-9}

Table 3.6. Common sequence motifs identified in the 1kb upstream sequence of *NOS1*, *NOS2*, *NOS3*, *NOS4*, *NIA1* and *NII1*. Motifs were predicted using MEME.

3.4 Discussion

3.4.1 Conclusions

- There is little similarity between the putative NOS genes' *NOS1*, *NOS2*, *NOS3* or *NOS4* sequences, gene intron-exon architectures and upstream promoters.
- Nos1, Nos2 and Nos3 are predicted to have a mitochondrial localisation, whilst Nos4 is predicted to locate within the cytoplasm.
- Nos1, Nos2, Nos3 and Nos4 carry the same domains, apart from Nos2 which is missing the FAD binding domain.

- *NIA1* and *NII1* exhibit close sequence similarity and gene intron-exon domain architecture to other phytopathogenic fungi: *B. cinerea*, *N. crassa*, *A. nidulans* and *F. oxysporum*.
- *NIA1* and *NII1* do not share any promoter motifs in the 1kb sequence upstream of the start codon.

3.4.2 Putative nitric oxide synthase, nitrate reductase and nitrite reductase genes

The identification of four putative *NOS* genes by Mary Illes represented the first instance of putative *NOS* genes being identified within the *M. oryzae* genome. The discovery of *NOS* in the Myxomycete *Physarum polycephalum* by Golderer et al. (2001) represents the only example of a non-animal eukaryote, with mammalian-like *NOS* genes (Golderer et al. 2001). To-date the most convincing observation of animal-like *NOS* in fungi was reported by Domitrovic et al. (2003). They identified putative *NOS* isoforms within *S. cerevisiae* via Western blotting using antibodies raised against mammalian *NOS* (Domitrovic et al. 2003). However, there are no annotated *NOS* genes within the *S. cerevisiae* genome.

The *in silico* analysis of Nos1, Nos2 and Nos3 protein sequences suggests a mitochondrial sub-cellular localization, which is significantly different from the predicted nuclear sub-cellular locations of *H. sapiens NOS*. However, Nos1, Nos2 and Nos3 predicted sub-cellular locations match that of mammalian mitochondrial *NOS* (mtNOS). mtNOS were simultaneously discovered by two separate groups (Kobzik et al. 1995; Bates et al. 1995). The existence of mtNOS is a contentious issue. Doubts have been expressed about the purity of extracted mtNOS samples; indeed, Kani et al. (2001) suggested that their extracts, which ranged in purity from 40 to 90%, may be contaminated with cytosolic *NOS* (Kanai et al. 2003). More recently, mtNOS have been isolated from animal liver, brain and kidney samples, where it is thought to associate with respiratory chain complex I (Mordhwaj et al. 2008). Therefore, the predicted sub-cellular localisations of Nos1, Nos2 and Nos3 align with animal mtNOS. The predicted subcellular

localization of *Nos4* is cytoplasmic/ cytosolic aligning closely to the mammalian inducible NOS isoform, iNOS. Both *Nia1* and *Nii1*, have cytoplasmic predicted subcellular localisations, which match plant NR and NIR sub-cellular positions (Ritenour et al. 1967).

Promoter analysis of the 1kb sequence 5' to ATG for each putative *NOS*, *NR* and *NIR* identified multiple statistically-significant consensus motifs, however the majority of these have no known function. Multiple TATA boxes are present upstream of *NOS1*, *NOS3*, *NOS4* and *NII1*. TATA boxes have been well-studied within *Aspergillus* species and are thought to regulate expression of the *Aspergillus NIAD-NIIA* gene cluster (Minetoki et al. 1996; Amaar and Moore 1998). It is likely therefore that they play a regulatory role within *M. oryzae*, moderating nitrate metabolism.

3.4.3 Nitric oxide synthase

The phylogenies constructed from whole NOS protein sequences and the oxygenase/reductase domain protein sequences exhibit strong similarity. This suggests that the complete NOS protein phylogeny provides an accurate estimation of evolutionary distances between proteins. However, the separate domain phylogenies allow for the alignment of single domain proteins, specifically bacterial NOS, giving insights into the evolutionary origins of NOS.

A tentative explanation for the observed NOS phylogenies can be found in the phenomena of gene fusion and gene fission. Gene fusions occur during genomic recombination events, that is, translocation, inversion or duplication, resulting in novel genes or gene clusters (Eichler 2001). The result of a successful gene fusion event is the combination of two or more single genes forming a single gene which encodes a multidomain protein, with novel functions. Gene fission, the reverse process of gene fusion, results in a single gene encoding multiple domains becoming split into multiple separate genes. The rate of fungal fusion/fission has been calculated as 1.28 : 1.0. This is unsurprising, as dividing a coding sequences is more likely to inhibit its protein function than the combination of separate genes (Durrens et al. 2008). The

increased fusion rate observed in larger genomes; results in an increased probability of a successful gene fusion event occurring in Eukaryote relative to Prokaryote genome (Durrens et al. 2008). This explains the greater number of multi-domain proteins found within higher Eukaryotes. Gene fusion is a common feature in fungal genomes, and has been studied within Ascomycetes (*S. cerevisiae*, *N. crassa* and *A. nidulans*) and to a lesser extent in Basidiomycetes (*Cryptococcus neoformans*) and Zygomycete (*Rhizopus oryzae*) (Durrens et al. 2008).

During the construction of the complete NOS protein sequence phylogeny, the following sequences failed to successfully align in the data matrix: *S. cerevisiae* SCRG_04751, *A. oryzae* AO090102000382, *B. subtilis* YP_004206779, *S. aureus* ZP_05693526, *A. thaliana* NP_850666, *N. benthamiana* BAF93184, *C. albicans* CAWG_03482.1 and *C. albicans* orf19.2672. These sequences fall into two domains of the three domains, that is higher Eukaryotes and Eubacteria. Analysis of their sequences with Pfam (<http://pfam.sanger.ac.uk/>) reveals they carry only a single oxygenase or reductase domain, explaining the poor alignment results (Finn et al. 2010). Single domain NOS have been studied in bacteria, the majority of which do not contain a multidomain NOS, but rather separate NOS oxygenase and reductase domains (Adak et al. 2002; Crane et al. 2010; Sudhamsu & Crane 2009). Based on this observation, I hypothesise that separate NOS domains represent the basal NOS isoform and that the evolution of the multidomain NOSs, present in higher Eukaryotes, is the result of gene fusion events between separate oxygenase and reductase proteins. *Sorangium cellulosum* is the only identified bacterial species, thus far, which contains a multi-domain NOS. This species may represent the intermediate evolutionary step between a discrete NOS domain and multi-domain Eukaryote NOSs (Schneiker et al. 2007). Examples of higher Eukaryotes with separate domain proteins may be the product of gene fission, for example *C. albicans* (Durrens et al. 2008). These hypotheses correlate with data generated by Durrens et al. (2008), these researchers used *in silico* analysis techniques to identify the frequency of fusion/fission events across 12 selected fungal genomes (Durrens et al. 2008). Of the 12 fungi studied, *N. crassa*, *A. nidulans* and *R. oryzae* are the three

species with the highest rates of gene fusion; approximately double that of *C. albicans*, indicating they are under diversifying selection

Proteins showing the highest number of gene fusion events include hydrolases, isomerases and ligases, all of which are well-represented within the *M. oryzae* secretome (Dean et al. 2005). Therefore, high rates of gene fusion may be beneficial for phytopathogenic fungi, which require expansive numbers of enzymes for host colonisation and invasion. The fatty acid synthase family is a well-reported example of protein evolution driven by gene fusion (McCarthy & Hardie 1984).

Guo et al. (2003) originally identified a putative animal-like *NOS* within *A. thaliana*. However, further experiments revealed it to be a cGTPase (Guo et al. 2003; Moreau et al. 2008). Genes from *A. thaliana* and *N. tabacum* were initially included within the *NOS* phylogenies. However, they were subsequently removed from the data matrix as they dramatically reduced the Bootstrap support for constructed phylogenies.

3.4.4 Nitrate reductase

The distribution of taxa within the NR phylogeny mirrors the relative evolutionary distances between fungal phyla: Ascomycota, Basidiomycota and Zygomycota, and Oomycetes (Blackwell 2010). *Nia1* is positioned within the monophyletic Ascomycota clade and lies alongside other phytopathogenic fungi, suggesting a close evolutionary relationship between phytopathogenic fungi. The fungal, animal, plant and bacterial NR phylogeny retained Ascomycota as a monophyletic group and placed plants and bacteria as additional monophyletic clades. The presence of separate fungi, plant and bacterial monophyletic groups within the phylogeny indicates an ancient evolutionary origin for NR, predating multicellularity. The relative absence of NR genes within the animals, suggests NR may have been the subject of a gene deletion in the evolutionary past, perhaps the result of animal life-style selection pressures. Mammalian enzymes with NR activity have been identified in animals but no true NR isoforms have been

identified in animals (Jansson et al. 2008; Lundberg et al. 2011). The abundance of multi-domain NR proteins within bacteria suggests that gene fusion events occurred in the distant evolutionary past.

Analysis of Ascomycota NR intron-exon architectures reveals strong conservation between *NIA1*, *N. crassa* and *F. oxysporum* NR genes, with a single intron located within the cytochrome b5-like heme/steroid binding domain sequence. It is unsurprising that *NIA1*, *N. crassa* and *F. oxysporum* are located alongside each other within the fungal specific NR phylogeny. *B. cinerea* and *A. oryzae* have similar domains but different intron-exon architectures to *NIA1*, and are situated within more distant clades in the Ascomycetes monophyletic group, suggesting a greater distance evolutionary relationship. Is the similar intron-exon architecture across the Ascomycota NR genes a reflection of a recent evolutionary species divergence or a life-style dictated purifying selection pressure?

3.4.5 Nitrite reductase

The fungi specific NIR phylogeny exhibits limited similarity with the fungi specific NR phylogeny. The Ascomycota fail to form a monophyletic group due to the presence of the Basidiomycete *U. maydis* and the Oomycete *P. infestans* within the clade. The close alignment of *P. infestans* and *U. maydis* following the addition of plant and bacterial protein sequences may represent convergent evolution, potentially resulting from their phytopathogenic specific life-style selection pressures. Equally the ‘jumbled’ positioning of taxa within the fungi, plant, bacteria and Oomycete NIR phylogeny may result from the multi-functionality of NIR proteins, as discussed in Chapter 1 (Frías et al. 1997; Takaya 2002). The presence of NIR genes in bacteria, fungi and plants, but absence in animals suggests a gene deletion in the distant evolutionary past. Nitrite reduction has been identified and is an important physiological pathway controlling NO levels in animals. Nitrite is reduced by deoxygenated ferrous haemoglobin, yielding NO and

methaemoglobin. However no plant/bacteria NIR homologues have been identified in animals (Shiva et al. 2011).

The intron-exon and domain architecture are conserved across *M. oryzae*, *N. crassa*, *F. oxysporum* and *A. nidulans*. Intron-exon architectures are similar, with single introns located within their nitrite and sulphite reductase 4Fe-4S and pyridine nucleotide-disulphide oxidoreductase coding sequences. *N. crassa* and *F. oxysporum* carry an additional intron in their pyridine nucleotide-disulphide oxidoreductase coding sequences, whilst *A. nidulans* contains three further introns. Relative to *NR*, the *NIR* intron-exon architectures are less conserved. This may be evidence of either an earlier *NIR* evolutionary divergence event than *NR*, or of reduced purifying selection on *NIR* genes.

3.4.6 The Ascomycota common ancestor

A common trend throughout the NOS, NR and NIR phylogenies are the close relationships between *M. oryzae* and *N. crassa*. This is unexpected as they exhibit significantly different life-styles, being phytopathogenic and saprotrophic, respectively. At the amino acid level, there is a 47% identity between *M. oryzae* and *N. crassa* homologues (Dean et al. 2005). Borkovich et al. (2004) estimated they evolved from a common ancestor 50 to 150 million years ago (Borkovich et al. 2004). This raises an interesting question about the life-style of their common ancestor. Was the common ancestor a saprotroph, meaning *M. oryzae* evolved phytopathogenicity or, was their common ancestor phytopathogenic with *N. crassa* subsequently losing its ability to infect plants? Current evidence based on genomic comparisons suggests the latter (Sánchez-Rodríguez et al. 2010). Annotation of the *N. crassa* genome reveals 9200 genes coding proteins greater than 100 amino acids, a number considerably less than *M. oryzae* (12,842 genes) but significantly greater than *S. pombe* (4800) and *S. cerevisiae* (6300) (Galagan et al. 2003). *N. crassa* was also shown to contain gene homologues encoding virulence factors associated with a phytopathogenic life-style. Sánchez-Rodríguez et al. (2010) reported that the majority of

genes required for pathogenicity were carried by the common ancestor of the Ascomycota. Species such as *N. crassa* and *A. nidulans* which are non-pathogenic exhibit reduced genome complexity, relative to pathogenic species. The expansion of gene families is essential for pathogenic species colonisation and invasion (Sánchez-Rodríguez et al. 2010).

Chapter 4: Generation of nitric oxide synthase, nitrate reductase and nitrite reductase replacement mutants in *Magnaporthe oryzae*

4.1 Introduction

This Chapter details the creation of targeted gene replacement mutants in *M. oryzae*; it includes the molecular techniques used to design and generate the DNA constructs for gene replacement and the PCR and Southern blots techniques used to confirm gene replacement.

4.1.1 Gene deletions within filamentous fungi

The recent boom in the number of fully sequenced fungal genomes has led to the discovery of increasing numbers of candidate genes associated with physiologically important processes, including metabolism and pathogenicity. However, facilitating gene study within filamentous fungi has proved problematic, with low rates (~5%) of successful targeted gene replacement (Weld et al. 2006). The process of targeted gene replacement relies on cellular proteins involved in the repair of DNA double-strand breaks (DSB). In Eukaryotes, this occurs through two separate pathways: first, by homologous recombination, where DSB are repaired through homologous DNA sequences combining; and second by, non-homologous end joining (NHEJ), where broken DNA ends are directly ligated together regardless of sequence homology, potentially leading to exogenous DNA becoming ectopically integrated within the genome (Li et al. 2010). In yeast, homologous recombination is the most common DSB repair method, however in filamentous fungi NHEJ is the predominant pathway, which explains the low frequency of targeted gene replacement observed (Takita et al. 1997; Daley et al. 2005). The NHEJ/DSB repair pathway requires formation of the Ku complex, a Ku70-Ku80 heterodimer, which binds directly to the broken ends of DNA strands (Koike 2002). Binding of the Ku complex

recruits a DNA dependent protein kinase, which in turn phosphorylates Artemis (DNA exonuclease) (Ma et al. 2005). The resulting complex recruits both DNA ligase IV and Xrcc4 which bind at the ends of the broken DNA strands and facilitate DNA repair (Hefferina & Tomkinson 2005). To combat the difficulties associated with NHEJ and successful targeted gene replacement, *Ku70* and *Ku80* genes have been deleted in multiple filamentous fungi, including *Neurospora crassa*, *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Aspergillus nidulans* (da Silva Ferreira et al. 2006; Nayak et al. 2006; Goins et al. 2006). The deletion of *Ku70* and *Ku80* results in a dramatic increase in the frequency (70-100%) of successful targeted gene replacements.

Targeted gene replacement in *M. oryzae* typically occurs at a 7% frequency (Talbot & Foster 2001). To improve the homologous recombination frequency of exogenous DNA, Villalba et al. (2008) generated two separate *Ku80* deletion mutants in wildtype strains P1.2 and Guy11. They reported that, following deletion of *Ku80*, targeted gene replacement frequencies jumped from 5-10% to 70-100% (Villalba et al. 2008). The increased rate of successfully disrupted genes using targeted gene replacement has important practical implications. The number of transformation experiments required to successfully generate mutants is reduced significantly, as are the number of false positive transformants, which result from ectopic recombination. Furthermore, Villalba et al. (2008) demonstrated that the disruption of *Ku80* had no detectable effect on mycelium growth, conidiation, conidia germination, mating or pathogenicity, as compared with P1.2 and Guy11 (Villalba et al. 2008). Li et al. (2010) and Ninomiya et al. (2004) similarly recorded no detectable differences in phenotypes following *Ku70* deletion in *N. crassa* and *Penicillium decumbens* relative to their wildtype strains (Ninomiya et al. 2004; Li et al. 2010). Similarly, Kershaw & Talbot (2009) successfully generated a *Ku70* deletion mutant in *M. oryzae* and showed no detectable differences in growth rate, sporulation or pathogenicity between $\Delta ku70$ and wildtype strain Guy11 (Kershaw & Talbot 2009).

4.1.2 Identification of *NOSs*, *NIA* and *NII*

As detailed in Chapter 3, 3.3.1, Mary Illes identified four putative *NOS* genes in the *M. oryzae* genome. Based on the expression profiles she generated for *NOS1*, *NOS2*, *NOS3* and *NOS4* in Guy11 germlings developing on barley leaves, she identified *NOS3* (MGG_07953) as the gene with the high level of expression at 12hpi, (Chapter 3, Figures 3.14 and 3.15). She generated a $\Delta nos3$ mutant via targeted gene replacement. Her qRT-PCR transcript profile revealed the next most highly expressed putative *NOS* gene, identified during Guy11 germling development was *NOS2* (MGG_05401). It was therefore selected for gene replacement in both $\Delta ku70$ and $\Delta nos3$ backgrounds.

Analysis of the *M. oryzae* genome reveals single gene copies for both nitrate reductase (*NIA1*) MGG_06062 and nitrite reductase (*NII1*) MGG_00634. Marketa Samalova (Postdoc) generated nitrate reductase mutants $\Delta nia1$ and $\Delta nos3\Delta nia1$ in the $\Delta ku70$ and $\Delta nos3$ backgrounds respectively. I disrupted *NII1* in both $\Delta ku70$ and $\Delta nia1$ backgrounds, generating $\Delta nii1$ and $\Delta nia1\Delta nii1$ mutants, and *NOS2* in both $\Delta ku70$ and $\Delta nos3$ backgrounds generating $\Delta nos2$ (M. Samalova also generated a $\Delta nos2$ mutant) and $\Delta nos2\Delta nos3$.

4.2 Experimental objectives

- a) To generate $\Delta nos2$, $\Delta nos2\Delta nos3$ deletion mutants.
- b) To generate $\Delta nii1$ and $\Delta nia1\Delta nii1$ deletion mutants.

4.3 Results

4.3.1 Generation of $\Delta nos2$ and $\Delta nos2\Delta nos3$ mutants

I amplified a 4664bp fragment containing the 3808bp *NOS2* coding sequence and its 476bp upstream and 380bp downstream flanking regions, from wildtype genomic DNA using NOS2-FL-F and NOS2-FL-R primers, (Chapter 2, Table 2.2). The amplicon was ligated into the multiple cloning site of the pGEMTEasy plasmid, generating pGEMTEasy-NOS2, Figure 4.1. The digestion of pGEMTEasy-NOS2 with *XhoI* released an 811bp fragment from the *NOS2* coding sequence. Similarly, the 945bp Bialaphos resistance cassette (*BAR*) was released from the pGEMTEasy-BAR plasmid, following digestion with *Sall*, and cloned into the *XhoI* restriction site in pGEMTEasy-NOS2, generating pGEMTEasy-NOS2/*BAR*. During the cloning of the *BAR* into pGEMTEasy-NOS2, it was cloned in reverse. This did not affect the functionality of the resistance marker as transformants grew successfully on DCM agar containing 100µg/ml (w/v) Bialaphos. The pGEMTEasy-NOS2/*BAR* construct was sequenced to confirm the integration of *BAR* into *NOS2*.

The linear 4798bp *NOS2/BAR* DNA fragment (Figure 4.2) was excised from pGEMTEasy-NOS2/*BAR* with *NotI* and introduced to $\Delta ku70$ and $\Delta nos3$ protoplasts, (as detailed in Chapter 2, 2.13). Transformed protoplasts were loaded into OCM agar and after 24 hours were overlaid with DCM agar containing 100µg/ml (w/v) Bialaphos. Colonies that successfully penetrated the upper DCM agar layer within 10 days were screened again for Bialaphos resistance through a second inoculation onto DCM agar containing 100µg/ml Bialaphos. Genomic DNA was extracted from putative transformants, (as detailed in Chapter 2, 2.14) and screened for both the presence and location of *BAR* using PCR analyses.

The presence of *BAR* was verified using *BAR-F* and *BAR-R* primers, which confirmed the integration of *BAR* within transformant genomes but not the site of *BAR* integration. Subsequently, NOS2-FL-F and NOS2-FL-R were used to amplify *NOS2* and its flanking regions.

The small size differences between WT and mutant amplicons (4664bp and 4798bp) meant restriction digests (*HindIII*, *PvuII* and *BamHI* and *XbaI*, data not shown) were required to confirm the presence of *BAR* within *NOS2*. Finally, the correct integration of *BAR* within *NOS2* was confirmed using primers NOS2-BAR-F and BAR-F. NOS2-BAR-F primer anneals upstream of NOS2-FL-F and BAR-F anneals within the *BAR* sequence (forward primer was required due to the reversed position of *BAR*). The 3875bp PCR amplicon was generated after the successful disruption of *NOS2* with *BAR*.

Transformants validated by PCR screening were analysed by Southern blot to confirm a single targeted gene replacement event, Figures 4.3, 4.4 and 4.5. Wildtype and transformant gDNA was extracted and digested with *KpnI*, separated and blotted onto Hybond N⁺ membrane, before being hybridised with the *BAR* probe (as detailed in Chapter 2, 2.14.2). Southern blot analysis confirmed a single targeted *NOS2* replacement in both $\Delta ku70$ and $\Delta nos3$; generating $\Delta nos2$ and $\Delta nos2\Delta nos3$.

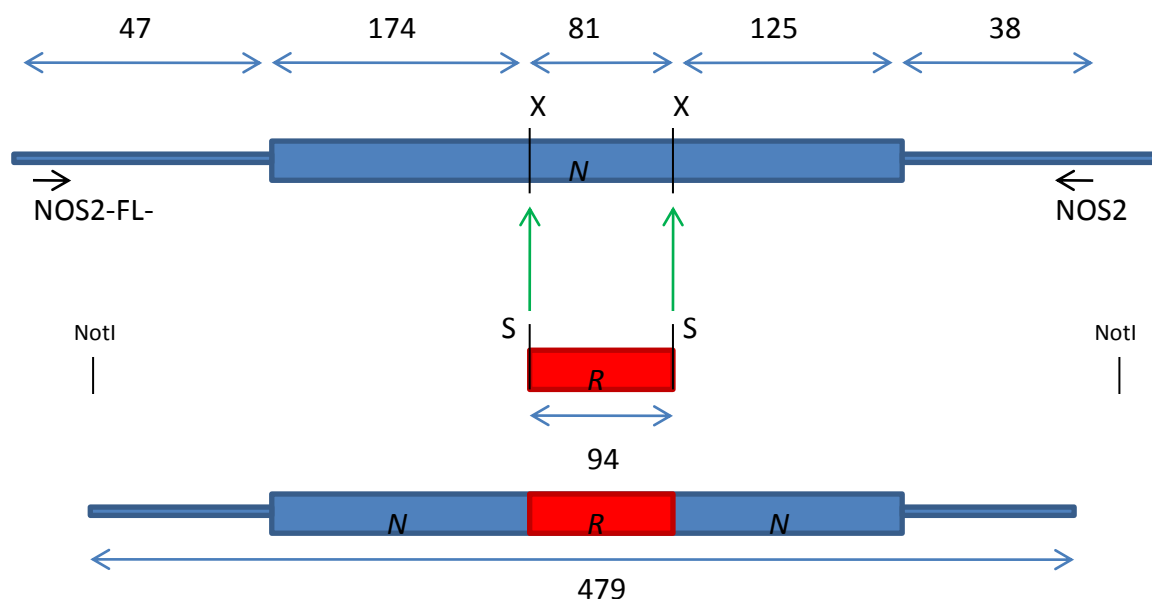


Figure 4.1. Design of DNA construct to replace *NOS2*. Primers NOS2-FL-F and NOS2-FL-R amplified a 4664bp amplicon from genomic DNA. This fragment was cloned into the multiple cloning site of pGEMTEasy plasmid and digested with *XhoI*, releasing an 811bp fragment from *NOS2*. The Bialaphos resistance cassette (*BAR*) was released from pGEMTEasy-BAR with *SalI* and cloned into the pGEMTEasy-NOS2 *XhoI* restriction site, generating pGEMTEasy-NOS2/*BAR* (*BAR* was cloned in reverse and is labelled *RAB*). The final linear DNA construct was excised from pGEMTEasy-NOS2/*BAR* with *NotI* prior to transformation.

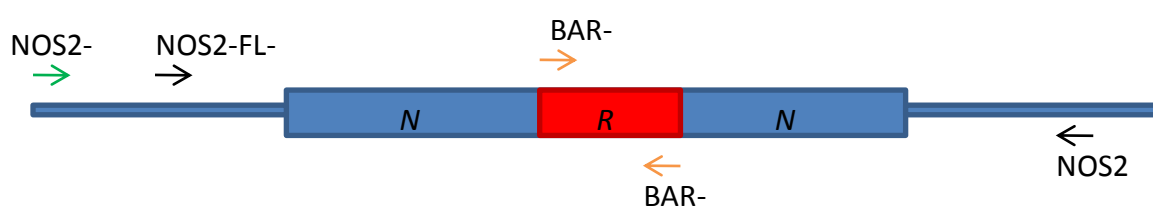


Figure 4.2. Screening of putative $\Delta nos2$ and $\Delta nos2\Delta nos3$ transformants by PCR amplification. Primers BAR-F and BAR-R were used to verify the presence of *BAR*. Primers NOS2-FL-F and NOS2-FL-R were used to amplify *NOS2*, and the presence of *BAR* was established with restriction digests of the amplicon. Primers NOS2-BAR-F and NOS2-FL-F were used to confirm both *BAR* presence and location within *NOS2*, with NOS2-BAR-F annealing upstream of NOS2-FL-F, and BAR-F annealing within reversed *BAR* (*RAB*).

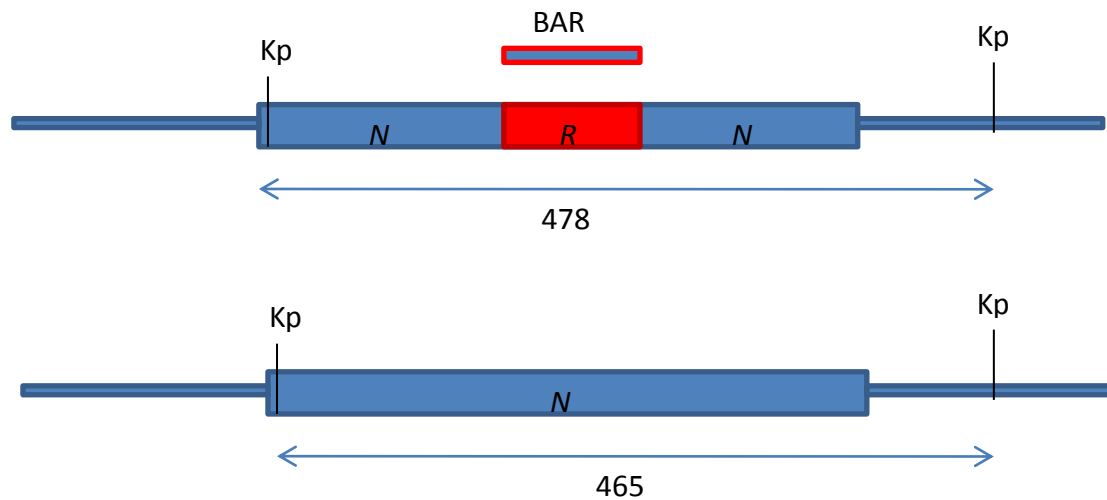


Figure 4.3. Disrupted and complete *NOS2* genes detailing the binding site of the *BAR* probe used in the Southern blot hybridisation assay and its *KpnI* restriction sites.

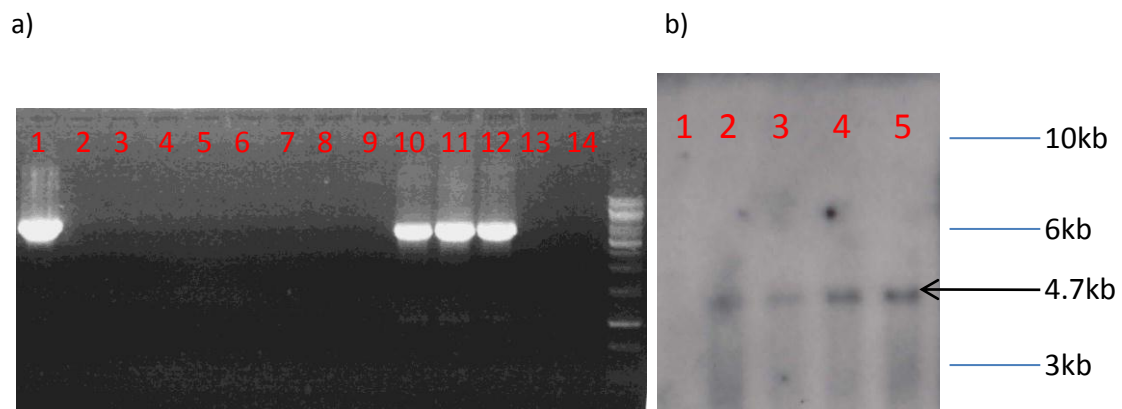


Figure 4.4. a) PCR analysis of putative $\Delta nos2$ transformants, amplification of the 3875bp fragment with primers *NOS2*-*BAR*-F and *BAR*-F confirmed the targeted replacement of *NOS2* with *BAR*. The 3875bp fragment was amplified from transformants in lanes 1, 10 and 11. Lanes 1 - 11 = $\Delta nos2$, 12 = positive control (a previously created $\Delta nos2$ mutant, carrying a *BAR* disrupted *NOS2* gene) and 13 = negative control (Guy11). The $\Delta nos2$ mutants were generated by both J. Johnson and M. Samalova.

b) Southern blot analysis of DNA from putative $\Delta nos2$ transformants (lanes 2-5) and negative control Guy11 (lane 1). The presence of a single 4.7kb fragment in lanes 2-3 verified a single *NOS2* replacement event. DNA was digested with *KpnI*, separated by gel electrophoresis and blotted onto Hybond N⁺ membrane. The membrane was then hybridised with the $\alpha^{32}\text{P}$ [dCTP] labelled probe, Figure 4.3. Southern blot hybridisations were completed by M. Samalova.

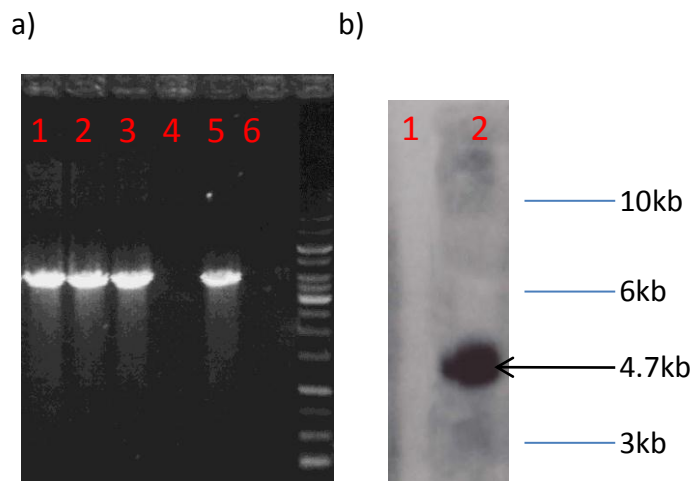


Figure 4.5. a) PCR analysis of putative $\Delta nos2\Delta nos3$ transformants, amplification of the 3875bp fragment with primers NOS2-BAR-F and BAR-F confirmed the successful targeted replacement of *NOS2* with *BAR*. The 3875bp fragment was amplified from transformants in lanes 1, 2 and 3. Lanes 1 - 4 = $\Delta nos2\Delta nos3$, 5 = positive control (a previously created $\Delta nos2$ mutant, carrying a *BAR* disrupted *NOS2* gene) and 6 = negative control (Guy11). The $\Delta nos2\Delta nos3$ mutants were generated by J. Johnson.

b) Southern blot analysis of DNA from putative $\Delta nos2\Delta nos3$ transformant (lane 2) and wildtype Guy11 (lane 1). The presence of a single 4.7kb fragment in lanes 2 verified a single *NOS2* replacement event. DNA was digested with *KpnI*, separated by gel electrophoresis and blotted onto Hybond N⁺ membrane. The membrane was then hybridised with the $\alpha^{32}\text{P}$ [dCTP] labelled probe, Figure 4.3. Southern blot hybridisations were undertaken by M. Samalova.

4.3.2 Generation of $\Delta nii1$ and $\Delta nia1\Delta nii1$ mutants

Primers NIR5-F and NIR5-HYG-R, and HYG-F and HYG-R were used to amplify two separate PCR products. Firstly, a 1564bp fragment was amplified from wildtype gDNA using primers NIR5-F and NIR5-HYG-R and contain both 789bp of upstream *NII1* sequence and the first 775bp of *NII1*. Secondly, the 1506bp Hygromycin B resistance cassette (*HYG R*) fragment was amplified from the pCB1636 plasmid using HYG-F and HYG-R primers. The 5' *NII1* and *HYG R* fragments were combined using overlapping PCR with NIR5-F and HYG-R primers, generating a 3070bp fragment. A third 1560bp fragment, consisting of 729bp of sequence downstream of *NII1* and

the 777bp 3' *NII1* sequence, was amplified from gDNA using NIR3-HYG-R and NIR3-R primers, Figure 4.6. The 3070bp 5' *NII1* and HYG R fragment was combined with the 3' *NII1* fragment by overlapping PCR, using NIR5-F and NIR3-R primers, and generated the complete 4630bp linear NIR5-HYG-NIR3 DNA construct, Figure 4.7. The NIR5-HYG-NIR3 construct was sequenced to confirm the successful integration of the three separate DNA fragments.

NIR5-HYG-NIR3 was introduced to $\Delta ku70$ and $\Delta nia1$ protoplasts, (as detailed in Chapter 2, 2.13.2). Transformed protoplasts were loaded into OCM agar and overlaid with MM agar containing 300 μ g/ml (w/v) Hygromycin B, 24 hours later. Colonies grew into the upper MM agar layer, within 10 days, were repeatedly screened through inoculation onto MM agar containing 300 μ g/ml Hygromycin B. Genomic DNA was extracted from putative transformants and screened for *HYG R* presence and its location, by PCR analysis.

The presence of *HYG R* was verified using NEW-HYG-F and NEW-HYG-R primers, (Chapter 2, Table 2.2), which confirmed the integration of *HYG R* within transformant genomes but not the site of *HYG R* integration. Subsequently, primers NIR5-F and NIR3-R were used to amplify *NII1* and its flanking regions; the small size difference between WT and mutant amplicons (4295bp and 4630bp) meant a *Sall* restriction digest (data not shown) was required to confirm the presence of *HYG R* within *NII1*. Finally, the integration site of *HYG R* in *NII1* was confirmed using primers Nitrite-Integration-F and NEW-HYG-R. Nitrite-Integration-F anneals upstream of NIR5-F and NEW-HYG-R annealed within the *HYG R* sequence. The 3601bp PCR amplicon was generated following the successful disruption of *NII1* with *HYG R*.

Putative transformants were analysed via Southern blot to confirm a single targeted *NII1* replacement event, Figures 4.8, 4.9 and 4.10. Wildtype and transformant DNA was extracted and digested with *EcoRI*, separated and blotted onto Hybond N⁺ membrane, before being hybridised with the radioactive *HYG R* probe. Southern blot analysis confirmed a single targeted *NII1* replacement in both $\Delta ku70$ and $\Delta nia1$ backgrounds, generating $\Delta nii1$ and $\Delta nia1\Delta nii1$.

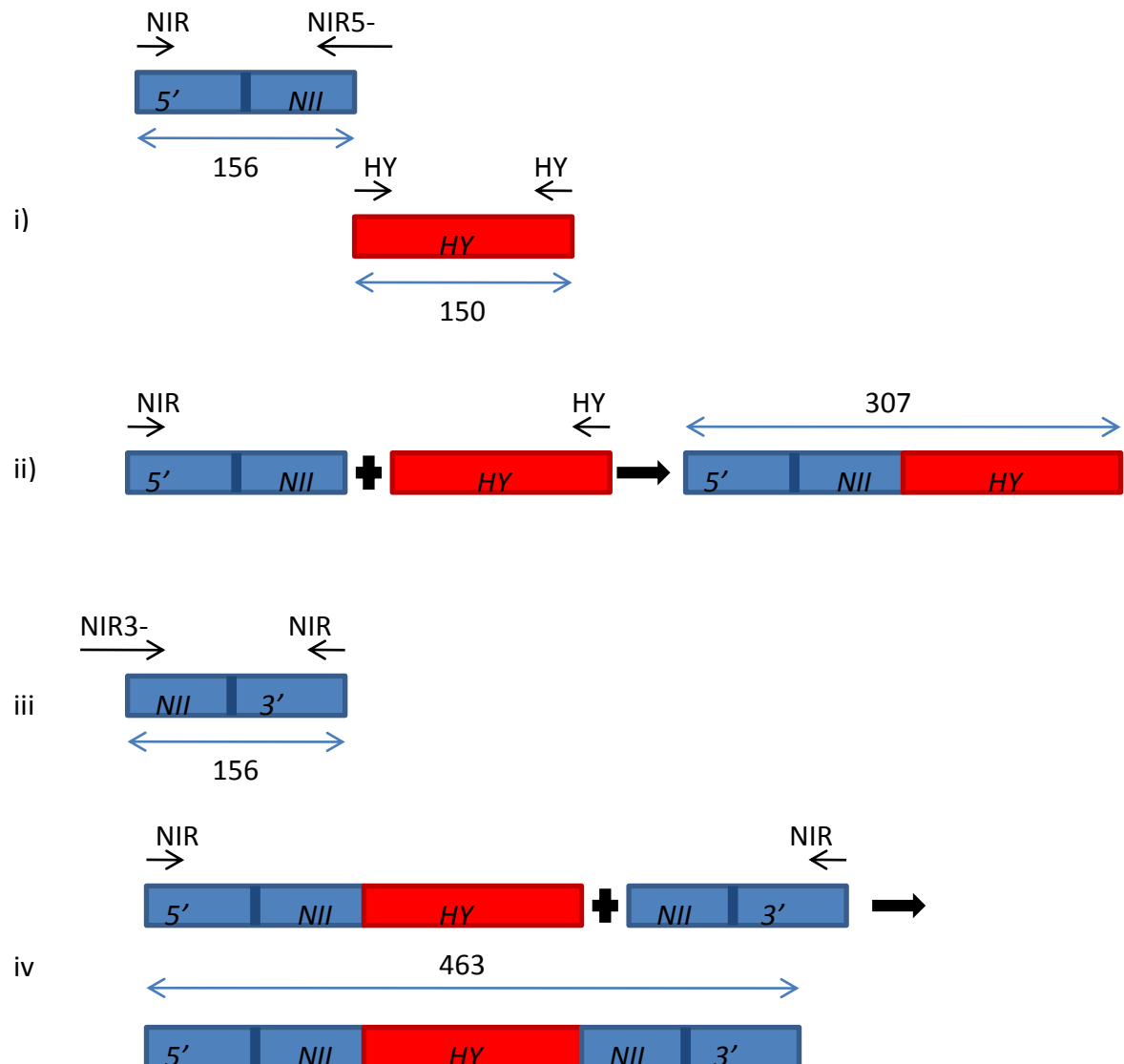


Figure 4.6. Design of the DNA construct to replace *NII1*. i) Two sets of primers, NIR5-F and NIR5-HYG-R and HYG-F and HYG-R were used to amplify two PCR products. Firstly, a 1.5kb amplicon containing *NII1* 5'coding and upstream sequence. Secondly, a 1.5kb amplicon containing Hygromycin B resistance cassette (*HYG R*) from the pCB1636 plasmid. ii) The two amplicons were combined by overlapping PCR, using primers NIR5-F and HYG-R, generating a 3070bp fragment. iii) A third 1.5kb amplicon, containing *NII1* 3' coding and downstream sequence was amplified using primers NIR-HYG-F and NIR3-R. iv) The 4.6kb DNA construct was generated following an overlapping PCR reaction, using primers NIR5-F and NIR3-R, and combined the 3kb *NII1* 5'and *HYG R* amplicon with the 1.5kb *NII1* 3' amplicon.

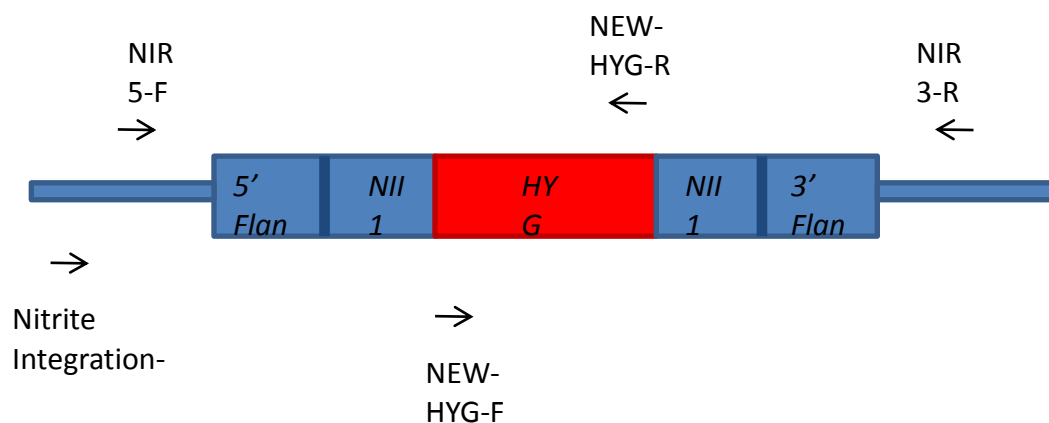


Figure 4.7. Screening of putative $\Delta nII1$ and $\Delta nia1\Delta nII1$ transformants by PCR amplification. Primers NEW-HYG-F and NEW-HYG-R were used to verify the presence of *HYG R*. Primers NIR5-F and NIR3-R were used to amplify *NII1*, and the presence of *HYG R* was established via restriction digests of the amplicon. Primers Nitrite-Integration-F and NEW-HYG-R were used to confirm *HYG R* presence and its site within *NII1*, with Nitrite-Integration-F annealing upstream of NIR5-F and NEW-HYG-R annealing within *HYG R*.

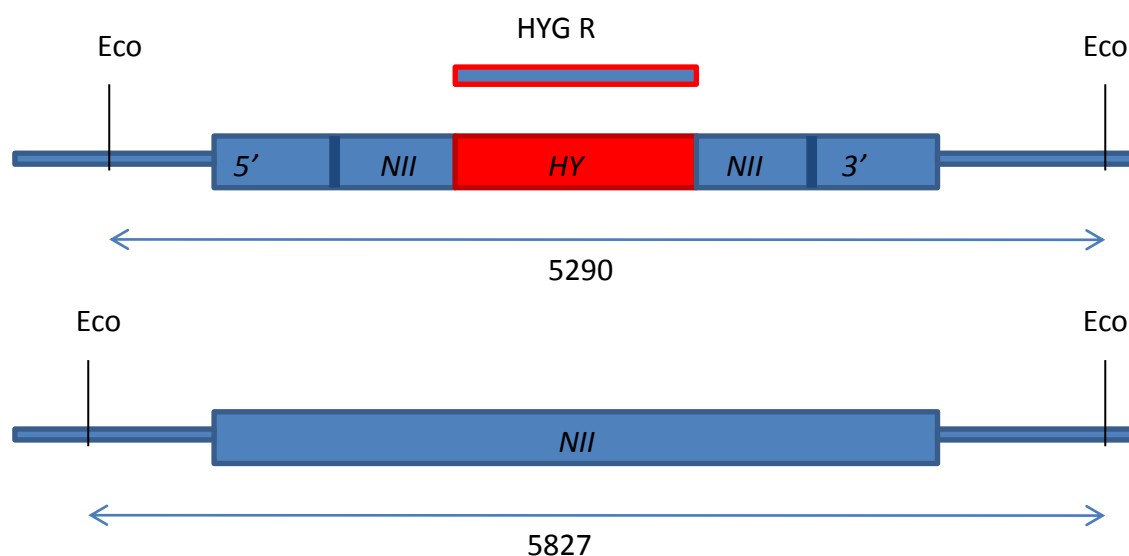


Figure 4.8. Disrupted and complete *NII1* genes detailing the binding site of the *HYG R* probe used in the Southern blot hybridisation assay and its *EcoRI* restriction sites.

a)

b)

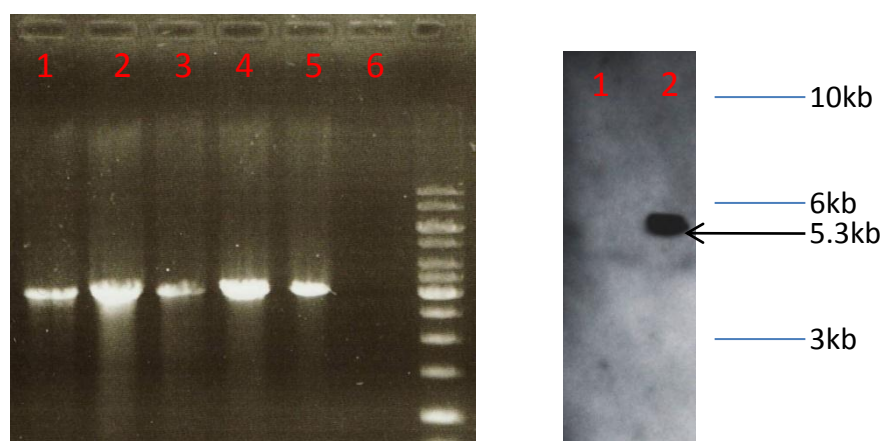


Figure 4.9 a) PCR analysis of putative $\Delta nii1$ transformants, amplification of the 3601bp fragment with primers Nitrite-Integration-F and NEW-HYG-R confirmed the successful targeted replacement of *Nii1* with *HYG R*. The 3601bp fragment was amplified from transformants in lanes 1, 2, 3 and 4. Lanes 1 - 4 = $\Delta nii1$, 5 = positive control (a previously created $\Delta nii1$ mutant, carrying a *HYG* disrupted *Nii1* gene) and 6 = negative control (Guy11). The $\Delta nii1$ mutants were generated by J. Johnson.

b) Southern blot analysis of DNA from putative $\Delta nii1$ transformant (lane 2) and wildtype Guy11 (lane 1). The presence of a single 5.3kb fragment in lane 2 verified a single *Nii1* replacement event. DNA was digested with *EcoRI*, separated by gel electrophoresis and blotted onto Hybond N⁺ membrane. The membrane was then hybridised with the $\alpha^{32}\text{P}$ [dCTP] labelled probe, Figure 4.8. Southern blot hybridisations were undertaken by M. Samalova.

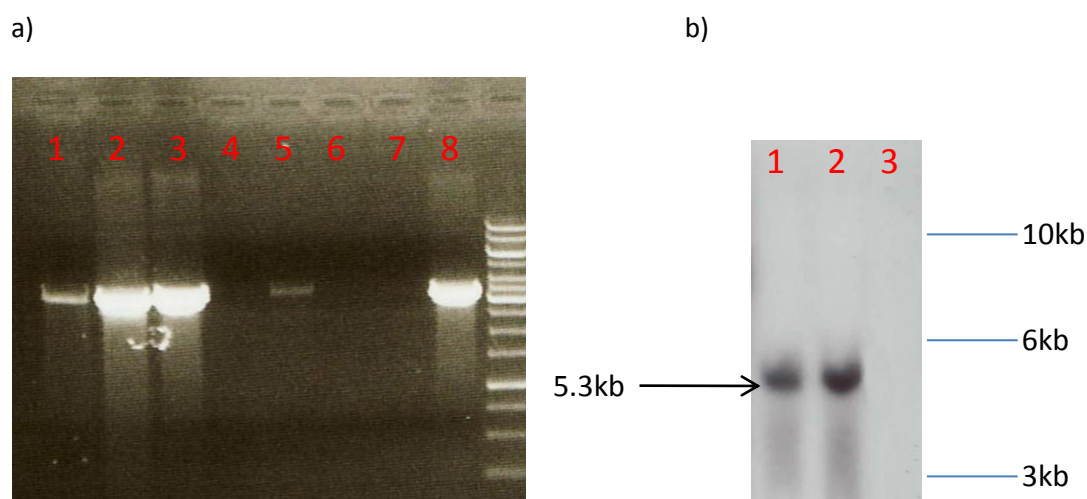


Figure 4.10 a) PCR analysis of putative $\Delta nia1\Delta nii1$ transformants, amplification of the 3601bp fragment with primers Nitrite-Integration-F and NEW-HYG-R confirmed the successful targeted

replacement of *NII1* with *HYG R*. The 3601bp fragment was amplified from transformants in lanes 1, 2, and 3. Lanes 1- 6 = $\Delta nia1\Delta nii1$, 7 = negative control (Guy11) and 8 = positive control (a previously created $\Delta nii1$ mutant, carrying a *HYG* disrupted *NII1* gene). The $\Delta nia1\Delta ii1$ mutants were generated by J. Johnson.

b) Southern blot analysis of DNA from putative $\Delta nia1\Delta nii1$ transformants (lanes 1 and 2) and wildtype Guy11 (lane 3). The presence of a single 5.3kb fragment in lanes 1 and 2 verified a single *NII1* replacement event. DNA was digested with *EcoRI*, separated by gel electrophoresis and blotted onto Hybond N⁺ membrane. The membrane was then hybridised with the $\alpha^{32}\text{P}$ [dCTP] labelled probe, Figure 4.8. Southern blot hybridisations were undertaken by M. Samalova.

4.4 Discussion

4.4.1 Construct generation

A combination of cloning and overlapping PCR techniques were used to generate two gene replacement constructs for the disruption of *NOS2* in $\Delta ku70$ and $\Delta nos3$ backgrounds and *NII1* in $\Delta ku70$ and $\Delta nia1$ backgrounds. The resulting mutant strains, $\Delta nos2$, $\Delta nos2\Delta nos3$, $\Delta nii1$ and $\Delta nia1\Delta nii1$, including $\Delta nos3$ (M. Illes), $\Delta nia1$ and $\Delta nos3\Delta nia1$ (M. Samalova), were created in the $\Delta ku70$ background which was chosen for its suppressed NHEJ activity.

Generating a gene replacement construct via a cloning based strategy proved to be both time consuming and problematic. Numerous problems were encountered with *E. coli* transformations, ligation reactions, restriction digests and sequencing reactions, the result of which meant the total time required to successfully disrupt *NOS2* using this strategy was protracted (>1 year). The efficiency of this transformation method was also particularly low (<5%) with numerous false positives identified. By contrast, using a gene replacement construct generated through a purely PCR based approach meant that within six weeks the construct could be generated, the transformation completed, putative transformants screened and the successful gene replacement confirmed by PCR analysis. The efficiency of this transformation technique was also significantly higher than the cloning method with a success rate of >60%.

The generation of $\Delta nos2$, $\Delta nos2\Delta nos3$, $\Delta nii1$, $\Delta nia1\Delta nii1$ by myself, and $\Delta nia1$, ($\Delta nos2$) and $\Delta nos3\Delta nia1$ by M. Samalova allows the function of these genes to be determined, specifically regarding their ability to generate NO. The introduction of the Bialaphos and Hygromycin B resistance markers into *NOS2* and *NII1* prevents a functional protein from being formed.

4.4.2 $\Delta Ku70$ as the background for mutant generation

The decision to use $\Delta ku70$ as the background for mutant generation has two associated disadvantages. Firstly, $\Delta ku70$ is a mutant; ideally it would have been preferable to use Guy11 as the background to generate the mutant strains. Secondly, only three antibiotic resistance markers are currently available for targeted gene replacements in *M. oryzae*: Hygromycin B, Sulfonylurea and Bialophos. Sulfonylurea was used as the resistance marker for the targeted replacement of *KU70* (Kershaw & Talbot 2009). This limits the number of separate gene replacements in a $\Delta ku70$ background to two compared to a possible three in Guy11. A fourth resistance marker, nourseothricin, has successfully been used within *Sclerotinia sclerotiorum* and *Cryptococcus neoformans*, however, to-date, it has not been tested in *M. oryzae* and is prohibitively expensive in comparison with Hygromycin B, Sulfonylurea and Bialophos (Shimizu et al. 2010; Levy et al. 2008). To ensure observed mutant phenotypes were the result of their targeted gene replacement and not the disruption of *KU70*, Guy11 was used as an additional control, to which $\Delta ku70$ and associated mutants could be compared.

4.4.3 Split-marker construct generation

The split-marker transformation technique, an alternative to the two approaches detailed above, allows successful targeted gene replacements in Guy11, enabling triple gene replacement mutants to be created. During split marker transformation technique, two linear DNA amplicons are introduced into *M. oryzae* protoplasts. Each amplicon contains an incomplete section of the gene replacement construct. Crucially, neither amplicon contains the complete resistance marker coding sequence. During the transformation process, simultaneous

recombination occurs between the two amplicons (generating a single linear fragment containing a complete resistance marker sequence), and homologous recombination into the genomic sequence. If a single amplicon recombines ectopically within the genome, the resulting transformant will not have a complete resistance marker cassette and will therefore fail to grow in the presence of the antibiotic. By comparison, any ectopic recombination which occurs whilst transforming using a single linear construct will result in a “false positive” transformant, which demonstrates antibiotic resistance without having replaced the gene of interest. Such transformants can only be identified through PCR and Southern blot analysis. Therefore, the use of the split-marker transformation technique greatly reduces the number of ectopic transformants generated allowing triple deletion mutants to be created and is significantly quicker than the *E. coli* based cloning strategy.

Chapter 5: Infection-related development of wildtype and mutant strains

5.1 Introduction

This Chapter seeks to address the question as to whether NO is required by *M. oryzae* for early germling morphogenesis and appressorium formation? The results presented herein exploit and compare wildtype strains Guy11 and $\Delta ku70$, with respect to the mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ germling differentiation, and melanised appressoria (MA) development on inductive and non-inductive surfaces.

5.1.1 Scoring

Phenotypic differences in the development of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ were assessed in morphological scoring assays. Conidia were harvested and inoculated onto TAAB plastic coverslips, a surface inductive to melanised appressoria (MA) formation, or glass microscope coverslips, a surface non inductive to MA formation (Chapter 2, 2.2.2 and 2.4). Each strain was scored for development 12 hours post inoculation (hpi), and categorised into one of four separate stages:

- Stage 1) Failed to germinate: conidia that by 12hpi had not developed an appressorium germ-tube.
- Stage 2) Germ-tube: conidia 12hpi which germinated and generated appressorium germ tubes, but failed to develop appressoria.
- Stage 3) Appressoria: germlings 12hpi with elongate appressorium germ tubes and immature un-melanised appressoria.

- Stage 4) Melanised appressoria: germings, 12hpi, which had elongate appressorium germ tubes and mature melanised appressoria.

Each independent scoring experiment was replicated at least three times and contained three biological replicates. For each biological replicate 120 germings were scored. Therefore, over 1000 conidia were scored per collective experiment.

5.1.2 Chemical genetics, linking NO and *NOS2*, *NOS3*, *NIA1* and *NII1*

To test whether NO plays a role in appressoria development, the NO scavenger PTIO was used to deplete any available NO in the germings. PTIO is a widely used NO scavenger which directly reacts with NO, removing it from biological systems (Miraglia et al. 2011; Meuchel et al. 2011; Melo et al. 2011). Conversely treating $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ with an exogenous supply of NO is an important test to confirm that any phenotypes result from an inability to produce NO, particularly as the proteins encoded by *NOS2*, *NOS3*, *NIA1* and *NII1* have not yet been directly observed to generate NO. DETANONOate was used to exogenously supply NO, it spontaneously decomposes, at neutral pH, releasing NO, specifically in its free radical form (see Chapter 7, 7.5.1.2 and 7.5.2.2 for detailed formation about PTIO and DETANONOate chemistries).

5.2 Experimental objectives

- 1) To assess germling morphogenesis of wildtype and mutant strains on artificial inductive surfaces.
- 2) To assess germling morphogenesis of wildtype and mutant strains on artificial non-inductive surfaces.
- 3) To investigate the effect of the NO scavenger PTIO on wildtype and mutant strains germination and development on artificial inductive surface.
- 4) To investigate the effect of the NO donor DETANONOate on wildtype and mutant strains germination and development on artificial inductive surface.

5.3 Results

5.3.1 Wildtype and mutant strain germling morphogenesis on an inductive surface

The ability of the TAAB coverslips to induce MA formation in the wildtype Guy11 was first tested (Figure 5.1) and was found to be strongly dependent on the batch of coverslips used. Therefore, each batch was tested for its ability to induce MA formation in Guy11, only those batches capable of doing so were used to assess the mutant strains' phenotypes.

Wildtype and mutant strains were assessed for their ability to develop infection-related structures on TAAB plastic coverslips. Conidia were inoculated in demineralised H₂O onto the plastic coverslips, (as detailed in Chapter 2, 2.2.4) and scored 12 hours post inoculation (hpi). Differences in MA development were assessed between strains, as this represents the final stage of infection-related morphogenesis and is considered the most accurate estimate of a strains ability to cause disease on an artificial host.

Quantitative scoring analyses demonstrated wildtype strains, Guy11 and $\Delta ku70$, developed high percentages of MA (>80%) at 12hpi, with no significant difference between the two strains, (p-value 0.62), Figure 5.2. By contrast, the mutant strains developed fewer MA, at this time point. Mutant strains $\Delta nos2$ and $\Delta nos3$, developed ~55% MA, a significant reduction relative to the wildtype strains, (p-values: 4.3×10^{-5} and 1.2×10^{-4} , respectively); $\Delta nos2 \Delta nos3$ developed ~30% MA (p-value 1.5×10^{-8}); $\Delta nia1$, $\Delta nii1$ and $\Delta nia1 \Delta nii1$ developed ~50% MA a (p-values: 2.7×10^{-5} , 6.1×10^{-5} and 4.3×10^{-6} , respectively). Finally, $\Delta nos3 \Delta nia1$ developed ~20% of MA, relative to the wildtype strains, (p-value 2.4×10^{-10}).

Visual inspection of mutant germlings on inductive TAAB coverslips, at 12hpi, suggested that their appressorium germ-tubes were longer than those of the wildtype strains, Figures 5.3 and 5.4. This observation was quantified and confirmed. The mean appressoria germ-tube lengths for wildtype strains Guy11 and $\Delta ku70$ were recorded as 31 and 26 μ m. By contrast the mean

appressoria germ-tube lengths of $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ were more than double that of $\Delta ku70$, Figure 5.3.

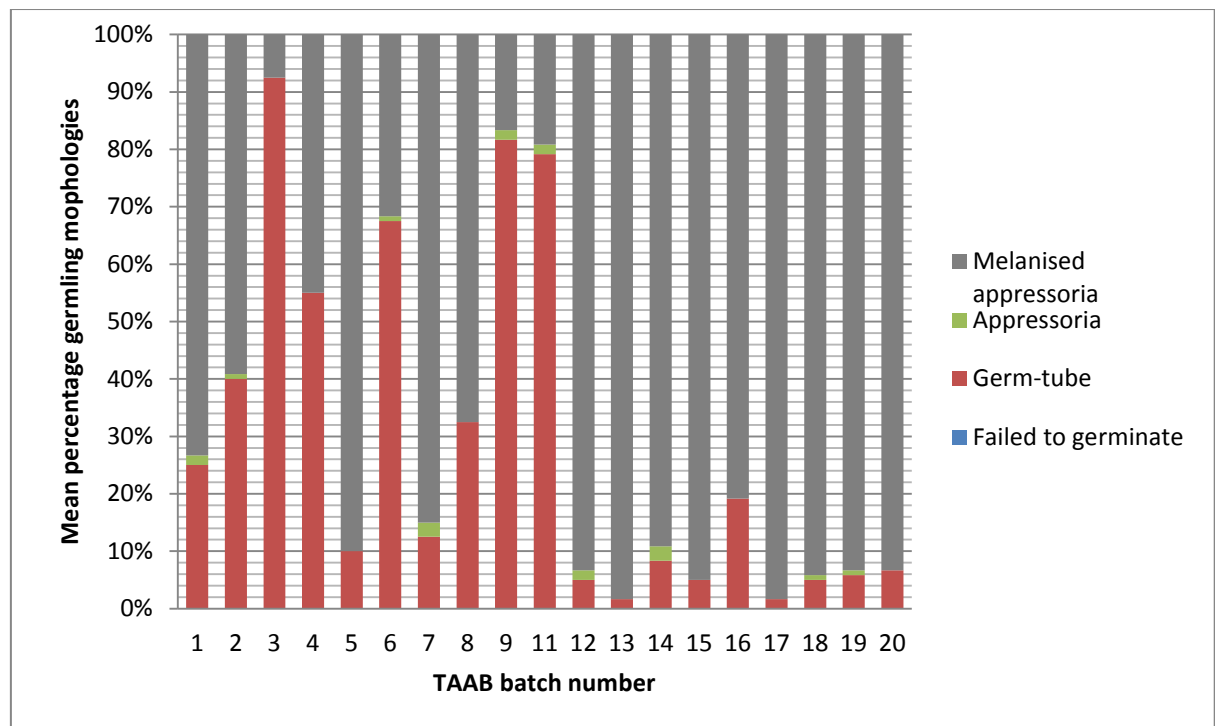


Figure 5.1. Wildtype Guy11 germling morphologies scored at 12hpi on various batches of TAAB plastic coverslips. Guy11 germlings were inoculated in demin H₂O and the mean percentage germling morphologies were calculated from 120 germlings.

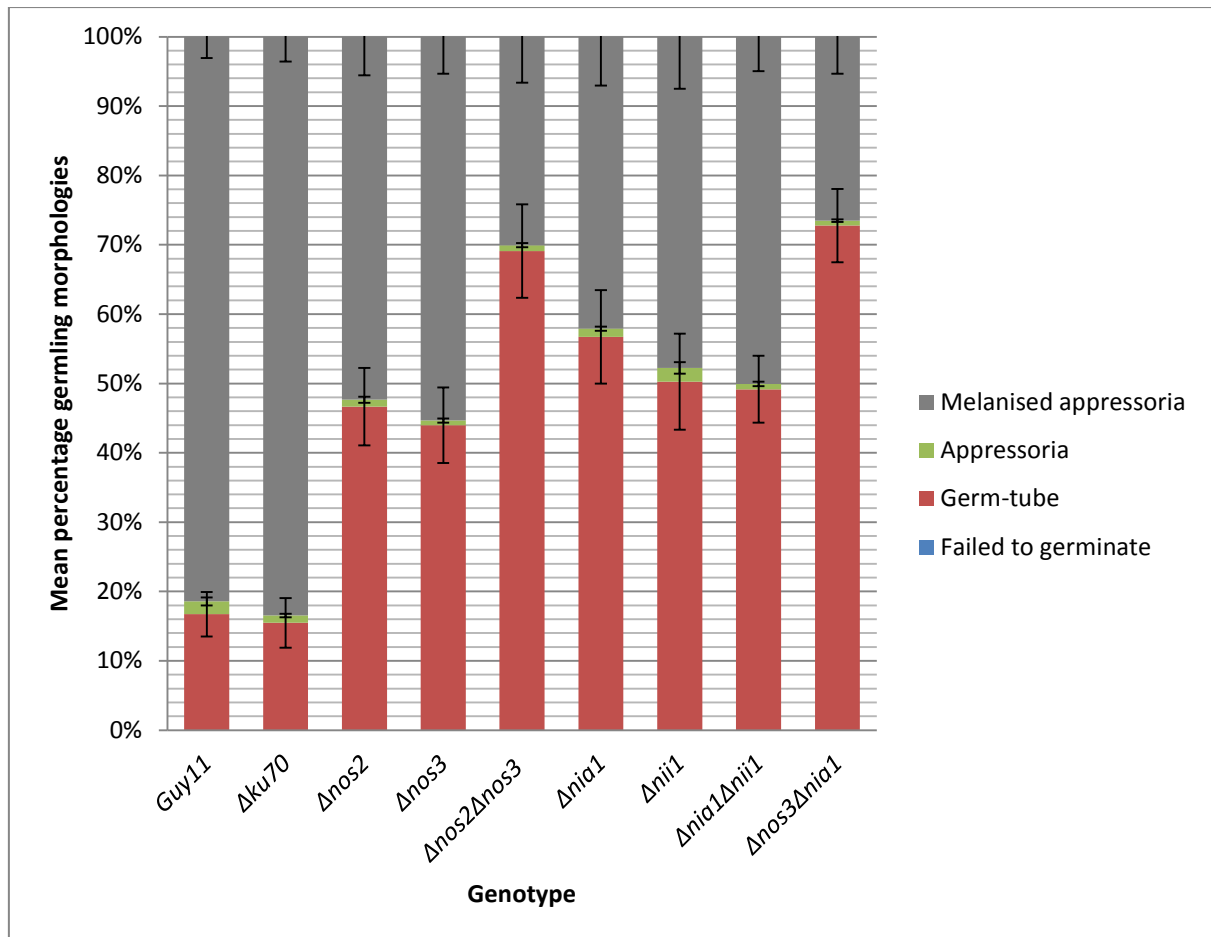


Figure 5.2. Wildtype and mutant germling morphologies scored at 12hpi on TAAB plastic coverslips, a surface inductive to MA formation in wildtype strain Guy11. Strains: Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ inoculated in demin H₂O. Mean percentage germling morphologies were drawn from five independent experimental replicates, each containing three biological replicates. For each biological replicate 120 germlings were assessed. Error bars represent the standard error of the mean.

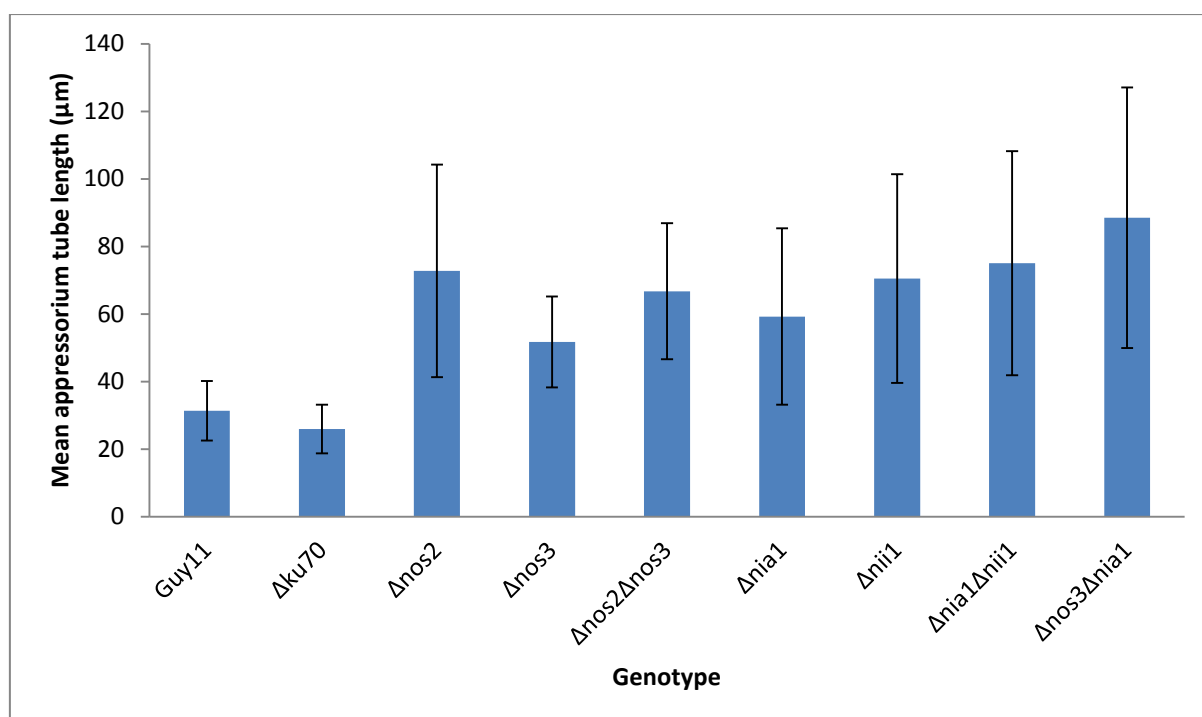
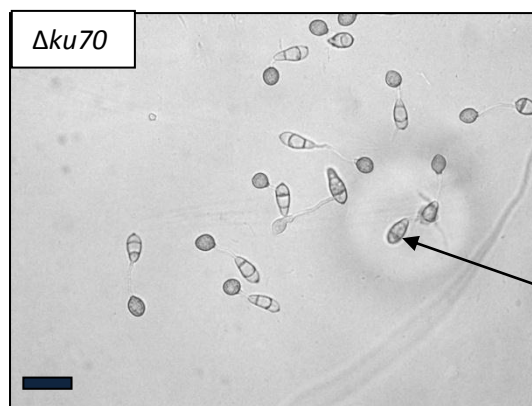
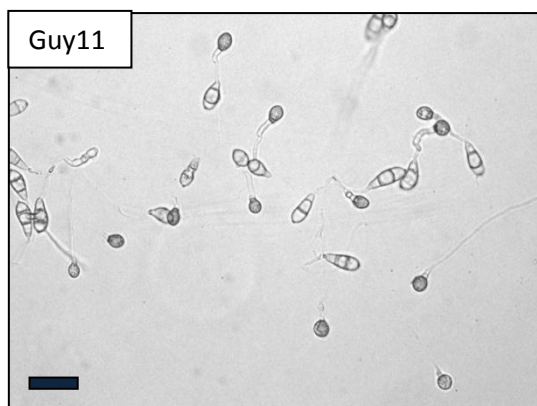
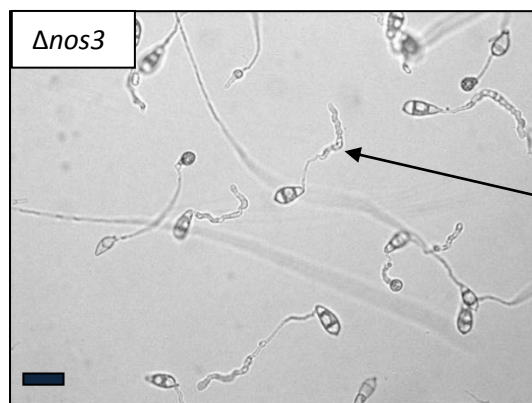
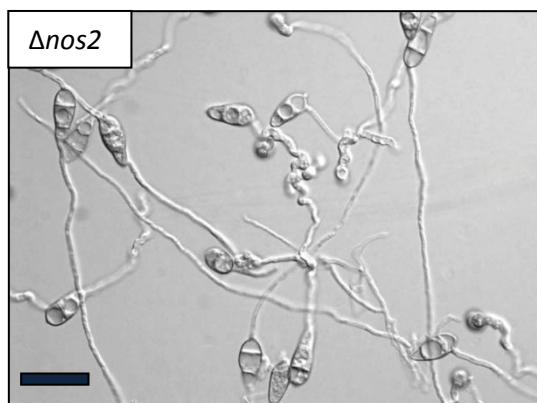


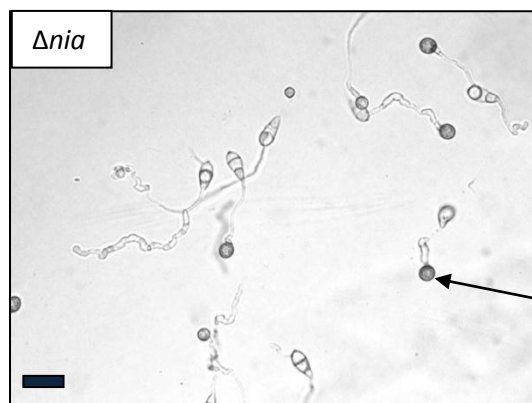
Figure 5.3. Wildtype and mutant germling appressorium germ-tube lengths 12hpi, on TAAB plastic coverslips, a surface inductive to MA formation in wildtype strain Guy11. Strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ inoculated in demin H₂O. Mean germling appressoria germ-tube lengths (μm) were calculated from a single experiment, containing three biological replicates. For each biological replicate 15 germlings were measured. Error bars represent the standard deviation.



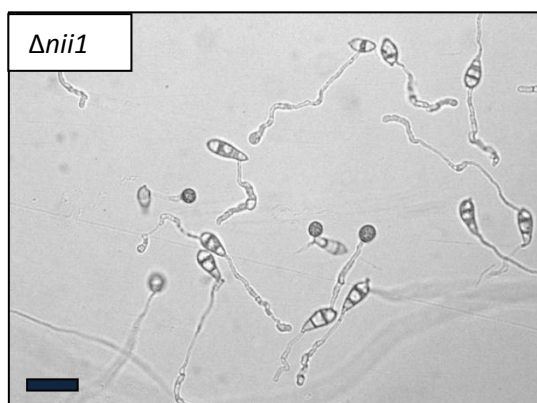
Conidium



Germ tube



Melanised
Appressorium



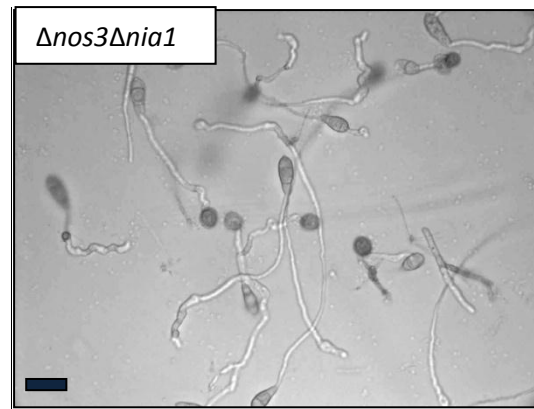
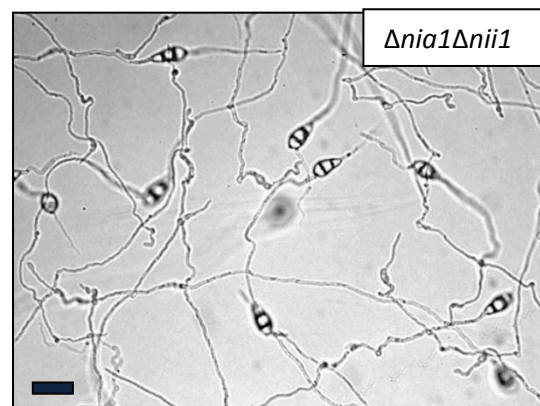
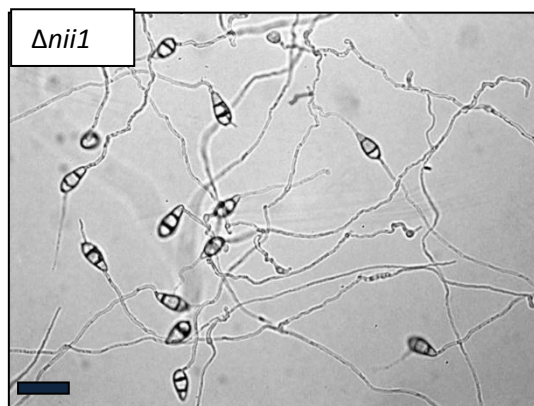
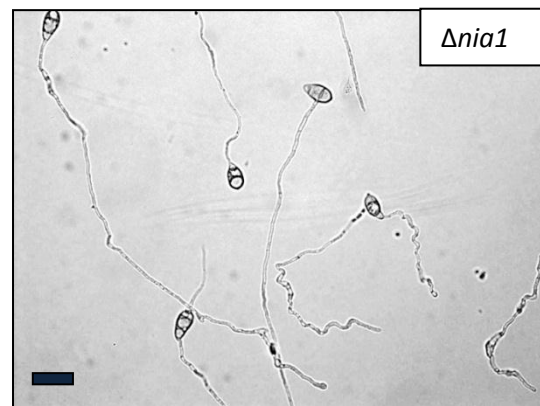
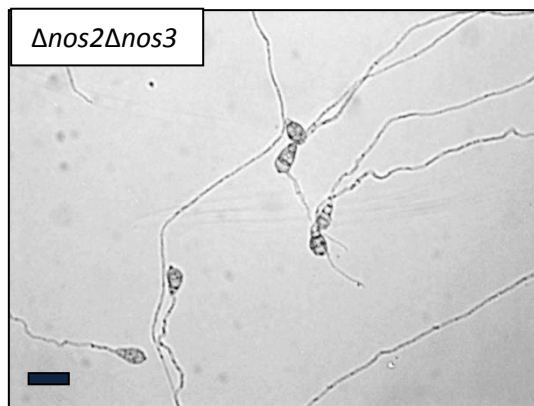
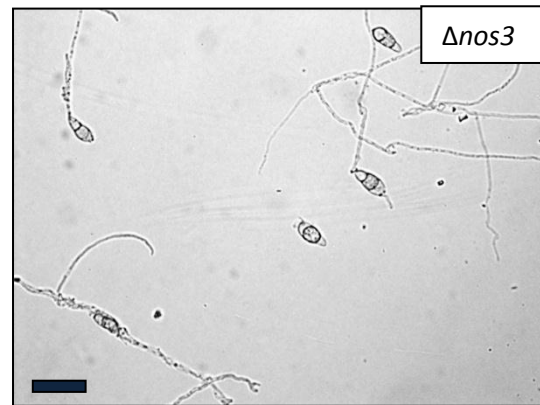
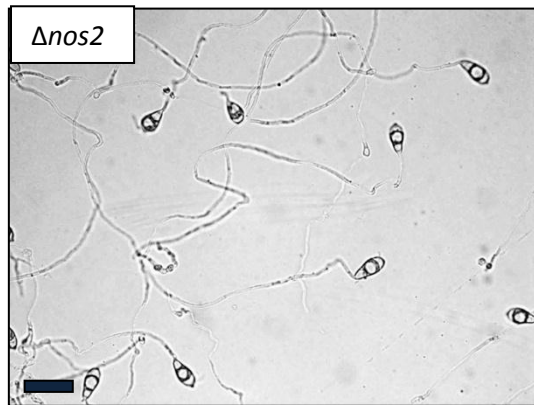
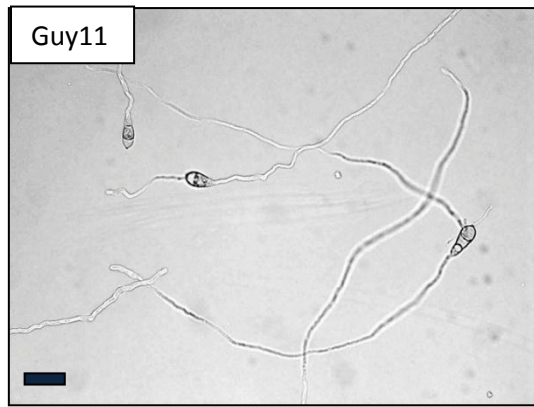


Figure 5.4. Wildtype and mutant germling morphologies, 12hpi on TAAB plastic coverslips, a surface inductive to MA formation in wildtype strain Guy11. Wildtype strains Guy11 and *Δku70* and mutant strains *Δnos2*, *Δnos3*, *Δnos2Δnos3*, *Δnia1*, *Δnii1*, *Δnia1Δnii1* and *Δnos3Δnia1* inoculated in demin H₂O. Scale bars represent 20μm.

5.3.2 Wildtype and mutant strain morphogenesis on a non-inductive surface

Development of wildtype and mutant strain were also assessed on a non-inductive surface, Figure 5.5. Conidia prepared in demineralized H₂O, were inoculated onto glass microscope coverslips, a surface non-inductive for MA formation in wildtype strain Guy11, due to its hydrophilic surface (as detailed in Chapter 2 2.4). No wildtype or mutant strains developed MA on this hydrophilic surface, Figure 5.6. Germlings' phenotypes were characterised by elongated appressorium germ-tubes (~160μm), with no clear differences in germ-tube length between wildtype and mutant strains



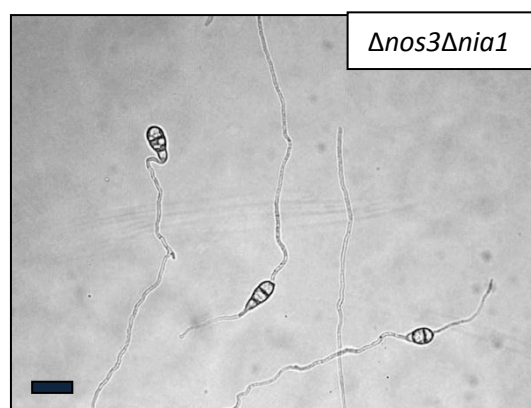


Figure 5.5. Wildtype and mutant germling morphologies 12hpi, on glass microscope coverslips, a non-inductive surface, which does not induce MA formation in wildtype Guy11. Wildtype strains Guy11 and $\Delta ku70$, and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ were inoculated in demineralized H_2O . Scale bars represent $20\mu m$.

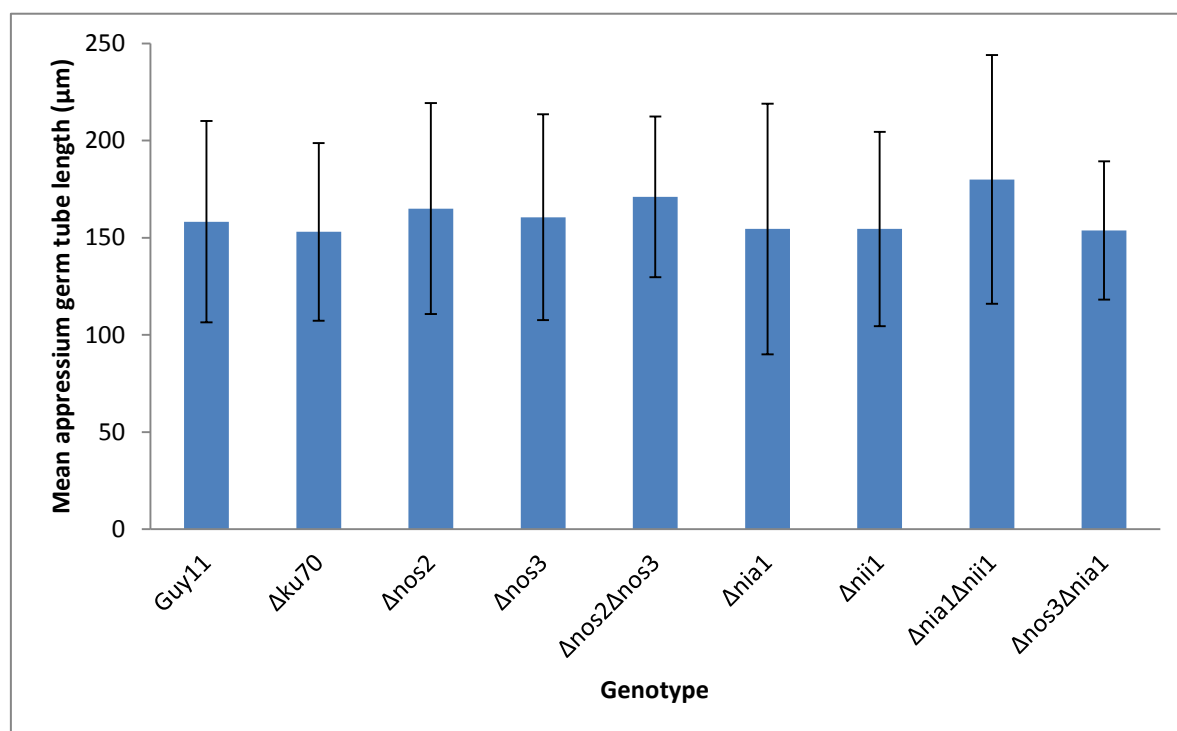


Figure 5.6. Wildtype and mutant germling mean appressoria germ-tube lengths 12hpi, on glass microscope coverslips, a non-inductive surface unable to induce MA formation in wildtype strain Guy11. Strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ were inoculated in H_2O . Mean germling appressoria germ-tube lengths were calculated from a single experiment, containing three biological replicates. For each biological replicate 15 germlings were measured. Error bars represent the standard deviation.

5.3.3 Wildtype and mutant strains germling morphogenesis on an inductive surface following treatment with the NO scavenger PTIO

The effect of the NO scavenger PTIO on germination and development of wildtype and mutant strains were assessed. PTIO (buffered in 10mM HEPES pH7) was applied to Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ conidia at the time of inoculation, onto inductive TAAB plastic coverslips, (as detailed in Chapter 2, 2.4). The resulting germling morphologies were scored at 12hpi, (as per Chapter 2, 2.4).

PTIO was buffered in 10mM HEPES pH7 to prevent pH-dependent side-effects from influencing conidia development. The effect of 10mM HEPES pH7 on wildtype Guy11 germination and development 12hpi, on inductive TAAB plastic coverslips, relative to demineralized H₂O was assessed, Figure 5.7. No significant differences in the percentage germination and formation of MA were observed with Guy11 germlings developing in demineralized H₂O or 10mM HEPES pH7, (p-values: 0.830 and 0.904, respectively). The buffer, 10mM HEPES pH7, was therefore considered to have no effect on *M. oryzae* germination and development, and was selected for use as the negative control to PTIO treatment (further information regarding 10mM HEPES is detailed in Chapter 7, 7.5.1.1).

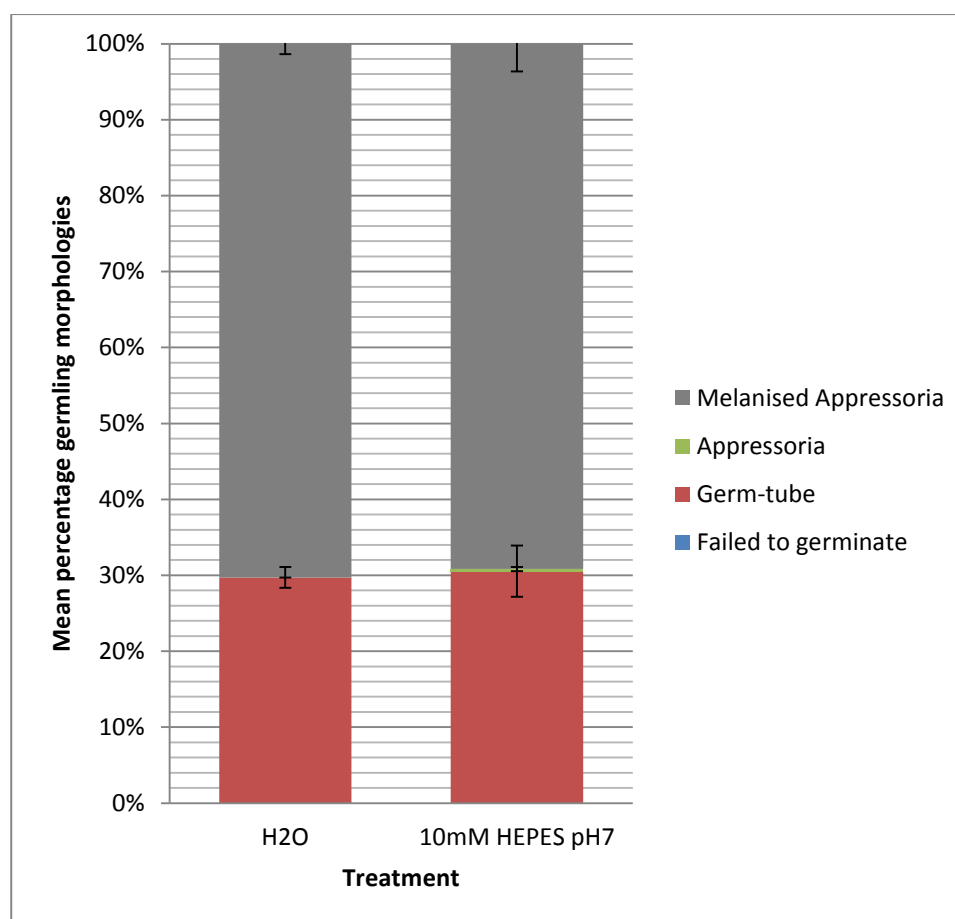


Figure 5.7. The effect of demineralised H₂O and 10mM HEPES pH7 on Guy11 germination and development at 12hpi, on TAAB plastic coverslips, a surface inductive to MA formation in wildtype strain Guy11. Mean percentage germling morphologies were calculated from three independent experimental replicates, containing three biological replicates; for each biological replicate 120 germlings were assessed. Error bars represent the standard error of the mean.

A suitable PTIO concentration for scavenging NO from wildtype and mutant strains was determined by inoculating Guy11 conidia with increasing concentrations of PTIO in 10mM HEPES pH7, Figure 5.8. The addition of PTIO dramatically reduced the percentage of Guy11 germlings forming MA: 200µM suppressed MA formation from ~70% to ~22%, relative to Guy11 conidia inoculated in 10mM HEPES. The addition of increasing concentrations of PTIO reduced the percentage of Guy11 germlings developing MA to ~20-12%, and suppressed germination by up to ~76%. Concentrations of PTIO at 450µM and above abolished MA formation (<2%) and prevented conidia germination. PTIO at 200µM was selected as the appropriate concentration

to apply to the wildtype and mutant strains. At this concentration PTIO compromised the ability of the wildtype to form MA without suppressing germination.

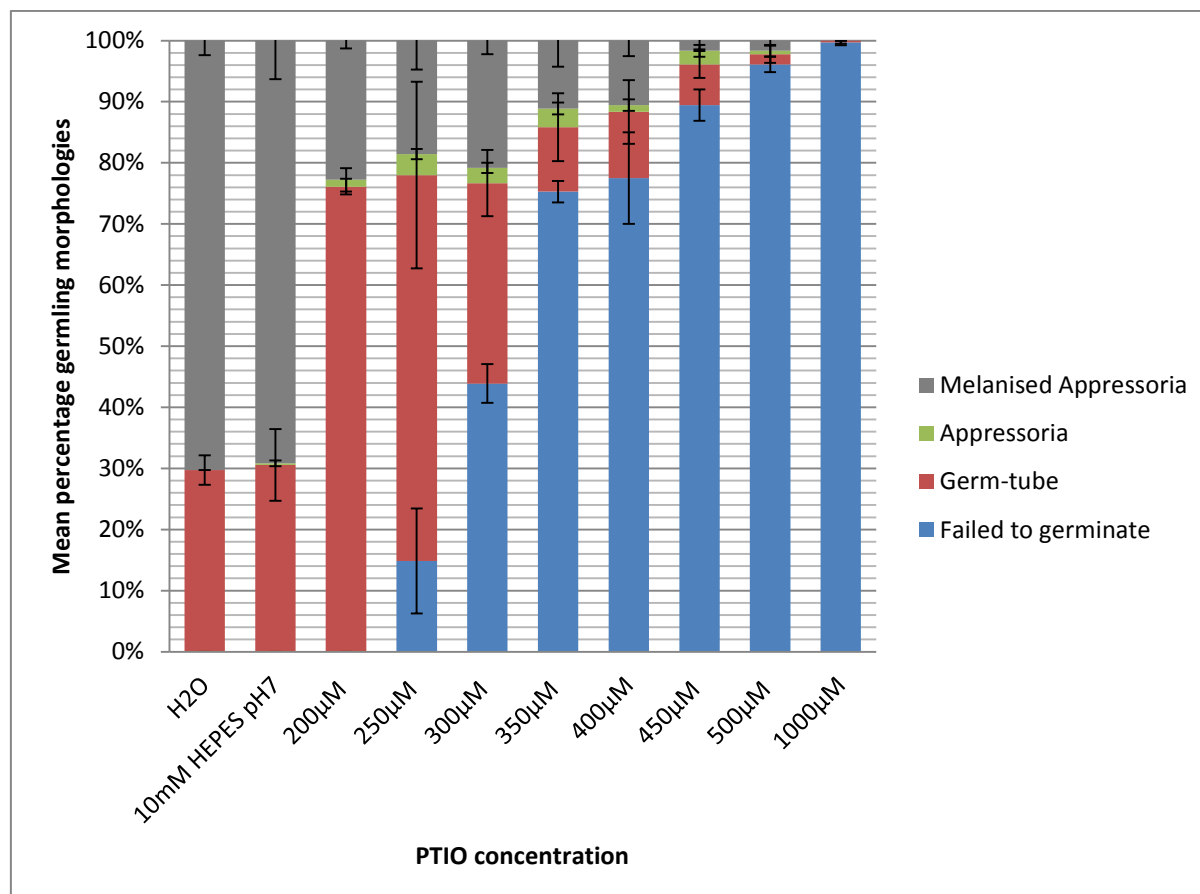


Figure 5.8. The effect of increasing PTIO concentrations (10mM HEPES pH7) on Guy11 conidia germination and development at 12hpi, on TAAB plastic coverslips, a surface inductive to MA formation in wildtype strain Guy11. Mean percentage germling morphologies were calculated from three independent experimental replicates, containing three biological replicates. For each biological replicate 120 germlings were assessed. Error bars represent the standard error of the mean.

Wildtype and mutant germlings inoculated in 10mM HEPES pH7 closely resembled the morphology seen following inoculation in demineralised H₂O, Figures 5.11 and 5.12. By contrast, the addition of 200µM PTIO to wildtype strains Guy11 and $\Delta ku70$ significantly reduced the percentage of germlings developing MA by 60%, (p-values: 9.4×10^{-7} and 0.01, respectively) and their morphologies resembled the mutant strain germlings inoculated in 10mM HEPES pH7, Figures 5.9 and 5.10. Such treatment also significantly suppressed the percentage of $\Delta nii1$ and $\Delta nia1\Delta nii1$ germlings forming MA by 30 and 20%, (p-values: 6.4×10^{-4} and 0.01, respectively). Treatment of $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$ and $\Delta nos3\Delta nia1$ conidia with 200µM PTIO slightly reduced the percentage of germlings developing MA by ~20%, an insignificant amount, (p-values: 0.5, 0.66, 0.5, 0.51 and 0.54, respectively). It also suppressed conidia germination by up to 20%. These observations, when combined with the effect of PTIO on the phenotype of Guy11 and $\Delta ku70$, indicate that NO has roles in both conidia germination and infection-related development in *M. oryzae*. Figure 5.11 details the mean decrease in the number of melanised appressoria following treatment with 200µM PTIO (10mM HEPES pH7).

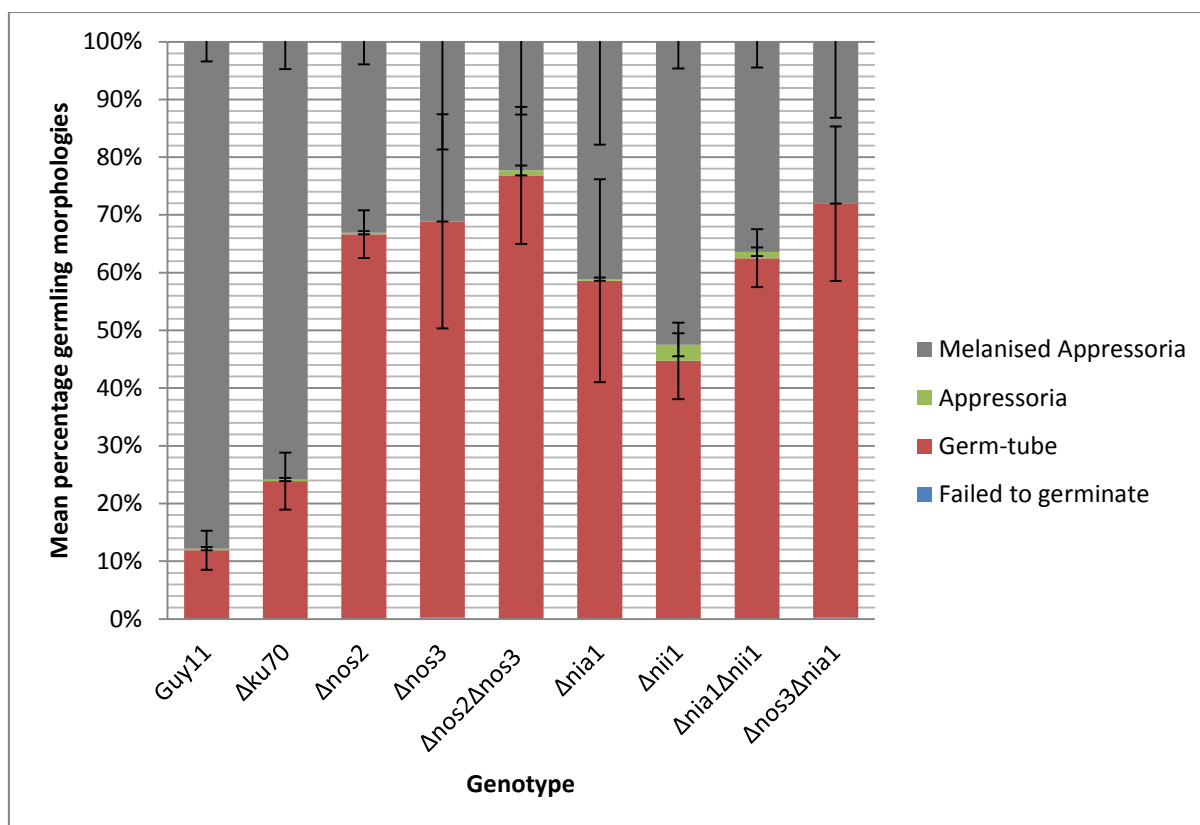


Figure 5.9. Wildtype and mutant strains inoculated in 10mM HEPES pH7, legend overleaf.

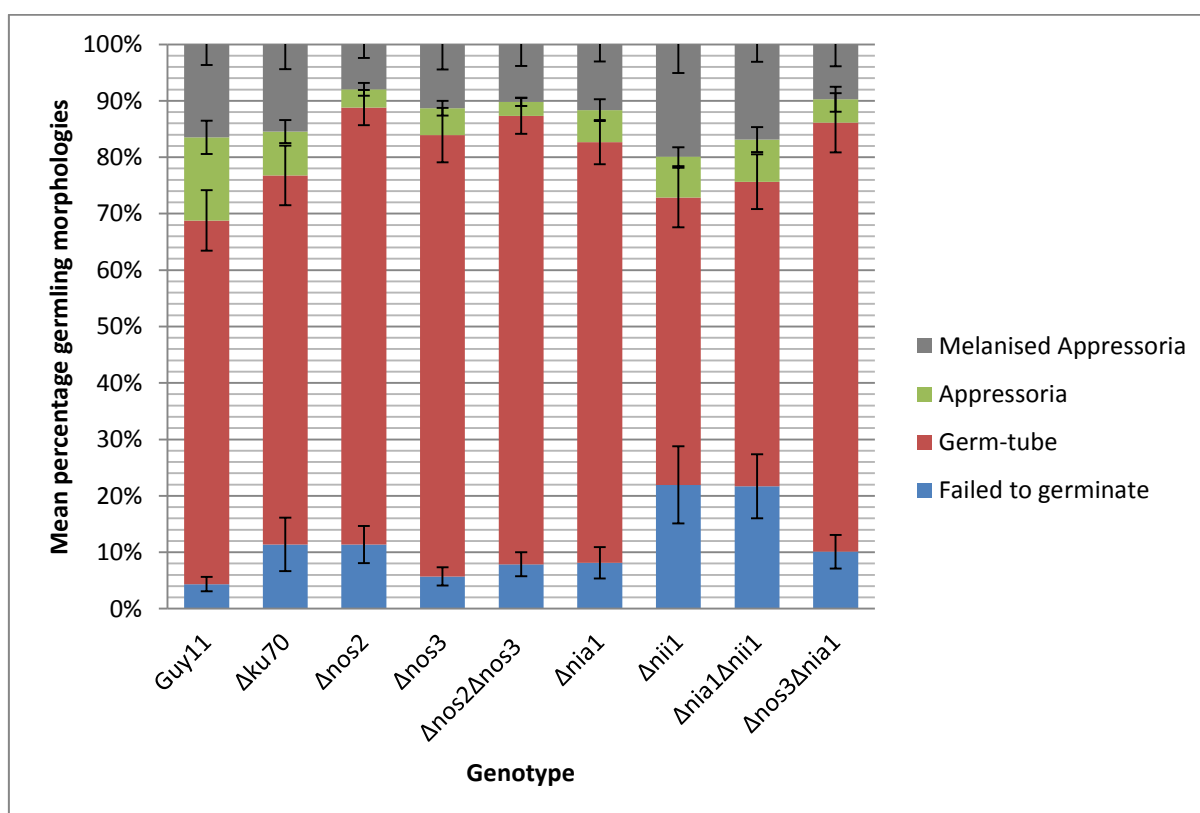


Figure 5.10. Wildtype and mutant strains inoculated in 200 μ M PTIO in 10mM HEPES pH7, legend overleaf.

Figure 5.9. Wildtype and mutant germling morphologies at 12hpi, on TAAB plastic coverslips, a surface inductive to MA formation in wildtype strain Guy11. Strains: Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ were inoculated in 10mM HEPES pH7. Mean percentage germling morphologies were calculated from three independent experimental replicates, containing three biological replicates; for each biological replicate 120 germlings were assessed. Error bars represent the standard error of the mean.

Figure 5.10. Wildtype and mutant germling morphologies at 12hpi, on TAAB plastic coverslips, a surface inductive to MA formation in wildtype strain Guy11. Strains: Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ were inoculated in 200 μ M PTIO (10mM HEPES pH7). Mean percentage germling morphologies were calculated from three independent experimental replicates, containing three biological replicates; for each biological replicate 120 germlings were assessed. Error bars represent the standard error of the mean.

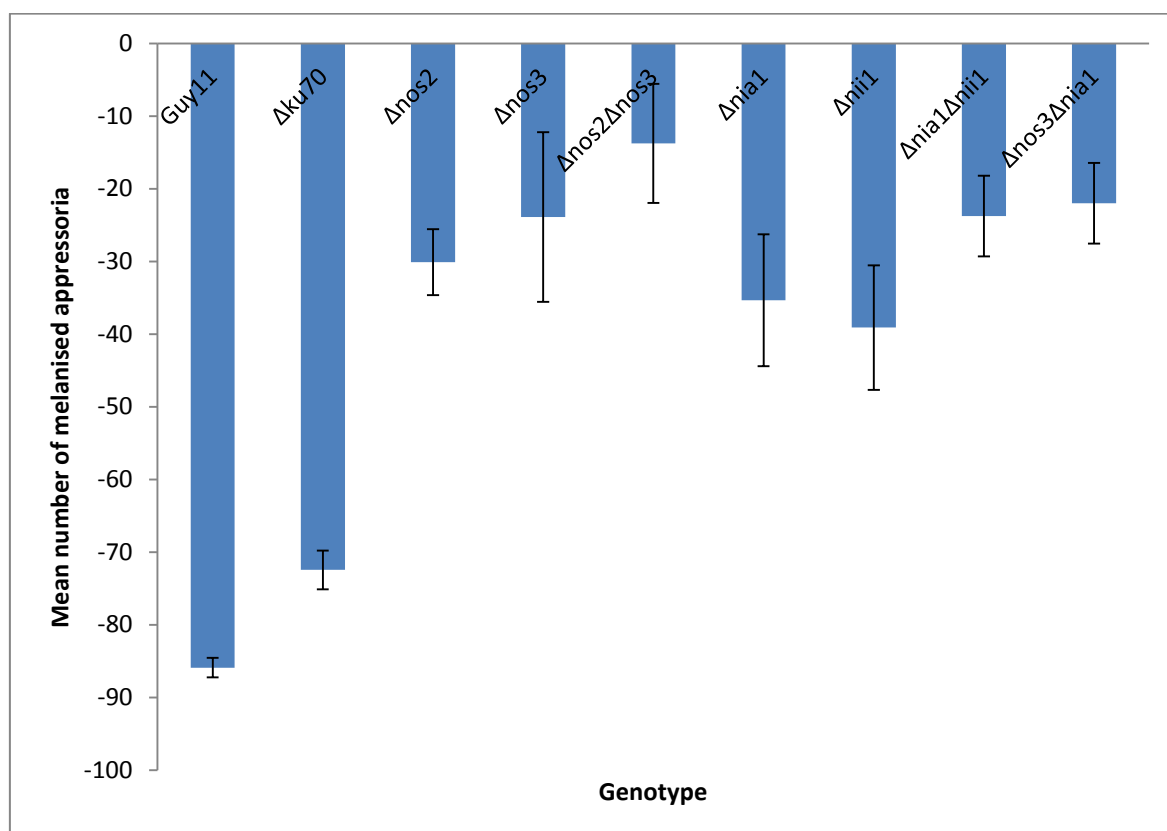
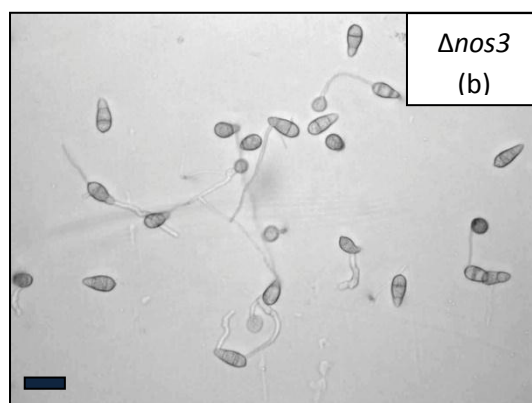
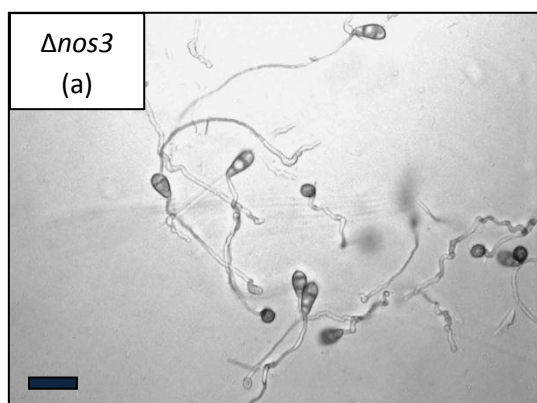
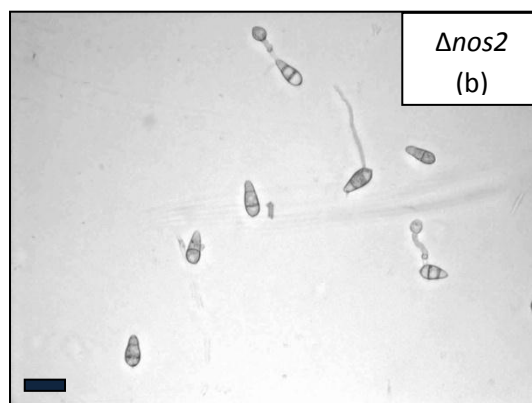
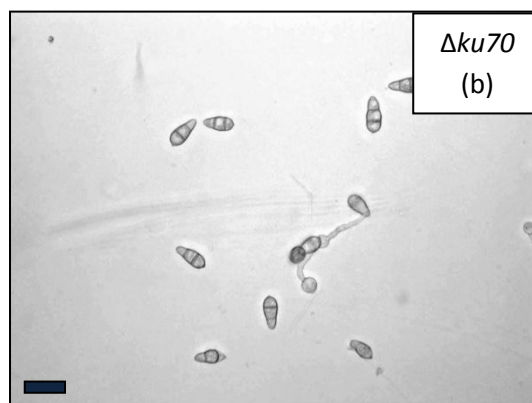
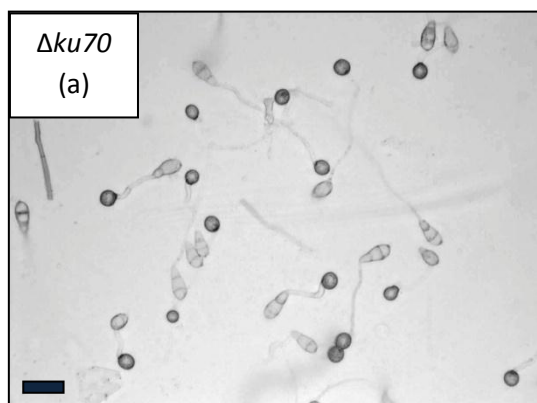
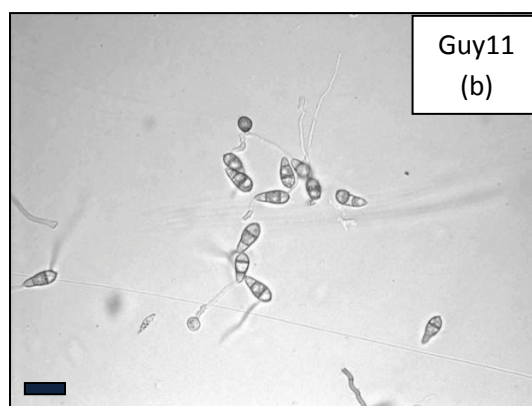
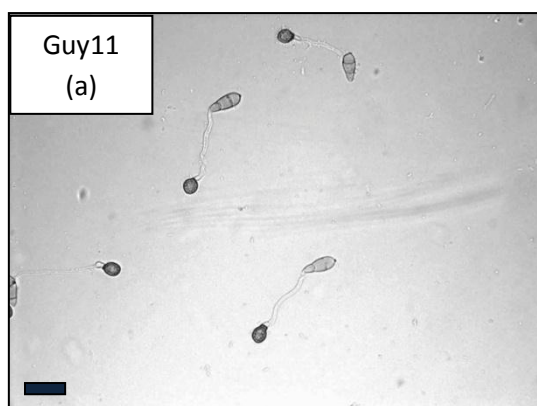
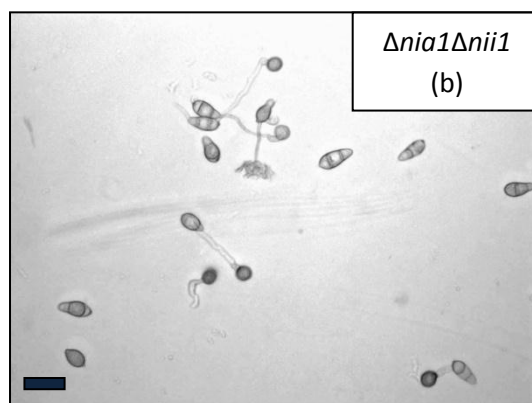
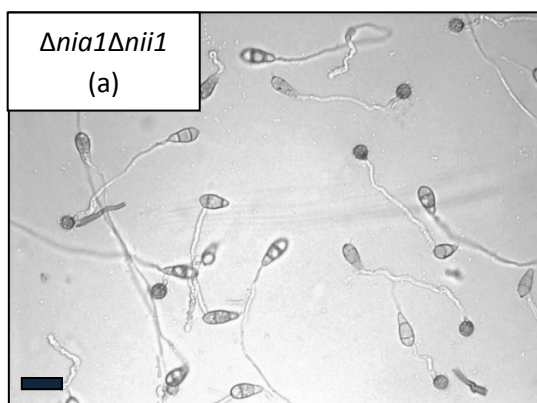
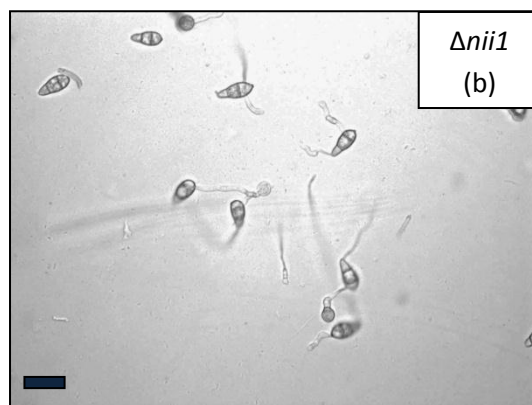
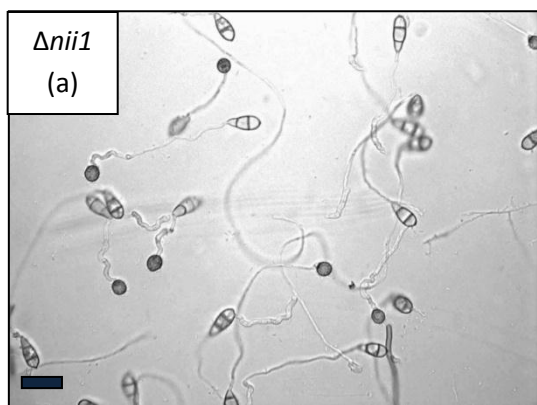
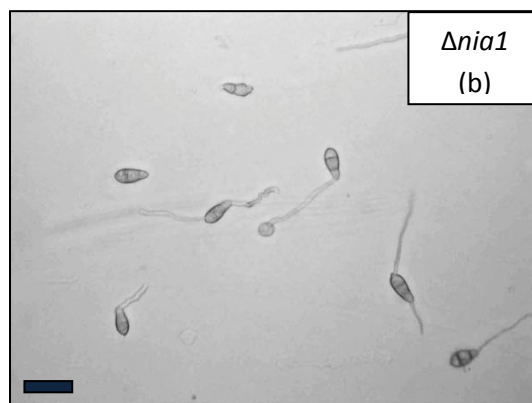
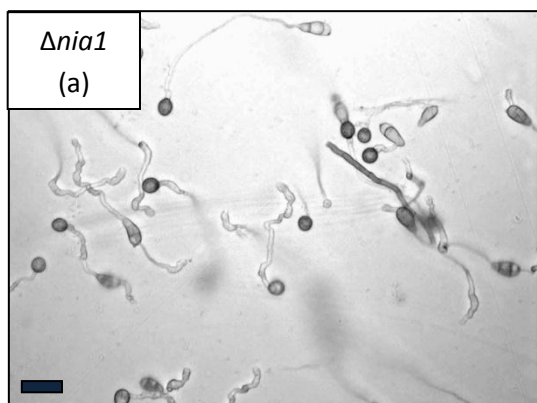
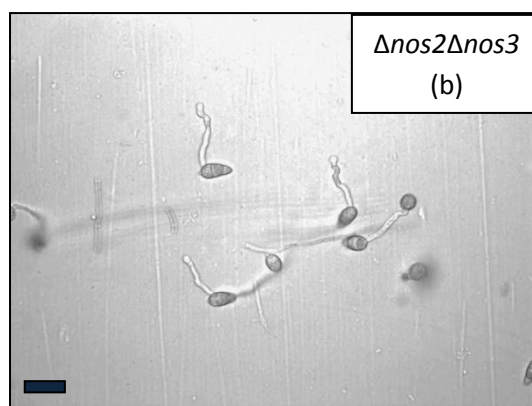
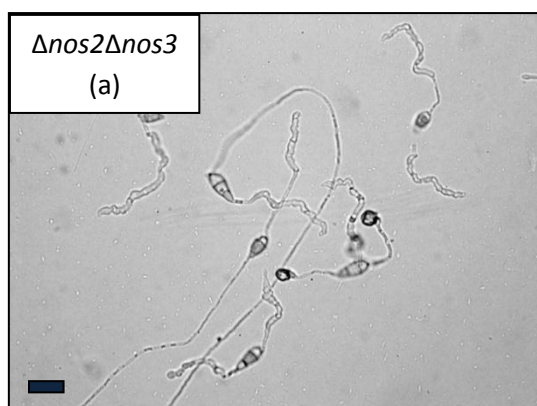


Figure 5.11. The mean difference in the number of melanised appressoria developed at 12hpi, by Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ germlings after treatment with 10mM HEPES pH7 and 200 μ M PTIO (10mM HEPES pH7) on inductive TAAB coverslips. Mean numbers of formed MA were calculated from three independent experimental replicates, containing three biological replicates; for each biological replicate 120 germlings were assessed. Error bars represent the standard error of the mean.





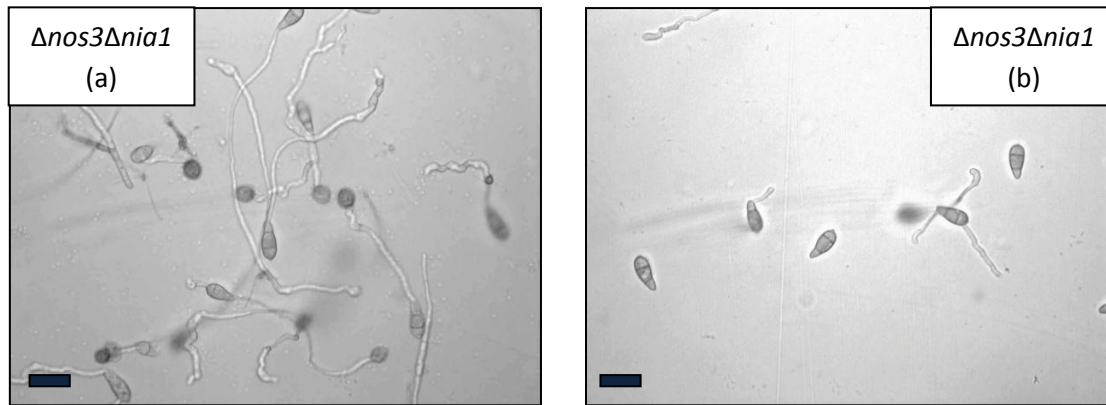


Figure 5.12. The effect of a) 10mM HEPES pH7 and b) 200μM PTIO in 10mM HEPES pH7 on wildtype Guy11 and $\Delta ku70$ and mutant $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ germling morphologies 12hpi, on TAAB plastic coverslips, a surface inductive to MA formation in wildtype strain Guy11. Scale bars represent 20μm.

5.3.4 Wildtype and mutant strains germlings morphogenesis on an inductive surface following treatment with the NO donor DETANONOate

The effect of the exogenous application of NO to Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ conidia 12hpi, was investigated on TAAB plastic coverslips, a surface inductive to MA formation in wildtype strain Guy11. Conidia were harvested and prepared, (as detailed in Chapter 2, 2.2.2 and 2.4), and the resulting germling morphologies were scored at 12hpi.

The impact of supplying exogenous NO on the wildtype and mutant strains' phenotypes represents an important experiment in confirming the role of *NOS2*, *NOS3*, *NIA1* and *NII1* in generating NO. If NO supplied through a donor restores a wildtype phenotype in $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ backgrounds this would provide strong evidence for their involvement in NO generation in *M. oryzae*.

DETANONOate spontaneously disassociates, releasing two molecules of NO in neutral and acidic solutions. To prevent its premature disassociation DETANONOate was prepared in a weak

alkaline solution (25 μ M NaOH) and stored at -80°C. Prior to its addition to conidia defrosted DETANONOate was diluted with 10mM HEPES pH7, lowering its pH and initiating NO release. The effects of 10mM HEPES pH7, 25 μ M NaOH and 10mM HEPES pH7 & 25 μ M NaOH, on Guy11 germling development at 12hpi, were compared to Guy11 inoculated in demineralised H₂O. 10mM HEPES pH7 had no significant effect on the percentage of MA developed by Guy11 germlings, relative to Guy11 conidia inoculated in demineralised H₂O, (p-value 0.99). Equally, 25 μ M NaOH alone or in 10mM HEPES pH7 had no significant effect on the percentage of MA developed by Guy11 germlings, only increasing the frequency on MA formation by an average of 6% and 10%, respectively (p-values: 0.98 and 0.38). Therefore, 10mM HEPES pH7 with 25 μ M NaOH was selected as the control for use with DETANONOate.

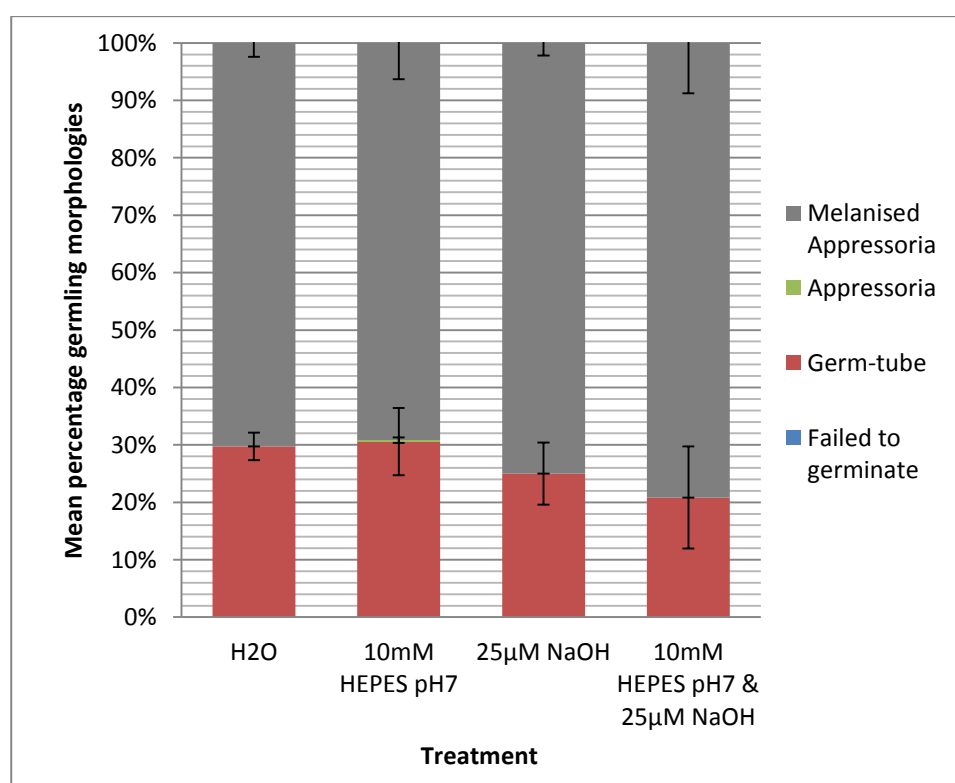


Figure 5.13. The effects of demineralized H₂O, 10mM HEPES pH7, 25 μ M NaOH and 10mM HEPES pH7 and 25 μ M NaOH on Guy11 conidia germination and morphogenesis at 12hpi, on TAAB plastic coverslips, a surface inductive to MA formation in wildtype strain Guy11. Mean percentage germling morphologies were calculated from three independent experimental replicates, containing three biological replicates; for each biological replicate 120 germlings were assessed. Error bars represent the standard error of the mean.

Δnos3 conidia were selected because previous chemical genetic assays conducted by M. Illes, using the NO donors SNP (sodium nitroprusside) and PAPANONOate were reported to partially restore the *Δnos3* phenotype to wildtype equivalent. Thus, *Δnos3* conidia were inoculated with increasing concentrations of DETANONOate in 10mM HEPES and 25μM NaOH on inductive TAAB plastic coverslips, to identify an NO concentration which reprieved the *Δnos3* phenotype. Treatment of *Δnos3* conidia with increasing concentrations of DETANONOate increased the percentage of *Δnos3* germlings developing MA, Figure 5.14, with a maximum effect at around 50μM. Higher concentrations of DETANONOate reduced the number of MA back towards control levels. Thus, the addition of DETANONOate clearly influences *Δnos3* germination and development. 100μM DETANONOate was selected as the most appropriate concentration to test with wildtype and mutant strains. It is important to note that the stoichiometry of NO disassociation from 100μM DETANONOate matches the NO scavenging stoichiometry of 200μM PTIO (Keefer et al. 1996).

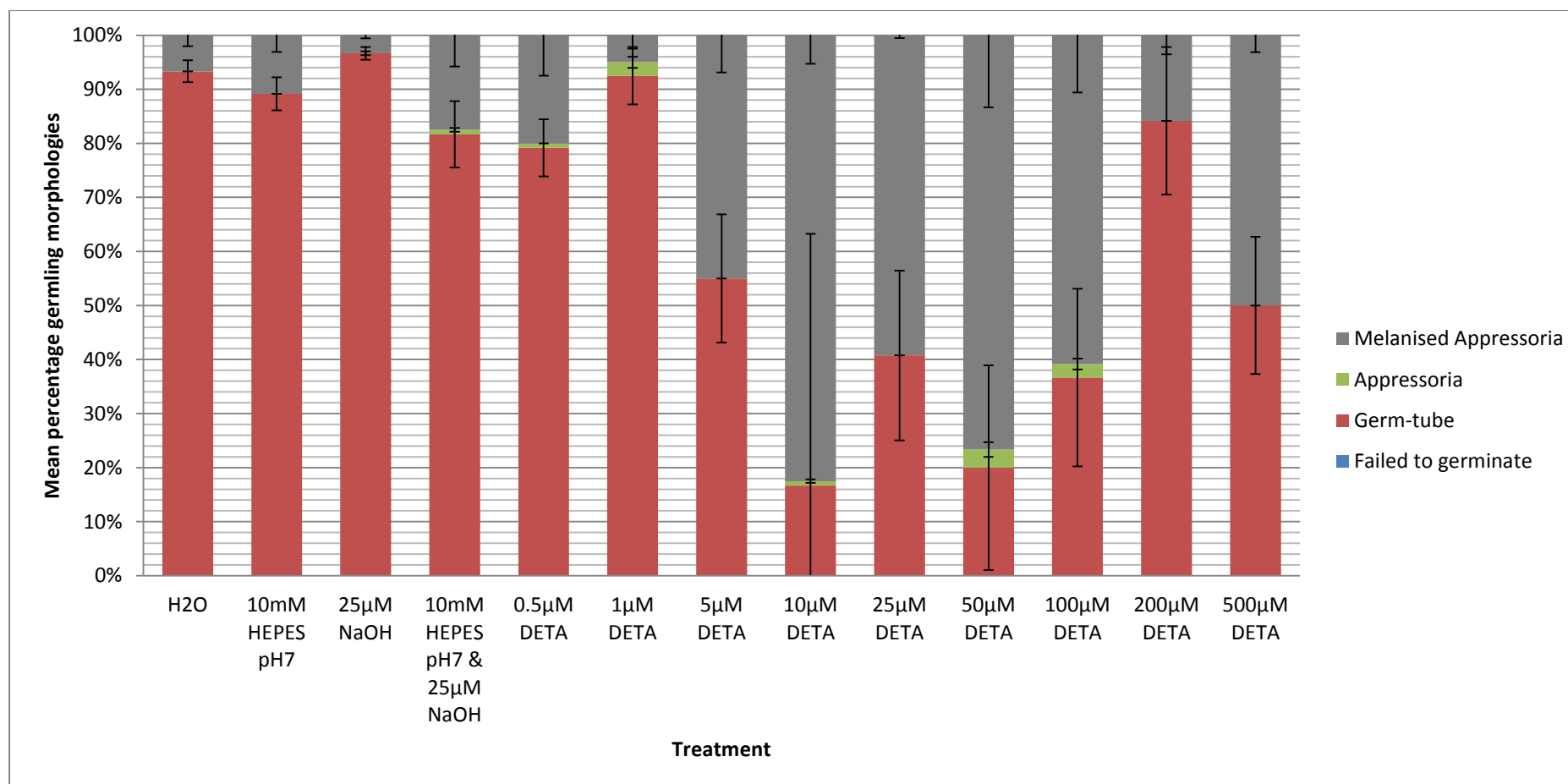


Figure 5.14. The effect of demineralised H₂O, 10mM HEPES pH7, 25µM NaOH, 10mM HEPES pH7 and 25µM NaOH, 0.5, 1, 5, 10, 25, 50, 100, 200 and 500µM DETANONOate (10mM HEPES pH7 and 25µM NaOH) on *nos3* conidia germination and development 12hpi, on TAAB plastic coverslips, a surface inductive to MA formation in wildtype strain Guy11. Mean percentage germling morphologies were calculated from three independent experimental replicates, containing three biological replicates; for each biological replicate 120 germlings were assessed. Error bars represent the standard error of the mean.

The application of 100 μ M DETANONOate in (10mM HEPES pH7 & 25 μ M NaOH) had no significant effect on the percentage MA developed by Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ germlings 12hpi, on inductive TAAB plastic coverslips relative to their 10mM HEPES pH7 and 25 μ M NaOH controls, Figures 5.15 and 5.16 (p-values: 0.2, 0.42, 0.93, 0.27, 0.65, 0.80, 0.40, 0.38 and 0.61, respectively). The treatment of the wildtype and mutant strains with 100 μ M DETANONOate (10mM HEPES pH7 and 25 μ M NaOH) partially increased the number developing MA in most strains, with the exception of Guy11 and $\Delta nos2$ germlings where a slight decrease was observed.

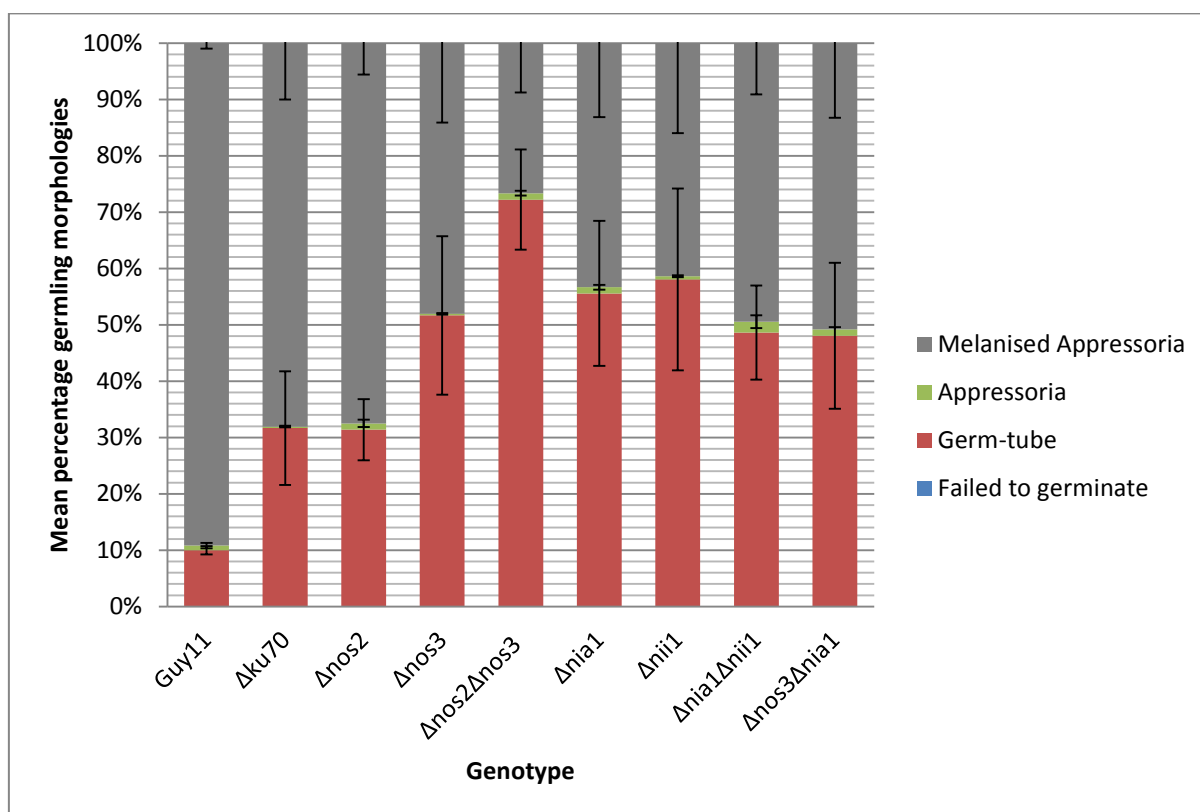


Figure 5.15. Strains inoculated in 10mM HEPES pH7 and 25µM NaOH, legend overleaf.

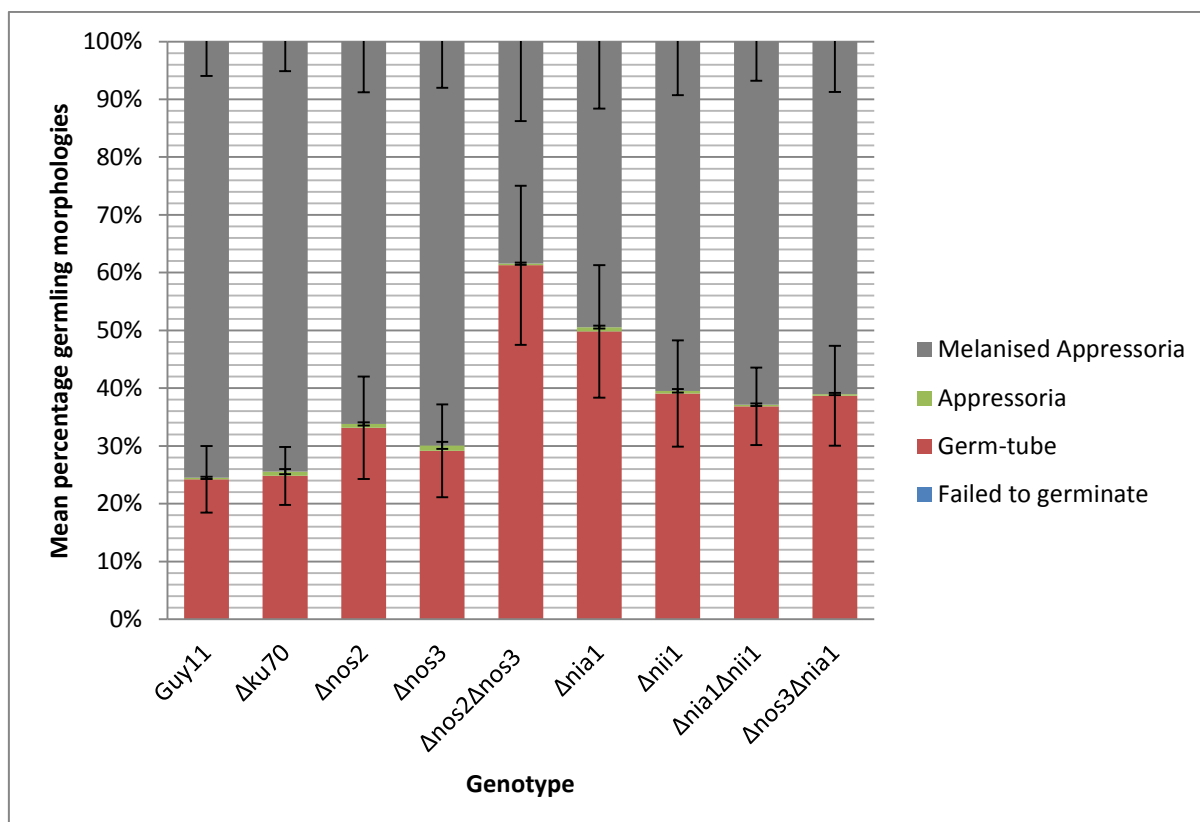


Figure 5.16. Strains inoculated in 100µM DETANONOate (10mM HEPES pH7 and 25µM NaOH), legend overleaf.

Figure 5.15. Wildtype and mutant germling morphologies 12hpi, on TAAB plastic coverslips, a surface inductive to MA formation in wildtype strain Guy11. Strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ were inoculated in 10mM HEPES pH7 and 25 μ M NaOH. Mean percentage germling morphologies were calculated from three independent experimental replicates, containing three biological replicates; for each biological replicate 120 germlings were assessed. Error bars represent the standard error of the mean.

Figure 5.16. Wildtype and mutant germling morphologies 12hpi, on TAAB plastic coverslips, a surface inductive to MA formation in wildtype strain Guy11. Strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ were inoculated in 100 μ M DETANONOate (10mM HEPES pH7 and 25 μ M NaOH). Mean percentage germling morphologies were calculated from three independent experimental replicates, containing three biological replicates; for each biological replicate 120 germlings were assessed. Error bars represent the standard error of the mean.

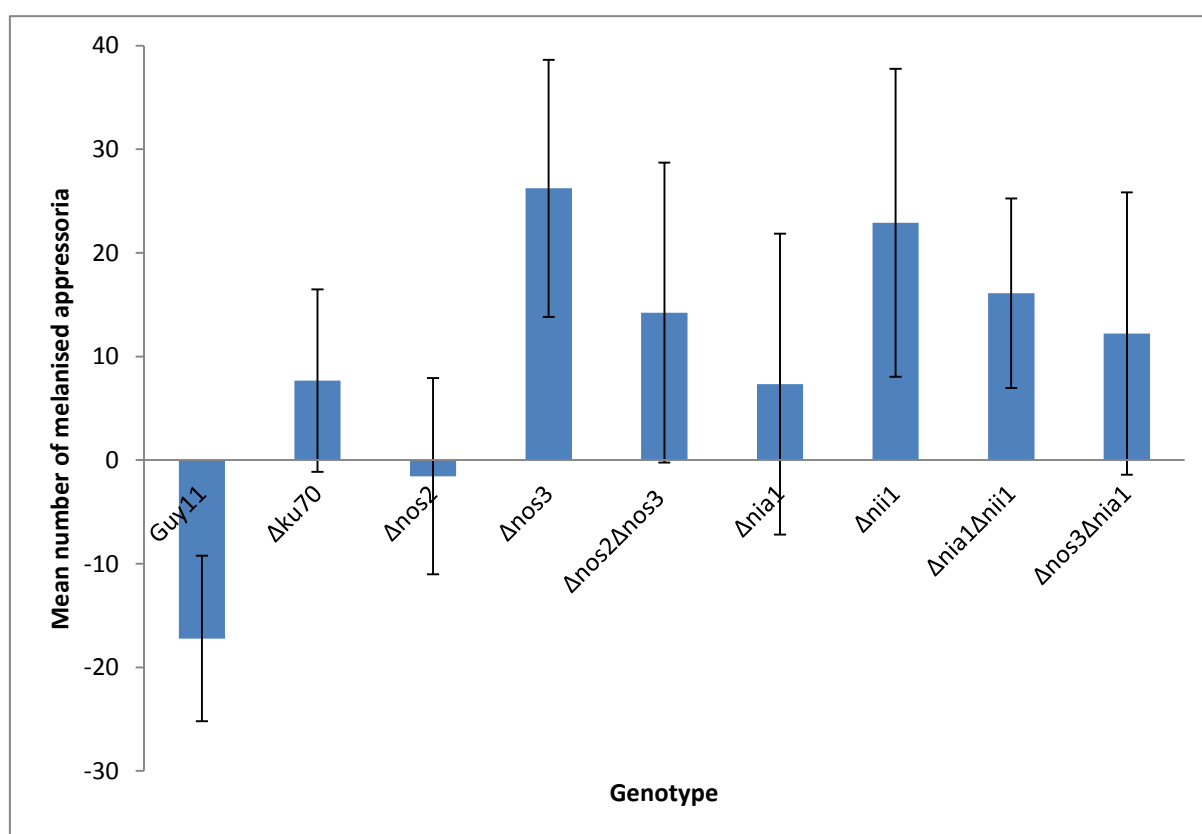
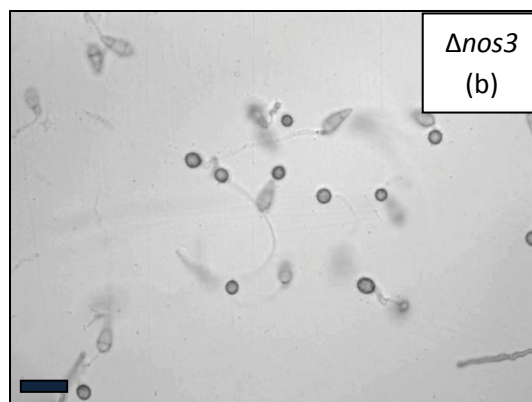
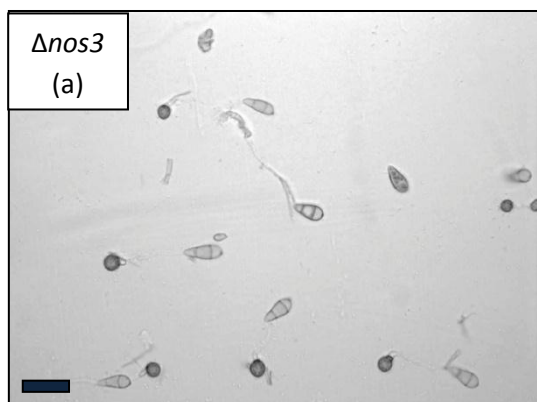
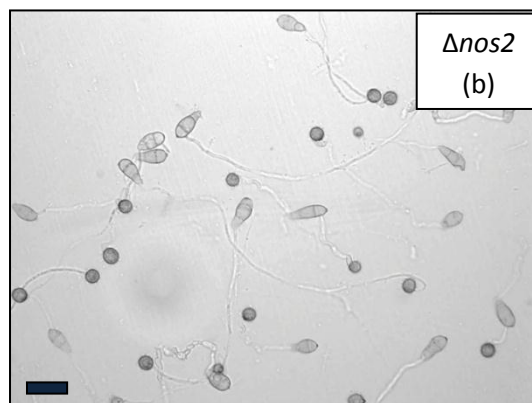
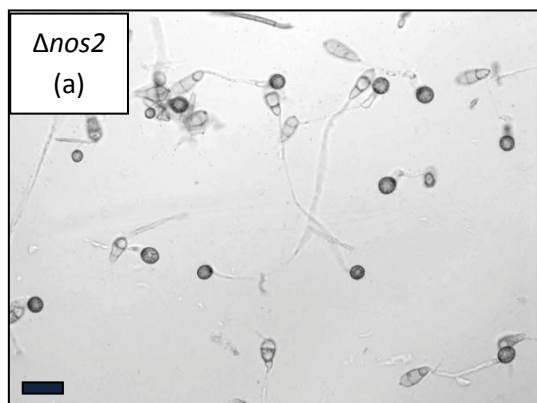
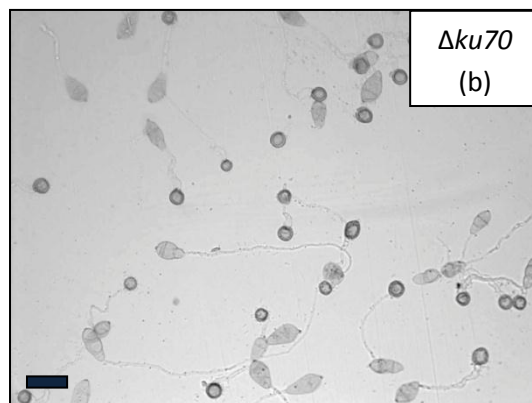
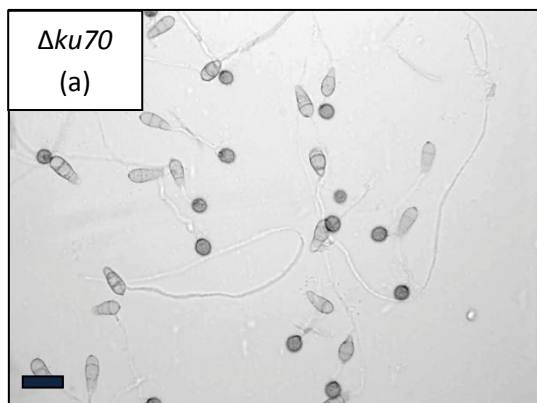
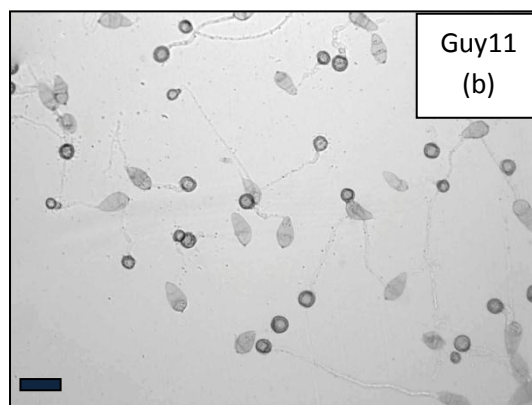
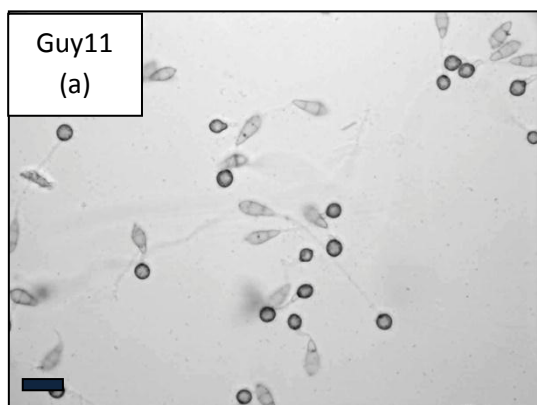
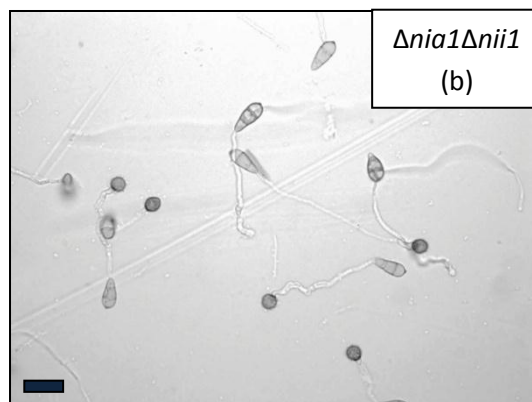
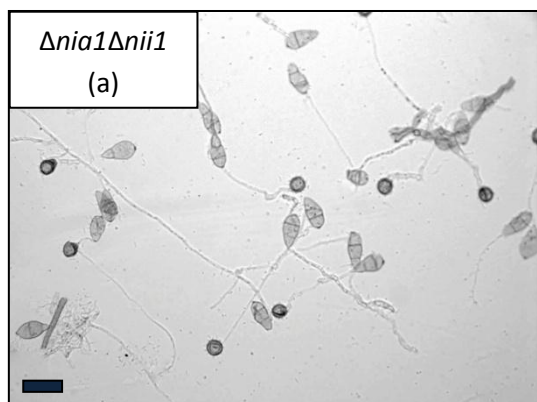
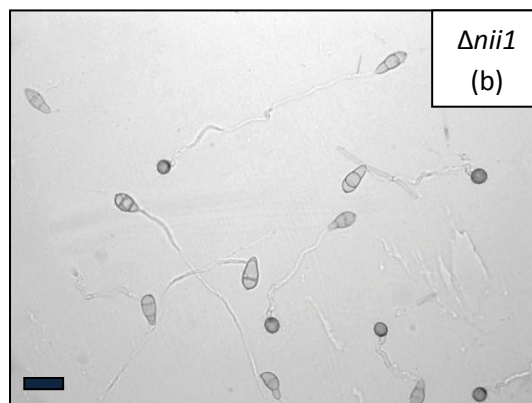
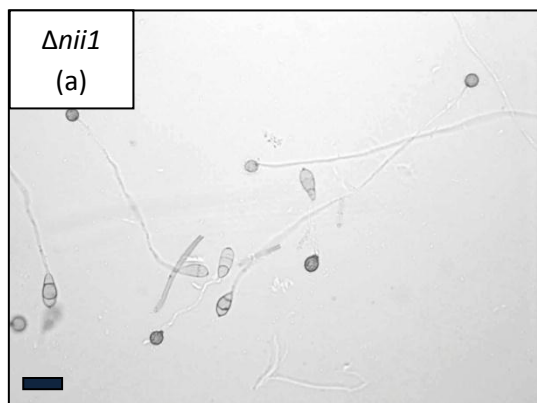
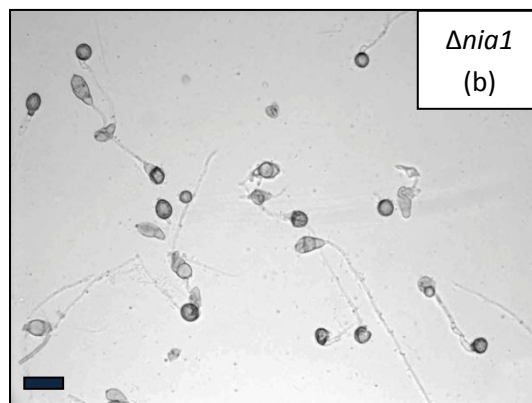
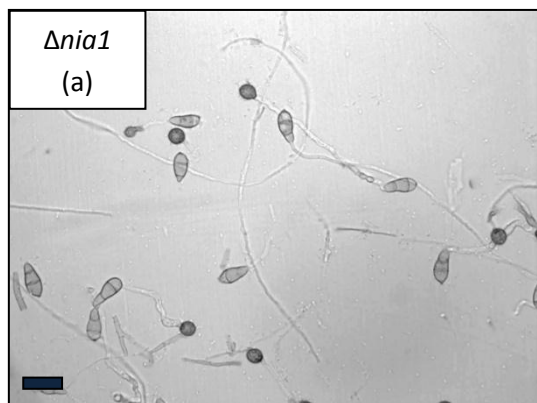
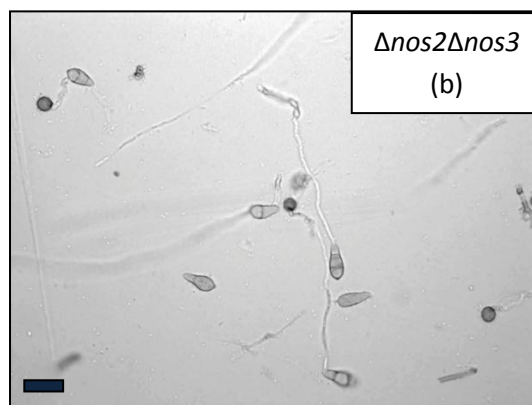
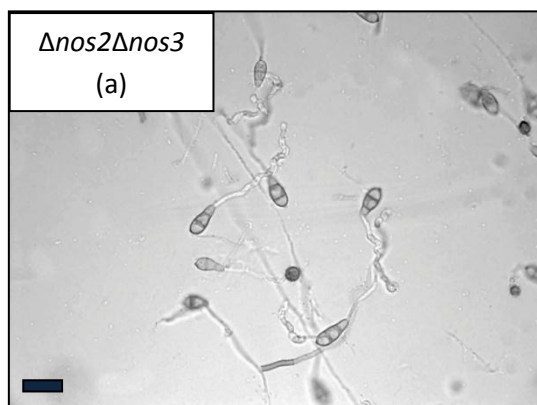


Figure 5.17. The mean increase/decrease in the number of melanised appressoria developed at 12hpi, by Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ germlings after treatment with 10mM HEPES pH7 and 25 μ M NaOH, and 100 μ M DETANONOate (10mM HEPES pH7 and 25 μ M NaOH). Mean numbers of formed MA were calculated from three independent experimental replicates, containing three biological replicates; for each biological replicate 120 germlings were assessed. Error bars represent the standard error of the mean.





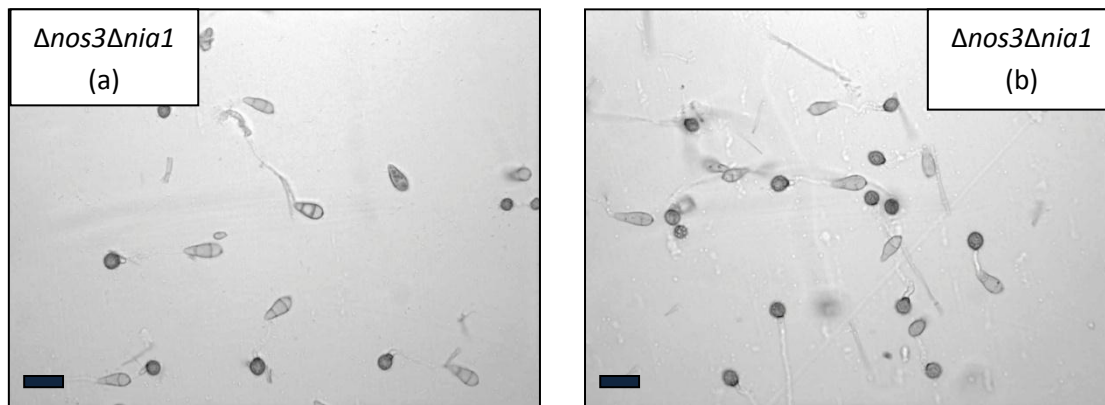


Figure 5.18, The effect of a) 10mM HEPES pH7 and 25μM NaOH and b) 100μM DETANONOate (10mM HEPES pH7 and 25μM NaOH) on wildtype Guy11 and $\Delta ku70$ and mutant $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ germling morphologies at 12hpi, on TAAB plastic coverslips, a surface inductive to MA formation in wildtype strain Guy11. Scale bars represent 20μm.

5.4 Discussion

5.4.1 Conclusions

- $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ exhibit a significantly reduced ability to form melanised appressoria on inductive coverslips relative to Guy11 and $\Delta ku70$.
- Their phenotypes represent elongate appressorium germ tubes and reduced numbers of melanised appressoria.
- $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ are not compromised in their ability to sense hydrophobicity.
- The addition of 200μM PTIO to Guy11 (10mM HEPES pH7) and $\Delta ku70$ significantly reduces their ability to form melanised appressoria and to a lesser extent reduces the levels of melanised appressoria formed by $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$.

- The addition of 200µM PTIO (10mM HEPES pH7) also inhibits the ability of Guy11, *Δku70*, *Δnos2*, *Δnos3*, *Δnos2Δnos3*, *Δnia1*, *Δnii1*, *Δnia1Δnii1* and *Δnos3Δnia1* to germinate.
- The addition of 100µM DETANONOate (10mM HEPES pH7 & 25µM NaOH) is unable to restore a wildtype phenotype to *Δnos2*, *Δnos3*, *Δnos2Δnos3*, *Δnia1*, *Δnii1*, *Δnia1Δnii1* and *Δnos3Δnia1*.

5.4.2 Wildtype and mutant strains morphogenesis on inductive and non-inductive surfaces

The morphology of *Δnos2*, *Δnos3*, *Δnos2Δnos3*, *Δnia1*, *Δnii1*, *Δnia1Δnii1* and *Δnos3Δnia1* germlings on inductive TAAB plastic coverslips demonstrate that they differ from the development of the wildtype strains Guy11 and *Δku70*. Whilst the mutant phenotypes vary, they are characterised by elongated appressorium germ-tubes, which are over twice the length of the wildtype *Δku70*, and their impaired ability to develop MA on inductive artificial surfaces. This ability indicates that *NOS2*, *NOS3*, *NOS2NOS3*, *NIA1*, *NII1*, *NIA1NII1* and *NOS3NIA1* are dispensable for MA formation and may be involved in surface sensing.

Low levels of appressoria formation and elongated appressorium germ tubes may arise from the disruption of surface-sensing signalling pathways. The phenotype has on multiple occasions been reported within the literature. Pth11 was the first GPCR identified in *M. oryzae*; its disruption led to the generation of a mutant whose phenotype mirrored that of wildtype germling on a non-inductive surface (DeZwaan et al. 1999). Similar to the mutant strains described here, *Δpth11* forms 10-15% appressoria on an inductive surface; with the majority of its germlings never developing beyond appressorium germ-tube generation. This indicates Pth11 is not required for appressoria development per se but most probably for surface recognition (DeZwaan et al. 1999). Similarly, the disruption of the adenylate cyclase gene,

MAC1, in *M. oryzae*, results in mutants which exhibit an inability to develop appressoria (Clergeot et al. 2001). cAMP was shown to play an essential role in both the morphogenesis and pathogenicity of *M. oryzae* (Choi & Dean 1997). Treatment of $\Delta mac1$ with cAMP recovered a wildtype phenotype, restoring appressoria formation and pathogenesis to wildtype levels. Furthermore, application of cAMP to wildtype conidia induced appressoria formation on non-inductive surfaces (Choi & Dean 1997). cAMP functions as a signalling molecule which activates cAMP dependent protein kinase A (PKA) through interaction with its regulatory subunit. Mutation of the inhibitory PKA regulatory subunit, coded by *CPKA*, resulted in the continuous activation of CpkA, and was able to restore appressoria formation in the $\Delta mac1$ mutant (Adachi & Hamer 1998).

Disruption of *MAGB* which encodes a GPCR α -subunit, by Liu and Dean (1997), also resulted in mutants unable to form appressoria. The exogenous addition of cAMP to $\Delta magb$ restored appressoria formation, which confirmed that MagB activates a cAMP dependent signalling pathway (Liu & Dean 1997). Generation of a dominant activated *MAGB* allele resulted in a mutant which formed appressoria on both hydrophilic and hydrophobic surfaces, highlighting the role MagB plays in surface sensing (Liu & Dean 1997). More recently Skamnioti & Gurr (2007) reported that following disruption of *CUT2*, mutants form elongate appressorium germ-tubes (~84 μ m) and low levels of MA (<25%) on inductive artificial surfaces (Skamnioti & Gurr 2007). Cut2, like Pth11 is thought to respond to specific surface cues to trigger a signalling cascade. It is hypothesised that these signalling cascades require additional environmental cues, such as hydrophobicity, before a threshold is reached and signal transduction to form MA occurs (Skamnioti & Gurr 2008). Disruption of surface sensing proteins, Cut2 and Pth11, or the signal cascade components, Mac1 and MagB, may prevent the necessary thresholds for signal transduction from being reached, resulting in atypical germling development.

Disruption of the putative NO synthesising genes *NOS2*, *NOS3*, *NIA1* and *NII1* in *M. oryzae*, may have a similar effect, by reducing or abolishing NO generation and therefore preventing

environmental cue thresholds from being overcome. However, as there are multiple putative NO generating genes within *M. oryzae*, the disruption of a single gene may not fully abolish the ability of *M. oryzae* to generate NO. Thus, even if NO is important, single gene replacement mutants may still form appressoria but at a suppressed level relative to the wildtype strains. Potential support for this hypothesis can be seen in Figure 5.2, where $\Delta nos2\Delta nos3$ and $\Delta nos3\Delta nia1$ demonstrated the lowest levels of melanised appressoria. Is this observation the synergistic effect of disrupting two versus one putative NO generating pathways in *M. oryzae*?

The elongated appressorium germ-tube phenotype observed following the inoculation of Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ onto non-inductive glass microscope coverslips confirmed that neither wildtype nor mutant strains were compromised in sensing hydrophobicity. If *NOS2*, *NOS3*, *NIA1* and *NII1* were linked to hydrophobicity surface sensing then a phenotype similar to the dominant activated *MAGB* allele would be observed, i.e. the formation of MA on non-inductive surfaces.

5.4.3 PTIO compromises the wildtype strains ability to form MA

The application of the NO scavenger PTIO to wildtype strains Guy11 and $\Delta ku70$ significantly impaired their ability to form melanised appressoria on inductive TAAB plastic coverslips. This phenotype closely resembles those of $\Delta nos2\Delta nos3$ and $\Delta nos3\Delta nia1$, in which only ~30% of germlings developed melanised appressoria. This observation would suggest the hypothesis that NO is required to induce MA formation in the wildtype strains. NO and its role in fungi appressoria formation has previously been suggested, Prats et al. (2008) observed that appressoria formation in *Blumeria graminis* was suppressed following the application of the mammalian NOS inhibitor L-NAME and the NO scavenger C-PTIO (Prats et al. 2008).

Intriguingly, the application of 200 μ M PTIO to $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ germlings did not totally abolish melanised appressoria formation.

This suggests the existence of an alternative, NO-independent signalling pathway capable of inducing melanised appressoria formation.

PTIO at high concentrations was also observed to inhibit germination in Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. A role for NO in fungal germination has previously been identified in *Colletotrichum coccodes* by Wang & Higgins (2005), they reported that NO negatively regulates *C. coccodes* germination, and that the application of mammalian NOS inhibitors L-NMMA and L-NNA accelerated conidia germination, whilst their inactive stereo isomers D-NMMA and D-NNA failed to do so (Wang & Higgins 2005). These findings are concordant with the role of NO in *M. oryzae* conidia germination found in this study. The conclusions of Wang & Higgins (2005) are based on the assumption that *C. coccodes* contains a mammalian like NOS which is sensitive to inhibition by L-NMMA and L-NNA. The use of PTIO to prevent NO-related activity in a biological system maybe more effective than the application of mammalian NOS inhibitors as PTIO, irrespective of the NO source, will scavenge and remove NO from the systems.

An important area that is frequently overlooked in chemical genetic analyses is environmental pH. Failure to control environmental pH during chemical genetic analyses may potentially result in biased data. Fungi are particularly sensitive to external pH, the role of pH in *Aspergillus*, *Colletotrichum* and *Phomopsis* species has been well documented (Bignell et al. 2005; Miyara et al 2010; Davidzon et al. 2010; Yakoby et al. 2000). The effect of external pH has been shown to impact on *Alternaria* and *Colletotrichum* species pathogenicity through regulation of gene expression. For example, the AaK1 endoglucanase gene in *Alternaria alternata* is significantly upregulated and is linked with maximum fungal virulence in environments with a pH value greater than 6, corresponding with the pH of decaying tissues (Eshel et al. 2002). In contrast, *Colletotrichum coccodes* and *C. gleosporioides* have been demonstrated to alkalinise decaying fruit tissue through the secretion of ammonia. Mutants defective in nitrogen utilisation, $\Delta nit1$ and $\Delta areA$ were suppressed in ammonia secretion and exhibited reduced pathogenesis (Alkan

et al 2008; Miyara et al 2010). It is therefore important to buffer any treatment applied to fungal conidia or germlings, to guarantee that the observed phenotype is pH independent and treatment dependent. Buffering of NOS inhibitors and PTIO is essential due to their acidic properties in aqueous solution. For this reason PTIO was buffered in 10mM HEPES pH7. Unfortunately, the majority of recently published fungal NO papers: Prats et al. (2008), Wang & Higgins (2005), Song et al. (2000), Xu et al. (2011), Golderer et al. (2001) and Li et al. (2010) failed to buffer their treatments in their chemical genetic analyses and do not report the pH of the treatments (Prats et al. 2008; Wang & Higgins 2005; Song et al. 2000; Xu et al. 2011; Golderer et al. 2001; Li et al. 2010).

5.4.4 Exogenous application of NO fails to restore wildtype melanised appressoria formation in the mutant strains

The treatment of $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ conidia with exogenous NO was insufficient to fully recover them to the level of wildtype melanised appressoria. A previous study using non-buffered NO donors SNP and PAPANONOate reported a partial recovery in the phenotype of $\Delta nos3$. The application of 1mM SNP restored the percentage of $\Delta nos3$ germlings forming appressoria to 65% (Mary Iles, PhD thesis 2007). The differences observed between $\Delta nos3$ treated with SNP and DETANONOate treatment may arise from the absence and presence of buffer or the rate of NO delivery.

However, the question, 'why does exogenously supplied NO fail to restore the mutant phenotypes to a wildtype equivalent' remains. With respect to the buffer, DETANONOate spontaneously disassociates, releasing two molecules of NO. At pH 7, NO release from DETANONOate maintains a 57 hour half-life, therefore during the 12 hour test period NO is released at a near-linear rate and germlings are exposed to a continuous background of NO. The level of NO released by DETANONOate resembles NO synthesis by mammalian iNOS, where NO has a cytotoxic rather than signalling role. NO synthesised by mammalian eNOS and nNOS is

associated with NO signalling and is strictly regulated through multiple pathways including intracellular calcium levels, calmodulin binding and protein phosphorylation (Nakane et al. 1991; Komeima et al. 2000). eNOS and nNOS produce small quantities of NO which activate signalling pathways. Therefore, for exogenous NO to recover a wildtype phenotype in the mutant background a small burst of NO rather than a continuous release may be required to activate downstream signalling cascades leading to MA development. This is especially relevant if, as previously suggested, NO is required to overcome environmental cue thresholds (Takahashi et al 2005; Skamnioti & Gurr 2007). In this instance, a specific NO burst would have to be coordinated with other environmental signalling responses, before an MA inducing signalling cascade is activated. This response is extremely unlikely to occur in an environment continuously exposed to NO. In contrast, SNP provides a rapid burst of NO but also produces toxic bi-products (Bisset et al. 1981)

Secondly, NO is a potentially toxic chemical: indeed, the sole purpose of NO synthesised by mammalian iNOS is to inflict nitrosative stress on invading pathogens. The continuous release of NO by DETANONOate may mirror the role of NO synthesised by mammalian iNOS, inflicting nitrosative stress on mutant conidia, which interferes with MA development. Choi & Dean (1997) disrupted the adenylate cyclase encoding gene *MAC1* in *M. oryzae* (Choi & Dean 1997; Adachi K & Hamer JE 1998). The resulting $\Delta mac1$ mutant displayed reduced conidiation, conidia germination, and vegetative growth and was unable to develop appressoria on an inductive surface. Appressoria development was restored through the addition of exogenous cAMP (Wilson & Talbot 2009). Importantly cAMP, unlike NO, is not cytotoxic and its exogenous supply successfully reprieves the $\Delta mac1$ mutants inability to synthesis cAMP without damaging the germings.

Chapter 6: Wildtype and mutant strains pathogenicity and growth

6.1 Introduction

In Chapter 5, the wildtype and mutant strains were assessed for their ability to develop infection related structures on TAAB plastic coverslips inductive to melanised appressoria (MA) formation in the wildtype Guy11. The data presented in this chapter details research investigating the ability of wildtype and mutant strains to infect barley and rice plants and their ability grow in different solid and liquid media.

6.1.1 Pathogenicity assays

To assess the pathogenicity of the wildtype and mutant strains, Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ conidia were aerosol inoculated onto 7 day old barley (*M. oryzae* susceptible cv. Golden Promise) and 21 day old rice (*M. oryzae* susceptible cv. CO39) leaves, (as detailed in Chapter 2, 2.5). After five days incubation, images of infected barley and rice leaves were captured and analysed for lesions using image analysis lesion detection software, written by Dr. Mark Fricker, University of Oxford. Five days was selected as the time point to measure the severity of disease on rice and barley leaves as this represents when lesions are sufficiently well developed as to enable easy detection but have not yet started to merge together hindering detection. The software analyses of disease lesions on barley and rice leaves, generated two measures for the severity of rice blast disease: i), lesion counts, in which the number of individual disease lesions were recorded; ii), mean percentage lesion area, in which the percentage surface area of each rice or barley leaf identified as disease lesion was calculated. These measures of rice blast disease provided a reasonable assessment of the pathogenicity of wildtype and mutant strains.

6.1.2 Nitrogen utilization in *M. oryzae*

The effect of nitrogen starvation has been well-documented in *M. oryzae*, having been demonstrated to induce the transcription of genes responsible for pathogenicity (Talbot et al. 1997). Expression of the putative *NOS* genes in *M. oryzae* may therefore be regulated by environmental nitrogen. Differences in growth between wildtype strains Guy11 and $\Delta ku70$, and mutant strains, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ may provide evidence for a link between environmental sensing and nitrogen metabolism. To test this hypothesis the growth of wildtype and mutant strains was assessed in a variety of environments containing different nitrogen sources.

The application of potassium chlorate to the wildtype and mutant strains was used to test their abilities to assimilate nitrate. Potassium chlorate is reduced by functional nitrate reductase to the toxic chlorite ion, which suppresses fungal growth, disruption of *NAI1* prevents the synthesis of *Nia1*, and therefore mutants which carry this disruption are unable to metabolise chlorate and their growth is not impaired (so long as nitrate isn't the sole nitrogen source) (Wilson et al. 2007; Cove 1976).

The effect of disrupting nitrate assimilation in wildtype and mutant strains was assessed using sodium tungstate. The tungstate ion is preferentially incorporated into *Nia1* over nitrate reductase molybdenum during protein synthesis. The resulting protein is non-functional, and prevents the reduction of nitrate to nitrite, suppressing nitrate assimilation (Lu et al. 2011; Horchani et al. 2010; Wang et al. 2010; Heimer et al. 1969).

6.1.3 Mycelial diameter growth assays

The diameter of mycelial growth of wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ was assessed on a variety of nitrogen sources and chemical inhibitors. Nine separate solid medias were prepared, (as detailed in Chapter 2, 2.1) including: complete medium (CM), minimal media (MN) nitrate salts, MN (non-

nitrate salts), MN (non-nitrate salts) and 10mM sodium nitrate, MN (non-nitrate salts) and 10mM sodium nitrite, MN (non-nitrate salts) and 300mM potassium chlorate, MN (non-nitrate salts) and 10mM ammonium sulphate, MN (non-nitrate salts) and 300mM potassium chlorate and 10mM ammonium sulphate and MN (nitrate salts) and 1mM sodium tungstate. The concentration of chemicals within each media was selected from previously published reports Wilson et al. (2007) and Jiang & Zhang (2002), who had already discerned appropriate concentrations for use with *M. oryzae* (Wilson et al. 2007; Jiang & Zhang 2002).

Conidia from wildtype and mutant cultures were harvested and a 20 μ l (1×10^4) droplet was inoculated into the middle of each agar plate, (as detailed in Chapter 2, 2.2.2). Inoculated plates were incubated at 24°C for 14 days under a 14/10 hour light-dark photoperiod, (as detailed in Chapter 2, 2.6).

Following 14 days incubation the total mycelia (TM) and melanised mycelia (MM) diameter growths were recorded for each strain. These measures, allowed differences in growth between genotypes to be more accurately recorded, compared to using a single growth measurement. TM diameter growth was defined as the maximum distance mycelia extended from the point of conidia inoculation. In normal or non-nitrogen limiting environments, the TM and MM diameter growth values closely aligned. By contrast, in stressed or nitrogen-limiting environments the MM diameter growth was substantially reduced relative to the TM diameter growth, and was characterised by its lack of melanin pigmentation, reduced mycelial density and absence of areal hyphae. Elevated MM diameter growth was associated with unstressed and non-nitrogen limiting conditions; the resulting mycelia was melanised, compactly spaced and exhibited aerial hyphae. Examples of the difference between TM and MM diameter growth are presented in Figure 6.1.

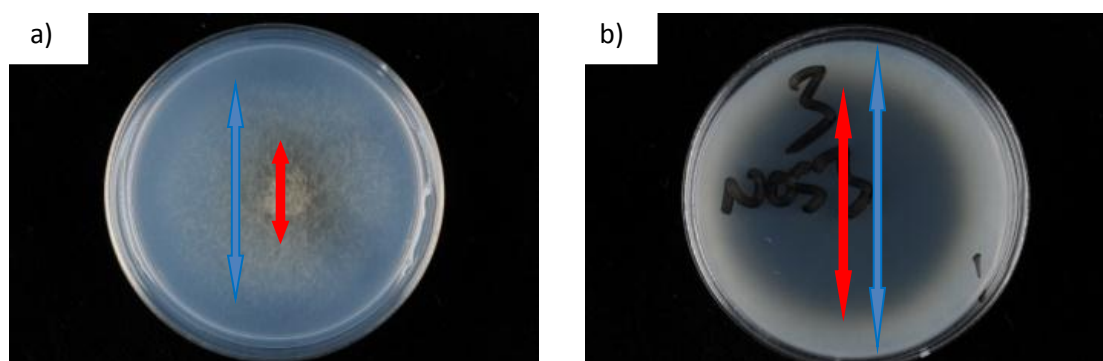


Figure 6.1. Differences in mycelial diameter growth between TM (blue arrow) and MM (red arrow), in 14 day old fungal cultures. Image a) represents a stressed or nitrogen limiting environment with a clear difference between TM and MM diameter growth. Image b) represents an unstressed or non-nitrogen limiting environment, with high TM and MM diameter growth.

6.1.4 Fungal biomass assays

Biomass assays provided an alternative more accurate technique for determining fungal biomass following growth in specific nutrient environments. Unlike growth assays, biomass assays allow the total fungal biomass to be measured, resulting in a more accurate measure of growth. The biomass of wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ were assessed in liquid media containing a variety of nitrogen sources and chemical inhibitors: CM, MN (nitrate salts), MN (non-nitrate salts), MN (non-nitrate salts) and 10mM sodium nitrate, MN (non-nitrate salts) and 10mM sodium nitrite, MN (non-nitrate salts) and 300mM potassium chlorate, MN (non-nitrate salts) and 10mM ammonium sulphate, MN (non-nitrate salts) and 300mM potassium chlorate and 10mM ammonium sulphate and MN (nitrate salts) and 1mM sodium tungstate. Conidia were harvested from 10 day old fungal cultures and a 20 μ l (1×10^4) droplet was inoculated into each liquid media, (as detailed in Chapter 2, 2.2.2). The inoculated media was incubated at 24°C, in darkness, shaking at 150 rpm for 14 days. The fungal biomass was then recorded, (as detailed in Chapter 2, 2.7).

6.2 Experimental aims

- 1) To investigate the pathogenicity of wildtype and mutant strains on barley.
- 2) To investigate the pathogenicity of wildtype and mutant strains on rice.
- 3) To investigate the growth of wildtype and mutant strains on a variety of solid media containing differing nitrogen sources and chemical inhibitors.
- 4) To investigate the growth of wildtype and mutant strains in a variety of liquid media containing differing nitrogen sources and chemical inhibitors.

6.3 Results

6.3.1 Barley pathogenicity assay

Wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ were spray inoculated onto 7 day old barley leaves (cv. Golden Promise) as detailed in Chapter 2, 2.5, Figures 6.2 and 6.4. Image analysis demonstrated no significant difference in the number of disease lesions between wildtype strains Guy11 and $\Delta ku70$, (p-value 0.587). The mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ developed differing numbers of disease lesions on barley leaves. However, there were no significant differences in the number of lesions between the wildtype $\Delta ku70$ and the mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$, (p-values: 0.33, 0.114, 0.511, 0.077, 0.558, 0.076 and 0.054, respectively).

Image analysis, recording the percentage lesion surface area on infected barley leaves revealed no significant difference between wildtype strains Guy11 and $\Delta ku70$, (p-value 0.176), Figures 6.3 and 6.4. Similarly, no significant differences in the percentage surface area of barley leaves classified as disease lesion were observed between $\Delta ku70$ and $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$, (p-values: 0.593, 0.051, 0.928, 0.058, 0.062, 0.202 and 0.655, respectively). No lesions were detected in the 0.2% gelatine control.

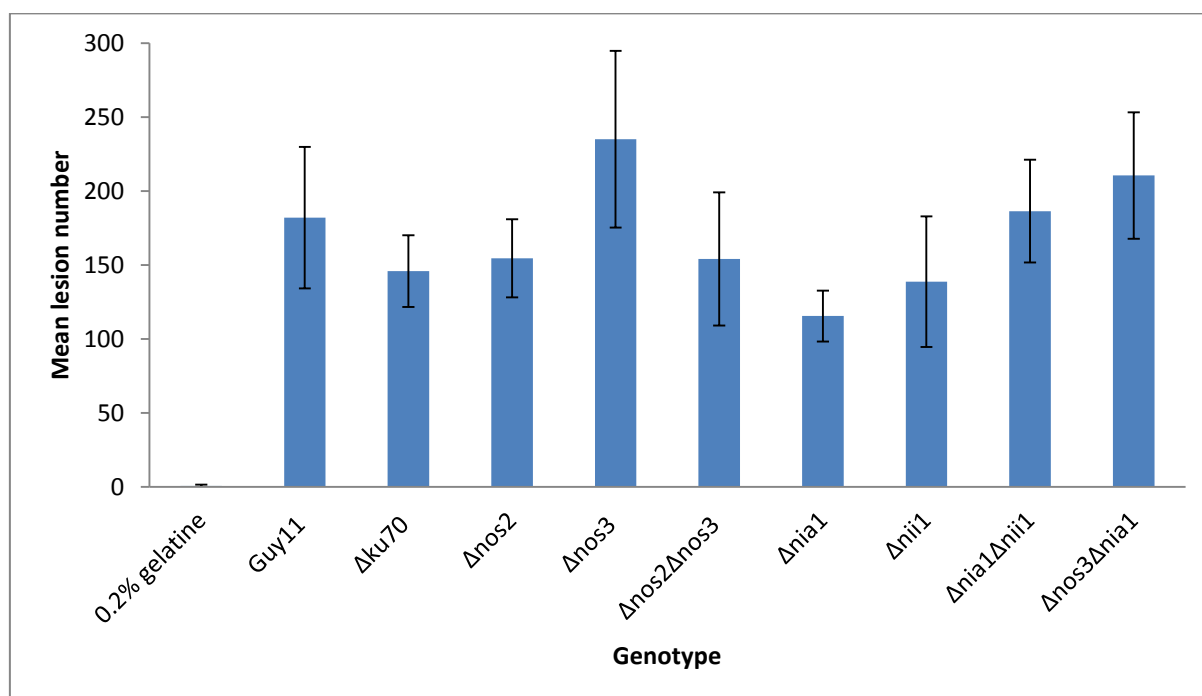


Figure 6.2. The number of disease lesions generated by wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$, $\Delta nos3\Delta nia1$ and gelatine control on 11 day old Barley leaves (cv. Golden Promise). Cut barley leaves were spray inoculated with 2ml 0.2% gelatine, 1×10^4 conidia suspension, and at 5 dpi image analysis software was used to determine the number of lesions per barley leaf. Mean lesion numbers were calculated from six independent experiments each containing ten biological replicates. Error bars represent the standard error of the mean.

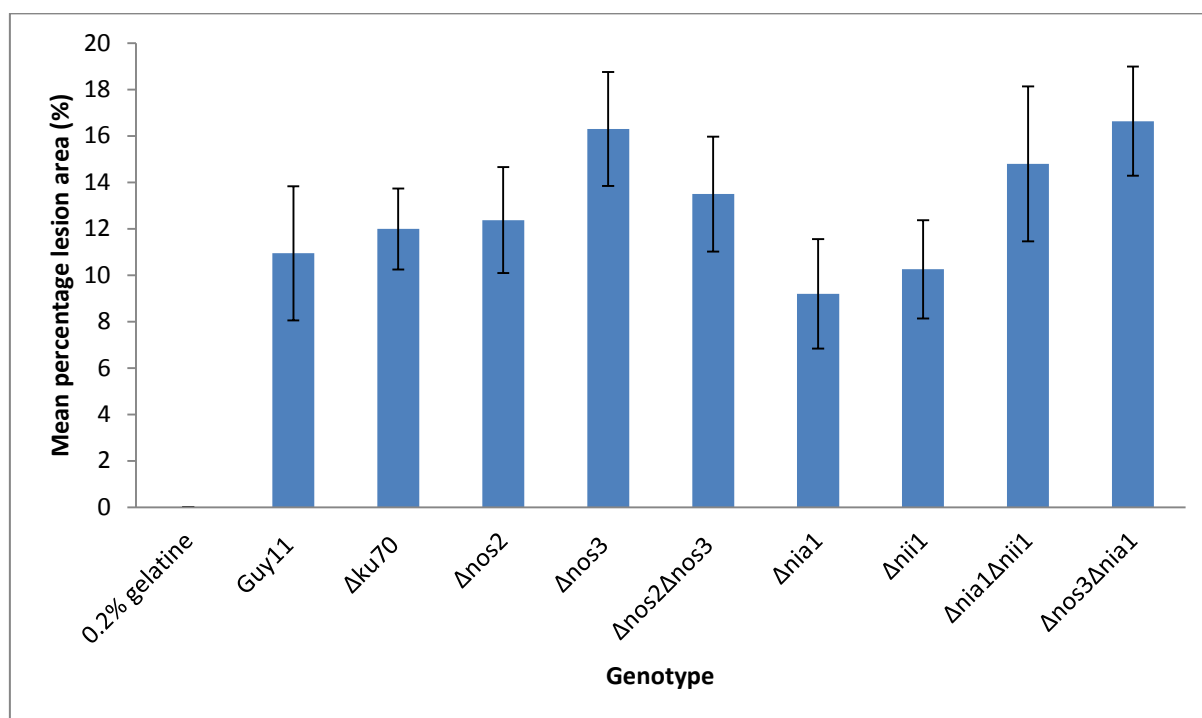


Figure 6.3. The percentage surface area of 11 day old barley leaves (cv. Golden Promise) identified as disease lesion following inoculation with wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$, $\Delta nos3\Delta nia1$ and gelatine control. Cut barley leaves were spray inoculated with 2ml 0.2% gelatine, 1×10^4 conidia suspension, and at 5dpi image analysis software was used to calculate the lesion percentage surface area per barley leaf. The mean lesion percentage surface areas were calculated from six independent experiments each containing ten biological replicates. Error bars represent the standard error of the mean.



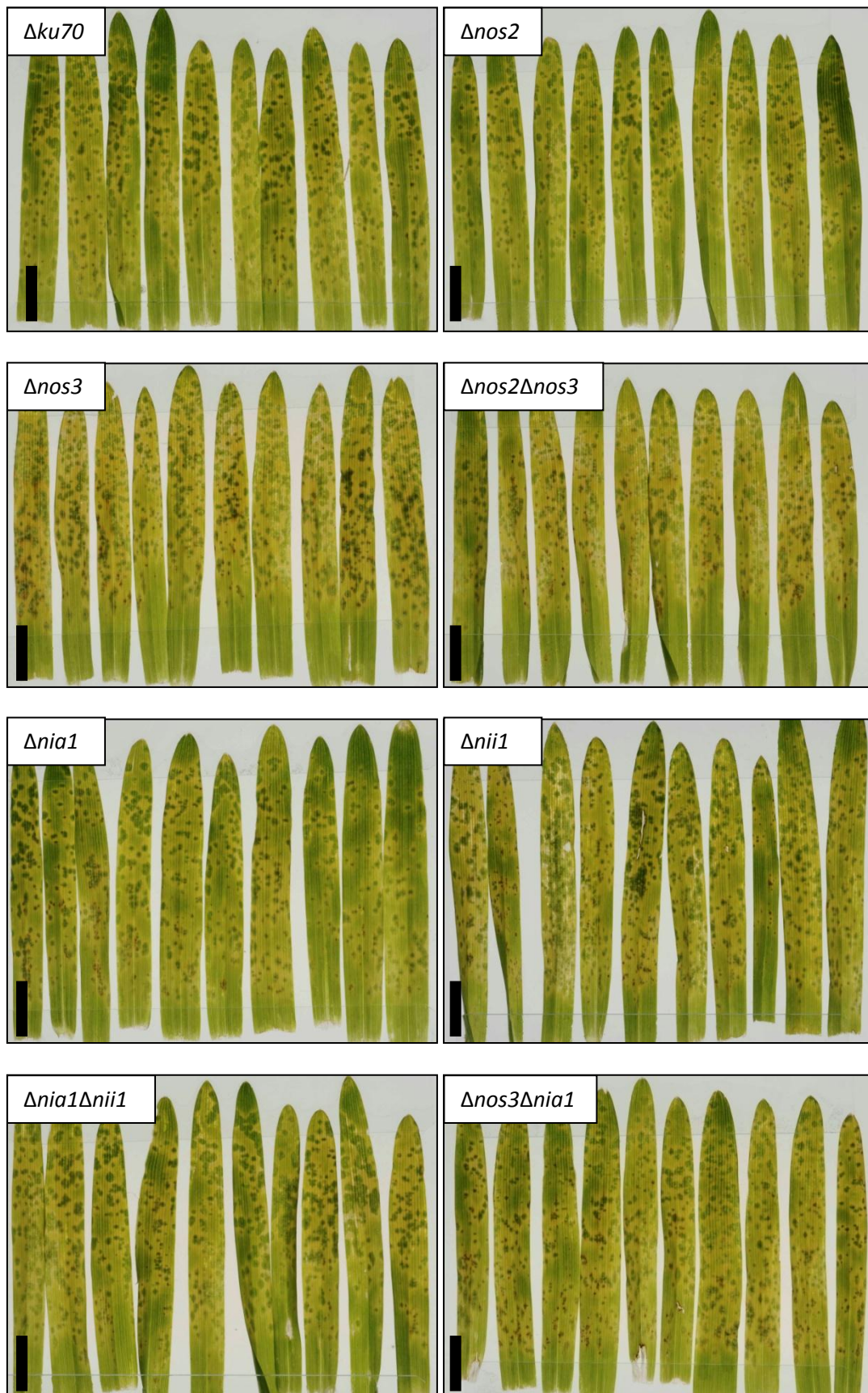


Figure 6.4. Disease lesions, 5 dpi on 11 day old barley leaves (cv. Golden Promise) following inoculation with wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$, $\Delta nos3\Delta nia1$ and gelatine control. Scale bar = 1cm.

6.3.2 Rice pathogenicity assays

Wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ were spray inoculated onto 21 day old rice (cv. CO39) leaves, (as detailed in Chapter 2, 2.5). Image analysis of the rice leaves 5 dpi revealed no significant difference in the number of disease lesions between the wildtype strains Guy11 and $\Delta ku70$, (p-value 0.93), Figures 6.4 and 6.6. Considerable variation in the number of disease lesions generated by the mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ were observed following the inoculation of rice leaves. However, T-test analysis revealed no significant difference between the mutant strains and the wildtype $\Delta ku70$, (p-values: 0.894, 0.794, 0.062, 0.089, 0.425, 0.709 and 0.975, respectively).

Image analysis of the percentage of lesion surface area on infected rice leaves revealed no significant difference between Guy11 and $\Delta ku70$, (p-value 0.328). Equally, no significant differences in the percentage surface area of rice leaves classified as lesion were observed between $\Delta ku70$ and $\Delta nos2$, $\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$, (p-values: 0.111, 0.919, 0.133, 0.895, 0.234 and 0.644, respectively), Figures 6.5 and 6.6. Image analysis revealed a significant reduction in the surface area of $\Delta nos2\Delta nos3$ infected rice leaves classified as lesion relative to rice leaves infected with $\Delta ku70$, (p-value 0.023). No differences in lesion appearance were observed between the wildtype strains and mutant strains.

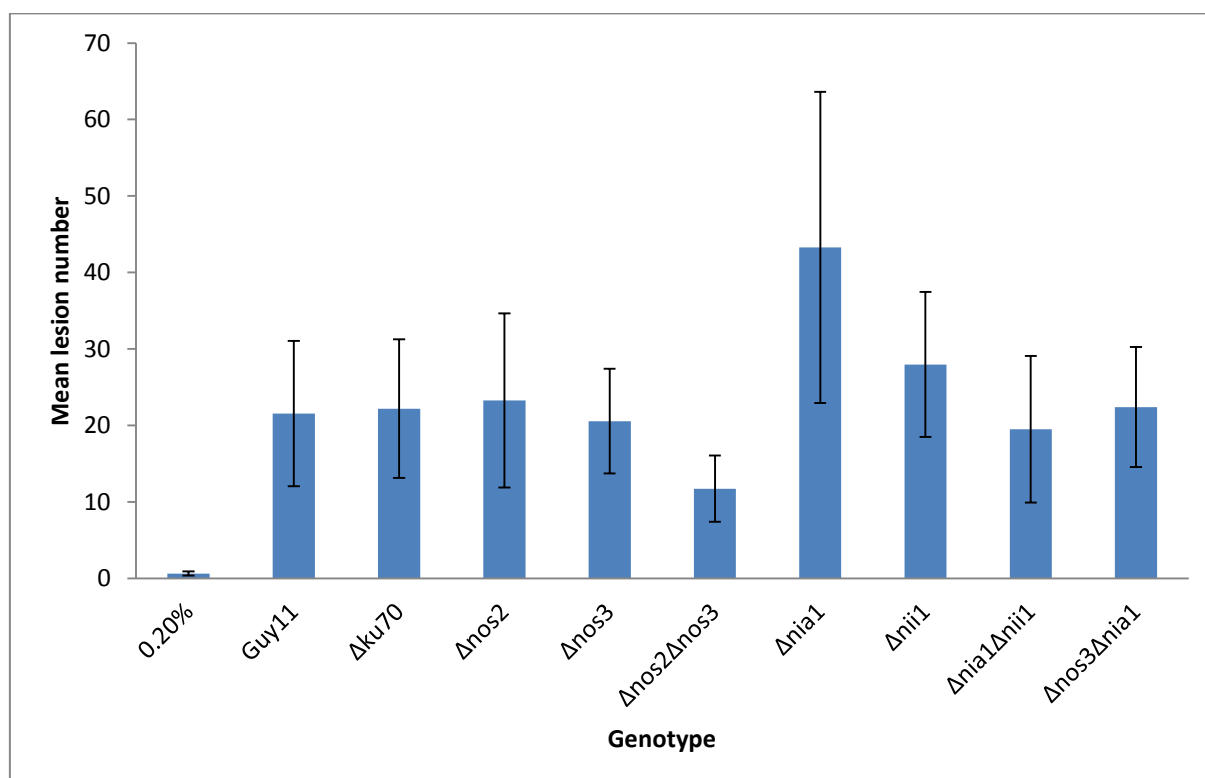


Figure 6.5. The number of disease lesions generated by wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$, $\Delta nos3\Delta nia1$ and gelatine control on 26 day old Rice leaves (cv. CO39). Cut rice leaves were spray inoculated with 2ml 0.2% gelatine, 1×10^4 conidia suspension, and 5 dpi image analysis software was used to determine the number of lesions per rice leaf. Mean lesion numbers were calculated from six independent experiments each containing ten biological replicates. Error bars represent the standard error of the mean.

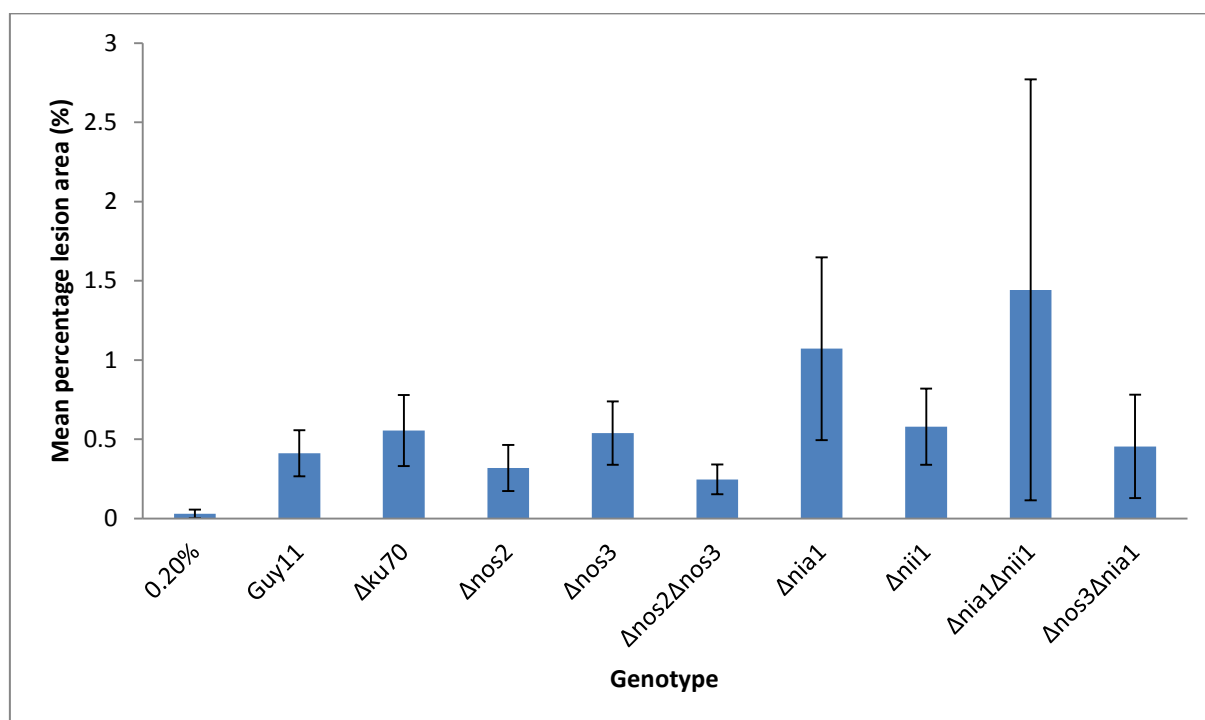
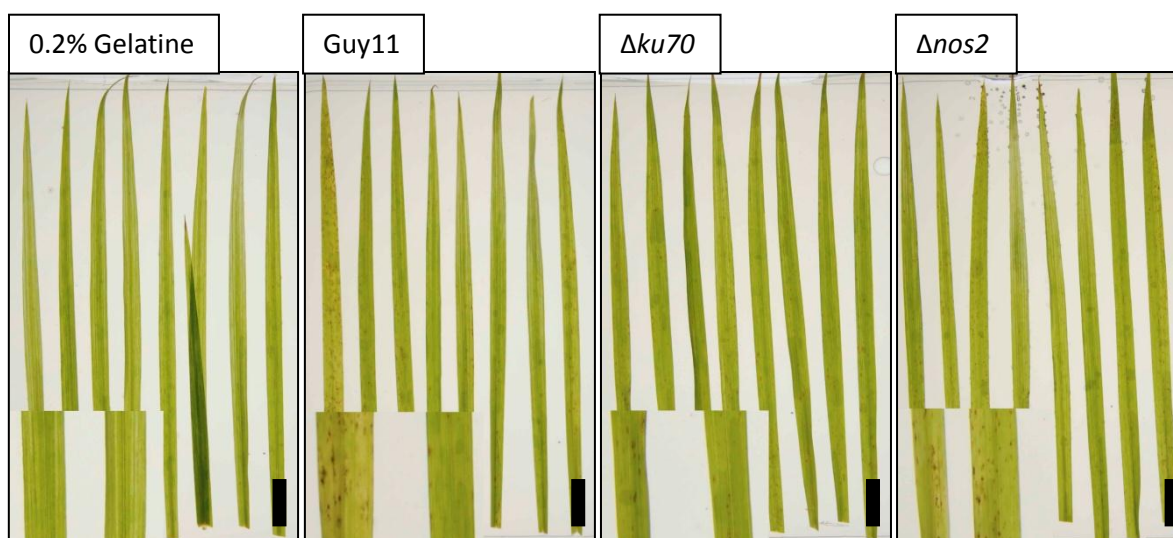


Figure 6.6. The percentage surface area of 26 day old Rice leaves (cv. CO39) identified as disease lesion following inoculation with, wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$, $\Delta nos3\Delta nia1$ and gelatine control. Cut rice leaves were spray inoculated with 2ml 0.2% gelatine, 1×10^4 conidia suspension, and at 5 dpi image analysis software was used to calculate the lesion percentage surface area per barley leaf. The mean lesion percentage surface areas were calculated from six independent experiments each containing ten biological replicates. Error bars represent the standard error of the mean.



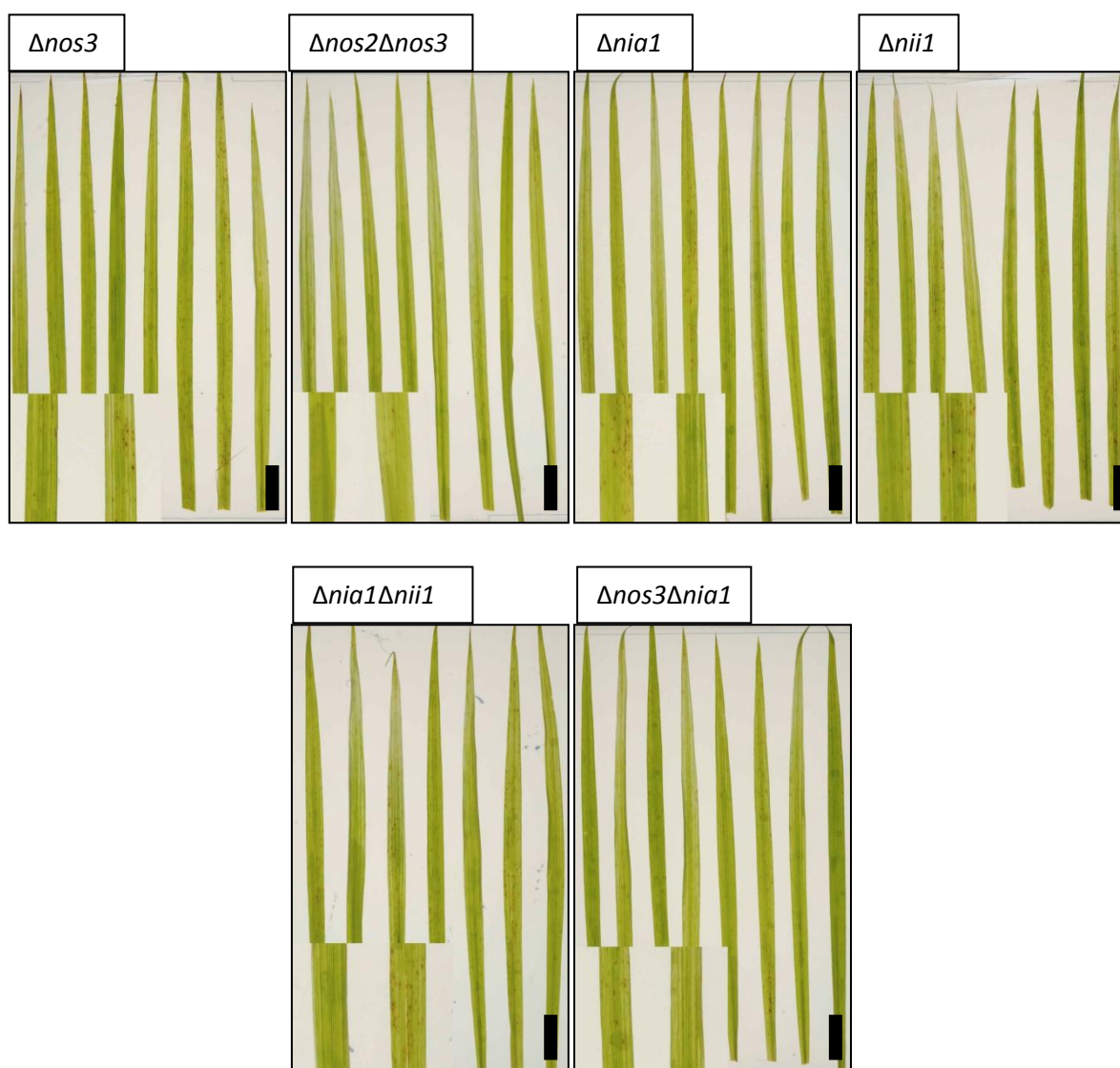


Figure 6.7. Disease lesions, 5 dpi of 26 day old rice leaves (cv. CO39), following inoculation with wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$, $\Delta nos3\Delta nia1$ and gelatine control. Scale bar = 1cm.

6.3.3 Growth assays

6.3.3.1 Wildtype and mutant strains growth on complete medium

The Total Mycelial (TM) and Melanised Mycelial (MM) diameter growth of wildtype and mutant strains were assessed on CM, (Chapter 2, 2.1). CM contains all the required nutrients for *M. oryzae* growth and therefore represents a positive control. All strains demonstrated high levels

of TM and MM diameter growth, Figure 6.7. No significant differences in TM or MM diameter growth were observed between Guy11 and $\Delta ku70$, (p-values: 1 and 0.056, respectively). No significant differences in TM or MM diameter growth were observed between $\Delta ku70$ and $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ (TM p-values: 1, 1, 0.155, 0.191, 0.34, 0.121 and 1, MM p-values: 0.327, 0.919, 0.09, 0.492, 0.848, 0.389 and 0.183, respectively.) Images of each strain following 14 days growth on CM are presented in Figure 6.8. All strains grow well on CM, consistent to it containing all the nutrients required by *M. oryzae*.

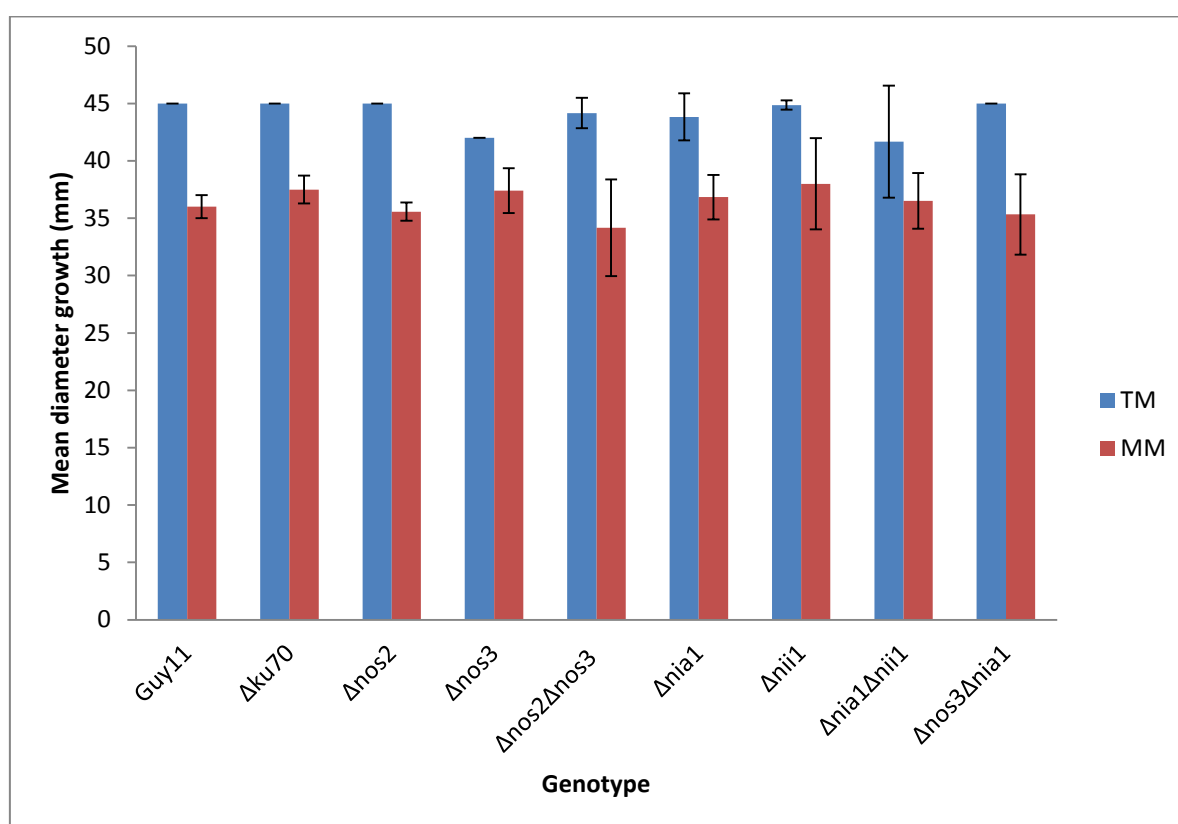


Figure 6.8. The impact of complete medium (CM) on total mycelia (TM) and melanised mycelia (MM) diameter growth of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated CM plates were incubated at 24°C under a 14/10 hour light/dark photoperiod for 14 days, following which, TM and MM diameter growths were recorded. Mean diameter growths were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.



Figure 6.9. Wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ following 14 days growth on CM, incubated at 24°C.

6.3.3.2 Wildtype and mutant strains growth on minimal media (nitrate salts)

Wildtype and mutant strains TM and MM diameter growths were assessed on Minimal Media (MN) containing nitrate salts as the sole nitrogen source (Chapter 2, 2.1). MN contains all the carbon sources required by *M. oryzae* but no nitrogen sources, here nitrogen is supplied as nitrate salts. No significant difference in TM or MM diameter growth was identified between Guy11 and $\Delta ku70$, (p-values: 0.299 and 0.186, respectively), Figure 6.9. Similarly, no significant differences in TM growth were observed between $\Delta ku70$ and $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$, (p-values: 0.181, 0.4, 0.253, 0.876, 0.513, 0.09 and 0.54, respectively). No significant differences in MM diameter growth were observed between $\Delta ku70$

and $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, (p-values: 0.382, 0.497 and 0.209, respectively). However, a significant reduction in MM diameter growth was identified between $\Delta ku70$ and $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$, (p-values: 4.57×10^{-4} , 1.16×10^{-5} , 1.18×10^{-4} and 2.02×10^{-4} , respectively). Images of each strain following 14 days growth on MN nitrate salts are presented in Figure 6.10. Guy11 and $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$ all grow well on MN containing nitrate salts, they carry a functional *NIA1* gene and can therefore metabolise nitrate. $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ carry disrupted nitrate assimilation pathways and therefore cannot metabolise nitrate, hence the reduced growth observed.

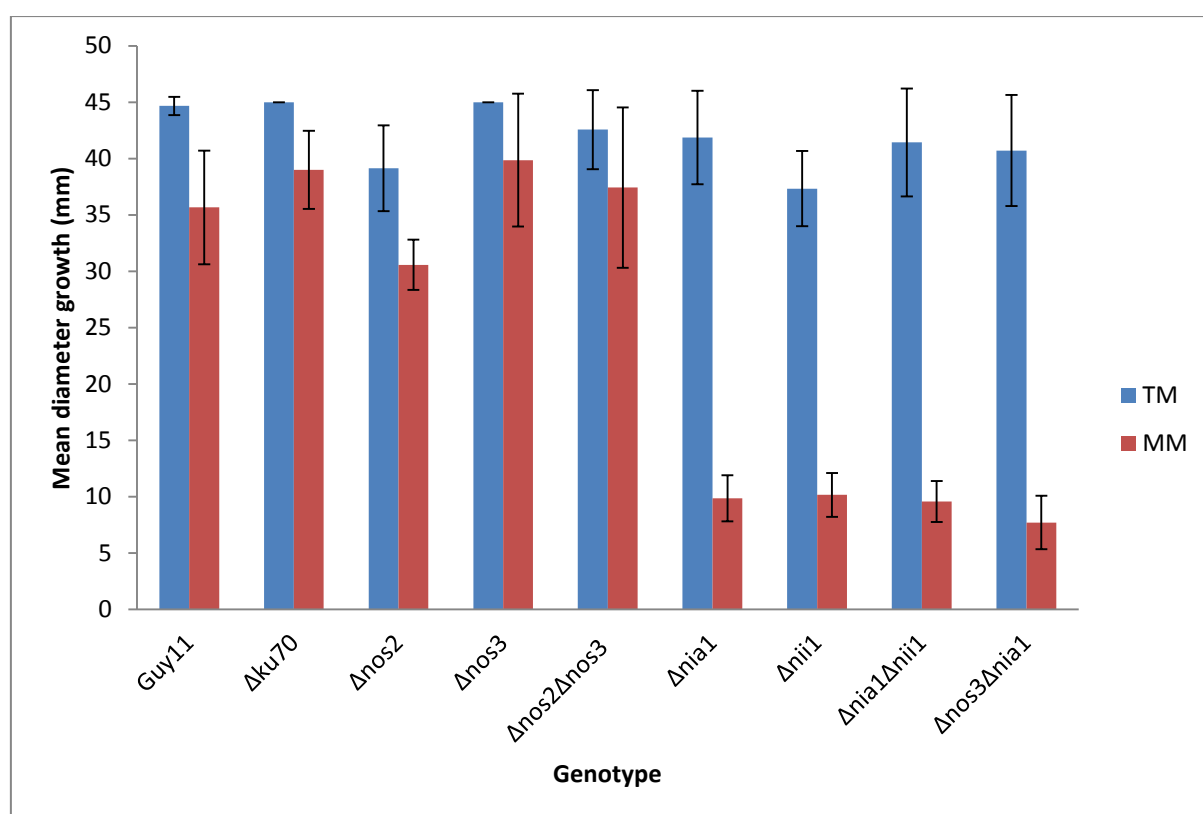


Figure 6.10. The impact of minimal media (MN) containing nitrate salts on TM and MM diameter growth of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated MN plates were incubated at 24°C under a 14/10 hour light/dark photoperiod for 14 days, following which, TM and MM diameter growths were recorded. Mean diameter growths were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.

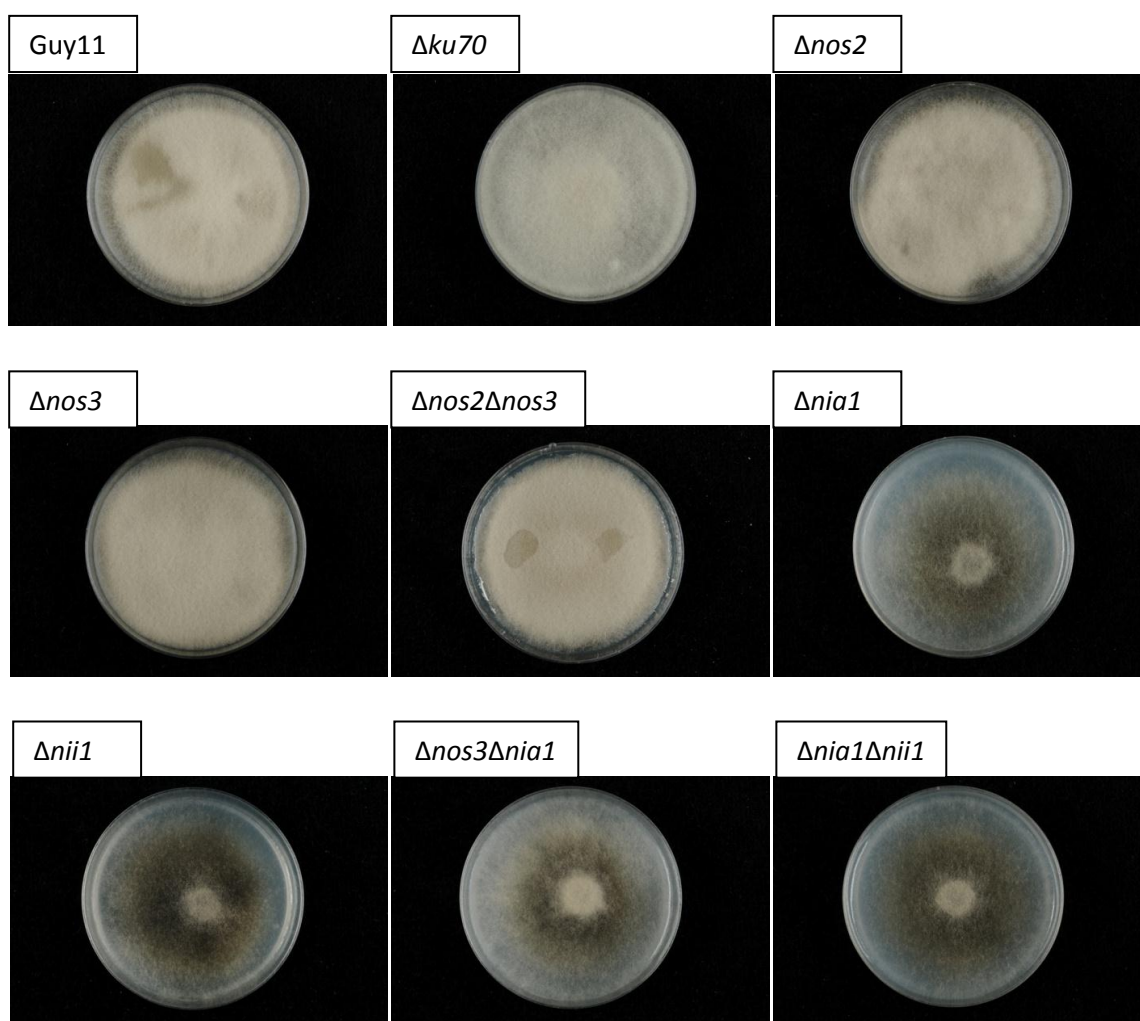


Figure 6.11. Wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ following 14 days growth on MN containing nitrate salts, incubated at 24°C.

6.3.3.3 Wildtype and mutant strains growth on minimal media (non-nitrate salts)

Wildtype and mutant strains TM and MM diameter growths were assessed on MN (non-nitrate salts), containing no additional nitrogen sources (Chapter 2, 2.1). MN with no supplemented nitrogen sources is a disparate environment for *M. oryzae* to grow and represents the negative control. No significant differences in TM or MM diameter growth were observed between wildtype strains Guy11 and $\Delta ku70$, (p-values: 0.377 and 0.801) or between $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$, (TM p-values: 0.15,

0.337, 0.337, 0.393, 0.114, 0.296 and 0.391, MM p-values: 0.287, 0.783, 0.645, 0.152, 0.187, 0.689 and 0.857, respectively), Figure 6.11. Images of each strain following 14 days growth on MN (non-nitrate salts) are presented in Figure 6.12. All strains grow poorly on MN (non-nitrate salts) as there is no available nitrogen for the fungi to metabolise.

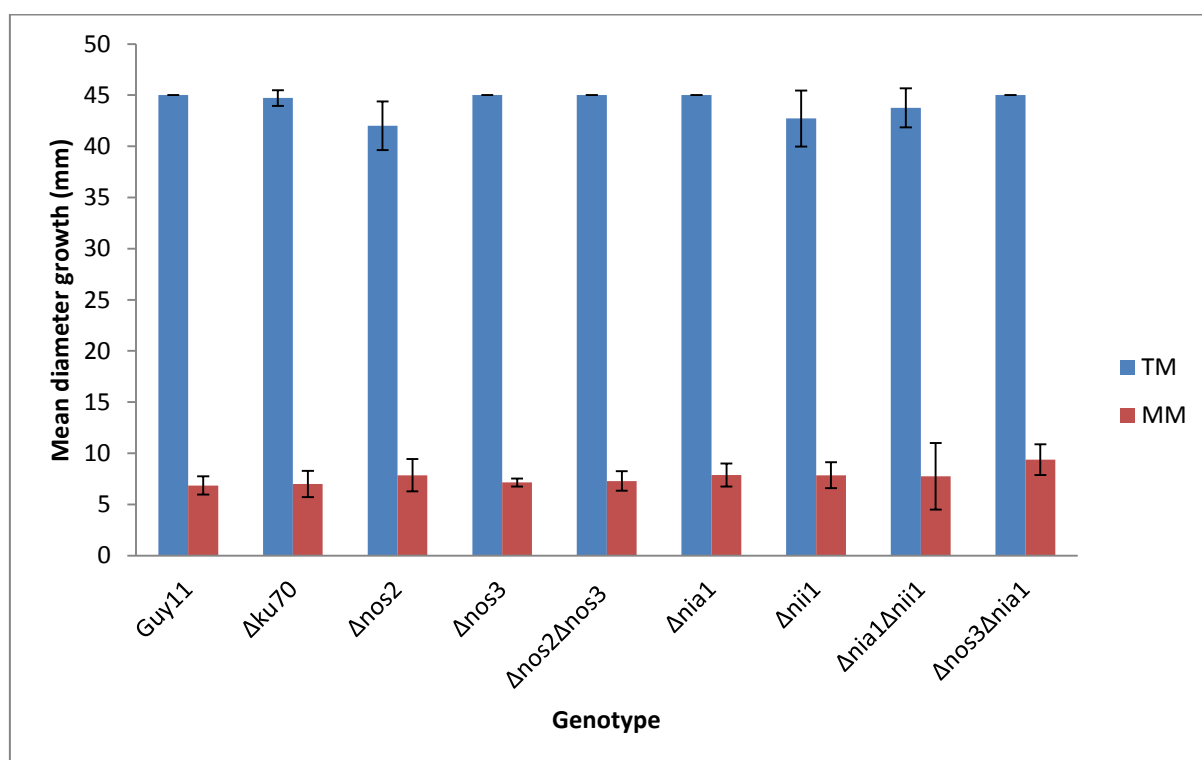


Figure 6.12. The impact of MN (non-nitrate salts) on TM and MM diameter growth of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated MN plates were incubated at 24°C under a 14/10 hour light/dark photoperiod for 14 days, following which, TM and MM diameter growths were recorded. Mean diameter growths were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.



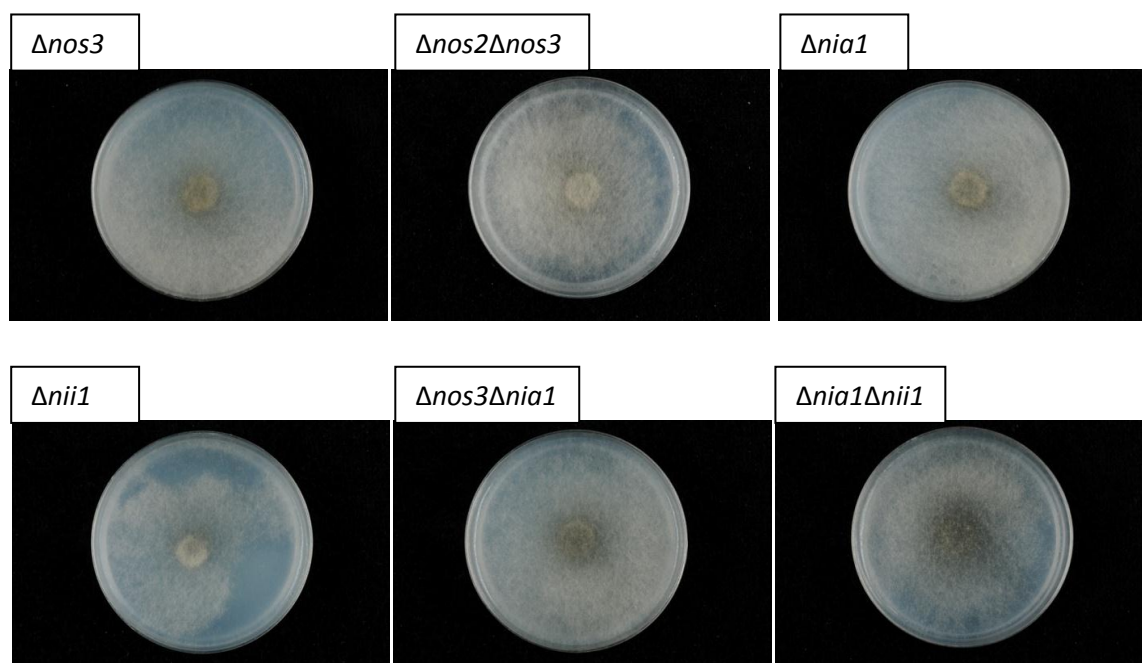


Figure 6.13. Wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ following 14 days growth on MN (non-nitrate salts), incubated at 24°C.

6.3.3.4 Wildtype and mutant strains growth on minimal media (non-nitrate salts) and 10mM sodium nitrate

Wildtype and mutant strains TM and MM growth were assessed on MN (non-nitrate salts) supplemented with 10mM sodium nitrate to test the mutant strains ability to metabolise nitrate (Chapter 2, 2.1). No significant differences in TM or MM were observed between wildtype strains Guy11 and $\Delta ku70$, (p-values: 0.072 and 0.271, respectively), Figure 6.13. No significant differences in TM or MM diameter growth were recorded between $\Delta ku70$ and $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, (TM p-values: 0.303, 0.646 and 0.672, MM p-values: 0.18, 0.41 and 0.668, respectively). The mutant strains $\Delta nia1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ demonstrated significantly increased TM diameter growth compared to $\Delta ku70$, (p-values: 5.71×10^{-4} , 0.013 and 0.021, respectively), but significantly reduced MM diameter growth, (p-values: 1.46×10^{-5} , 9.5×10^{-7} and 3.43×10^{-6} , respectively). No significant difference in TM diameter growth was

observed between $\Delta ku70$ and $\Delta nii1$, (p-value 0.245), mirroring $\Delta nia1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$; $\Delta nii1$ demonstrated a significant reduction in MM diameter growth relative to $\Delta ku70$, (p-value 2.03×10^{-8}). Images of each strain following 14 days growth on MN (non-nitrate salts) supplemented with 10mM sodium nitrate are presented in Figure 6.14. Guy11 and $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$ all grow well on MN containing non-nitrate salts and 10mM sodium nitrate, they carry a functional *NIA1* gene and can therefore metabolise nitrate. $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ carry disrupted nitrate assimilation pathways and therefore cannot metabolise nitrate, hence the reduced growth observed.

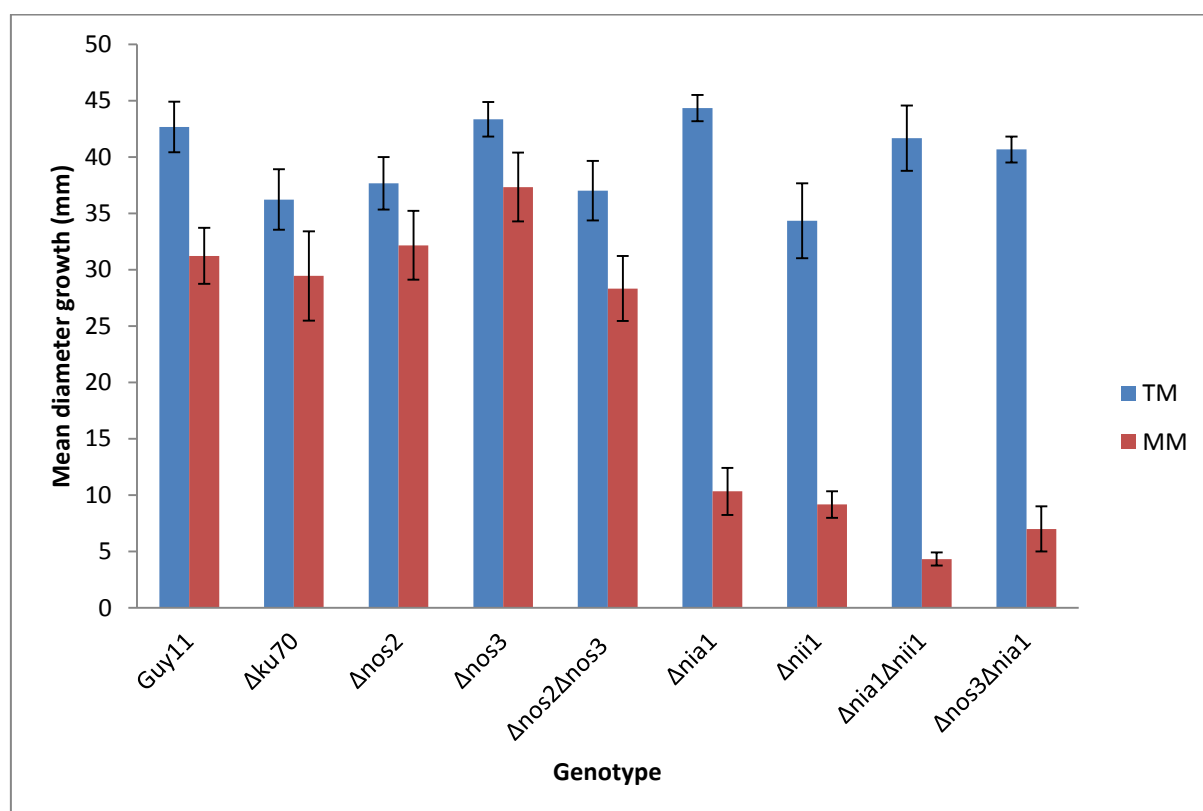


Figure 6.14. The impact of MN (non-nitrate salts) supplemented with 10mM sodium nitrate on TM and MM diameter growth of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated MN plates were incubated at 24°C under a 14/10 hour light/dark photoperiod for 14 days, following which, TM and MM diameter growths were recorded. Mean diameter growths were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.

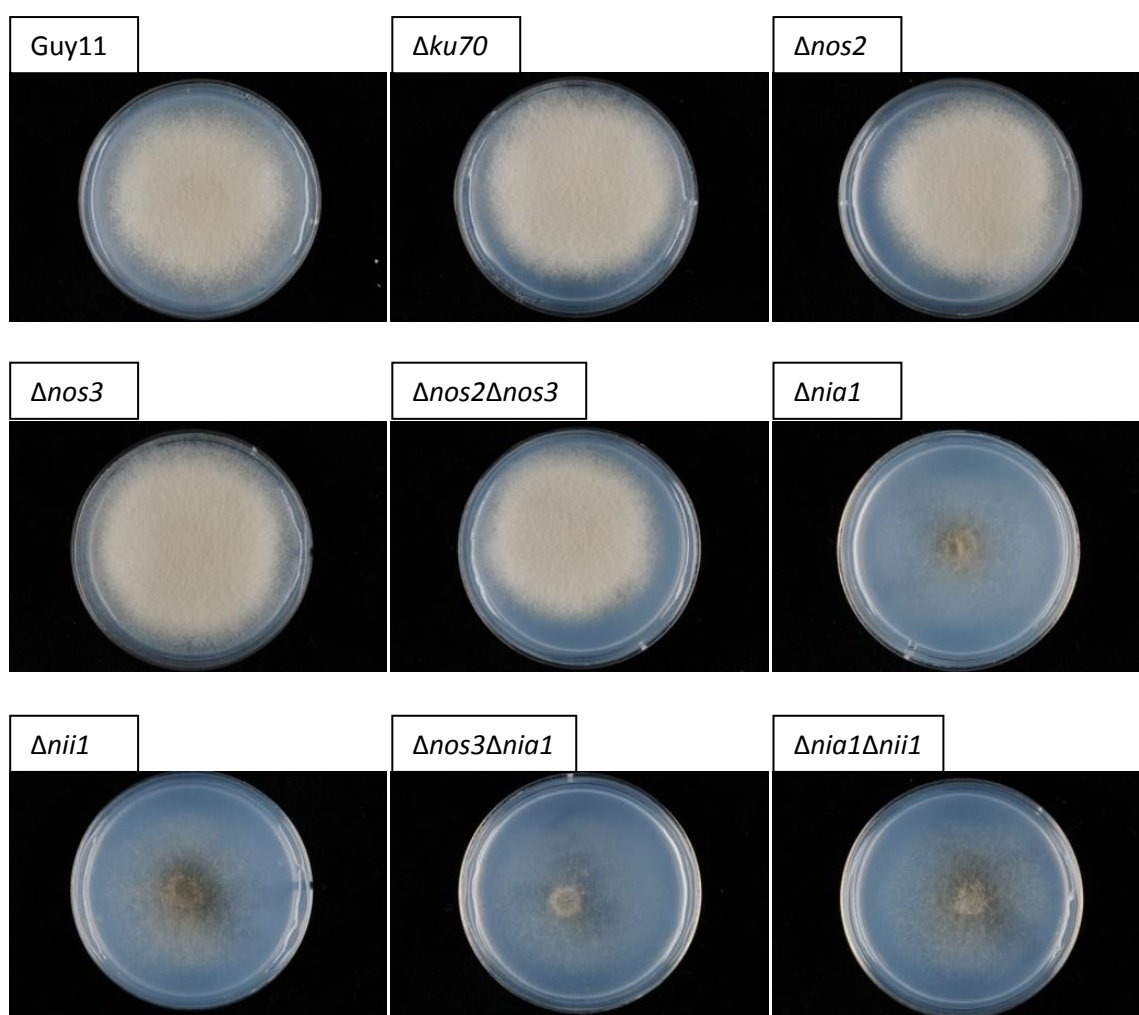


Figure 6.15. Wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ following 14 days growth on MN (non-nitrate salts) supplemented with 10mM sodium nitrate, incubated at 24°C.

6.3.3.5 Wildtype and mutant strains growth on minimal media (non-nitrate salts) and 10mM sodium nitrite

Wildtype and mutant strains TM and MM growth were assessed on MN (non-nitrate salts) supplemented with 10mM sodium nitrite, to assess the mutant strains ability to metabolise nitrite (Chapter 2, 2.1). A significant reduction in TM and MM diameter growths were observed between wildtype strains Guy11 and $\Delta ku70$, (p-values: 6.0×10^{-4} and 0.026, respectively), Figure 6.15. By contrast, no significant differences in TM growth were observed between $\Delta ku70$ and

Δnos2, *Δnos3*, *Δnos2Δnos3*, *Δnia1* and *Δnos3Δnia1*, (p-values: 0.938, 0.065, 0.146, 0.144 and 0.158). *Δnii1* demonstrated a significant reduction in TM diameter growth relative to *Δku70*, whilst *Δnia1Δnii1* exhibited significantly increased TM diameter growth, (p-values: 1.18×10^{-5} and 0.011, respectively). No significant differences in MM diameter growth were observed between *Δku70* and *Δnos2*, *Δnos3* and *Δnos2Δnos3*, (p-values: 0.236, 0.41 and 0.663, respectively). However, a significant reduction in MM diameter growth was observed between *Δku70* and *Δnia1*, *Δnii1*, *Δnia1Δnii1* and *Δnos3Δnia1*, (p-values: 4.48×10^{-4} , 1.93×10^{-7} , 1.14×10^{-5} and 0.002, respectively). Images of each strain following 14 days growth on MN (non-nitrate salts) supplemented with 10mM sodium nitrite are presented in Figure 6.16. Guy11 and *Δku70*, *Δnos2*, *Δnos3*, *Δnos2Δnos3* all grow well on MN containing non-nitrate salts and 10mM sodium nitrite, they carry a functional *NI11* gene and can therefore metabolise nitrate. *Δnii1*, *Δnia1Δnii1* and *Δnos3Δnia1* carry disrupted nitrate assimilation pathways and therefore cannot metabolise nitrate, hence the reduced growth observed. *Δnia1* carries a functional *NI11* gene yet is unable to metabolise nitrite.

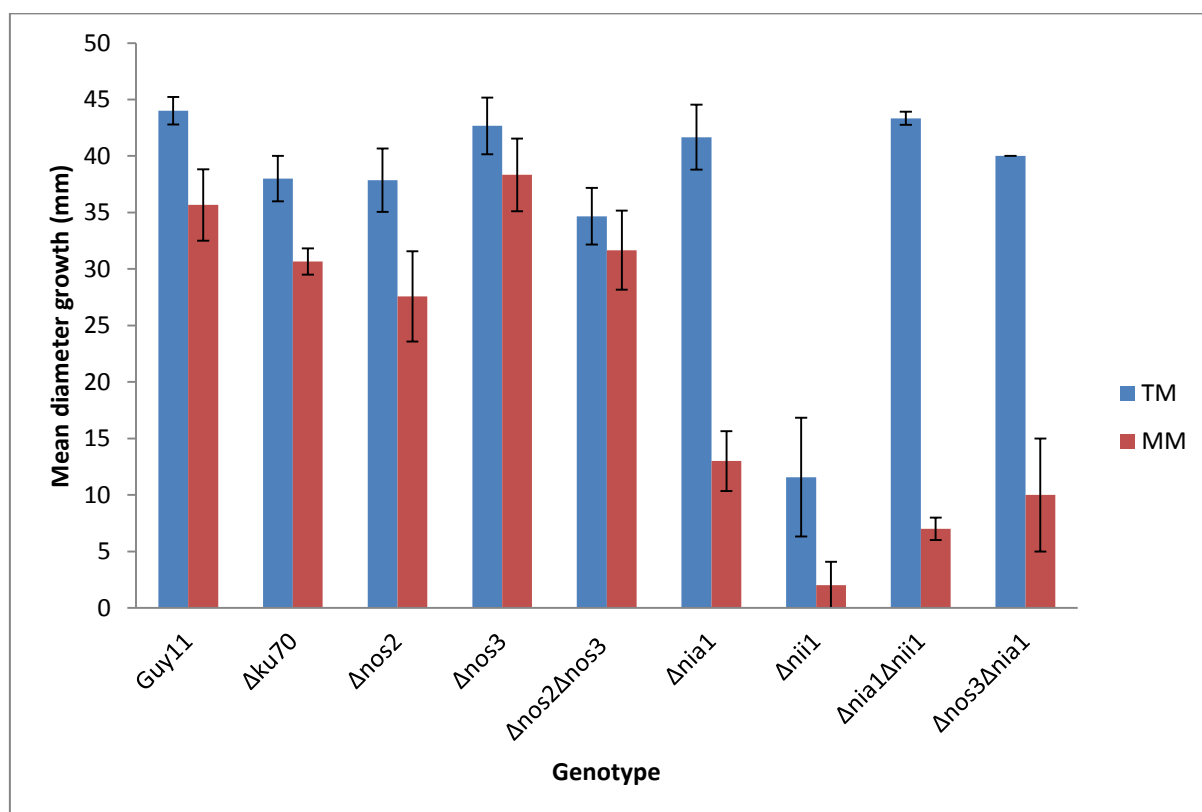


Figure 6.16. The impact of MN (non-nitrate salts) supplemented with 10mM sodium nitrite on TM and MM diameter growth of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated MN plates were incubated at 24°C under a 14/10 hour light/dark photoperiod for 14 days, following which, TM and MM diameter growths were recorded. Mean diameter growths were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.

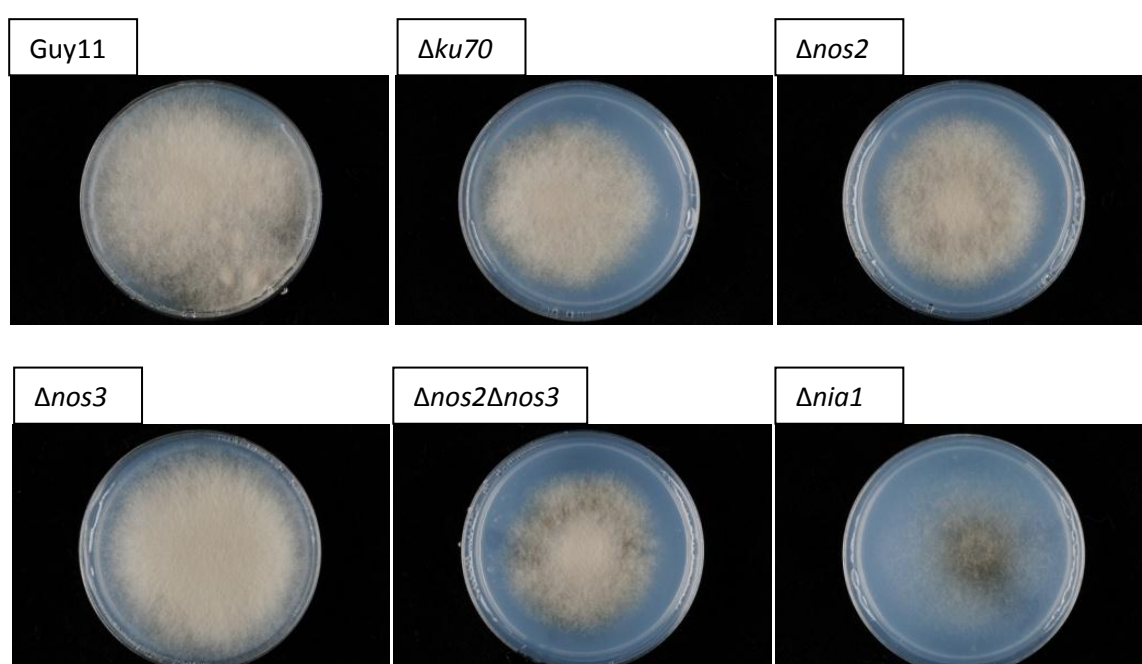




Figure 6.17. Wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ following 14 days growth on MN (non-nitrate salts) supplemented with 10mM sodium nitrite, incubated at 24°C.

6.3.3.6 Wildtype and mutant strains growth on minimal media (non-nitrate salts) and 10mM ammonium sulphate

The TM and MM diameter growth of wildtype and mutant strains were assessed on MN (non-nitrate salts) supplemented with 10mM ammonium sulphate, to assess the ability of the mutant strains to metabolise ammonium. No significant differences in TM or MM diameter growth were observed between Guy11 and $\Delta ku70$, (p-values: 0.632 and 0.632, respectively), or between $\Delta ku70$ and $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ (TM p-values: 0.821, 0.794, 0.251, 0.279, 0.138, 0.348 and 0.0871, MM p-values: 0.821, 0.794, 0.251, 0.263, 0.138, 0.222 and 0.087, respectively), Figure 6.17. Images of each strain following 14 days growth on MN (non-nitrate salts) supplemented with 10mM ammonium sulphate are presented in Figure 6.18. All strains grow well on MN containing non-nitrate salts and 10mM ammonium.

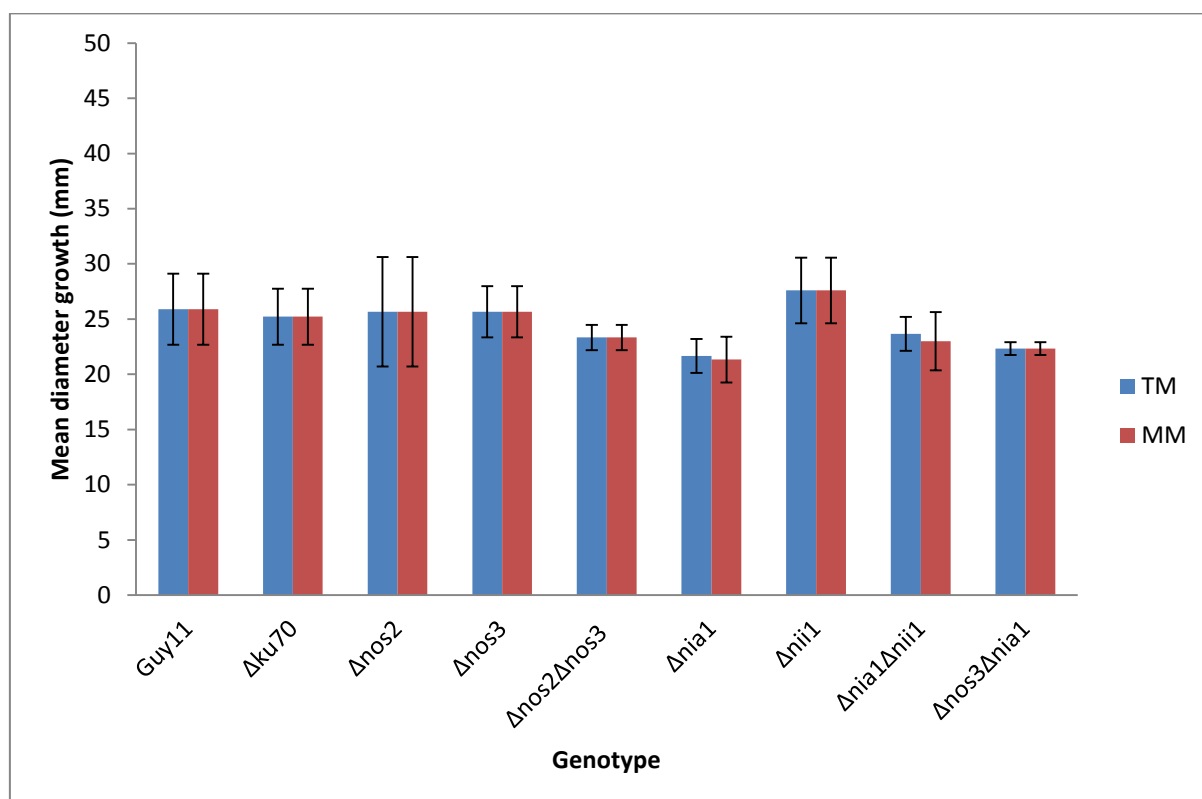


Figure 6.18. The impact of MN (non-nitrate salts) supplemented with 10mM ammonium sulphate on TM and MM diameter growth of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated MN plates were incubated at 24°C under a 14/10 hour light/dark photoperiod for 14 days, following which, TM and MM diameter growths were recorded. Mean diameter growths were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.

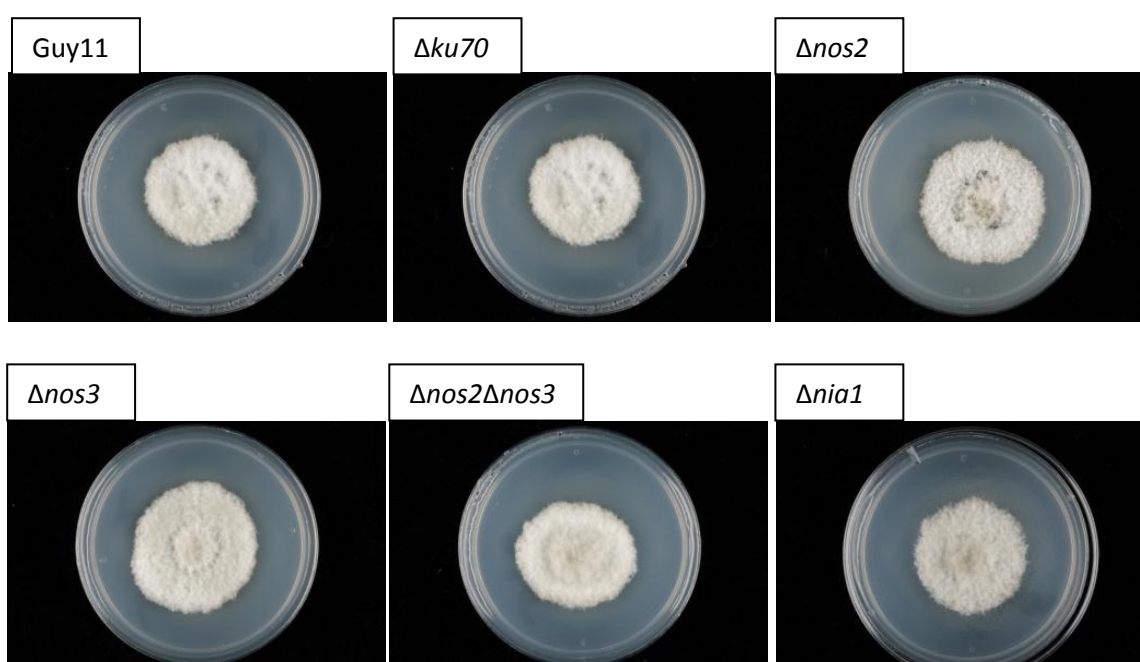




Figure 6.19. Wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ following 14 days growth on MN (non-nitrate salts) supplemented with 10mM ammonium sulphate, incubated at 24°C.

6.3.3.7 Wildtype and mutant strains growth on minimal media (nitrate salts) and 300mM potassium chlorate

Wildtype and mutant strains TM and MM were assessed on MN (nitrate salts) supplemented with 300mM potassium chlorate, to test whether the mutant strains carry a functional nitrate reductase protein. No statistical differences in TM or MM diameter growth were observed between wildtype strains Guy11 and $\Delta ku70$, (p-values: 0.108 and 0.34, respectively), Figure 6.19. No significant differences in TM or MM diameter growth were recorded between $\Delta ku70$ and $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nii1$ and $\Delta nia1\Delta nii1$, (TM p-values: 0.543, 0.111, 0.272, 0.767 and 0.111, MM p-values: 0.446, 0.299, 0.299, 0.446 and 0.299, respectively). $\Delta nia1$ and $\Delta nos3\Delta nia1$ demonstrated significantly increased TM and MM growths relative to $\Delta ku70$, (TM p-values: 1.33×10^{-4} and 1.74×10^{-9} , MM p-values: 3.97×10^{-10} and 5.73×10^{-8} , respectively). Images of each strain following 14 days growth on MN (non-nitrate salts) supplemented with 300mM potassium chlorate are presented in Figure 6.20. All strains with the exception of $\Delta nia1$ and $\Delta nos3\Delta nia1$ exhibit limited levels of growth.

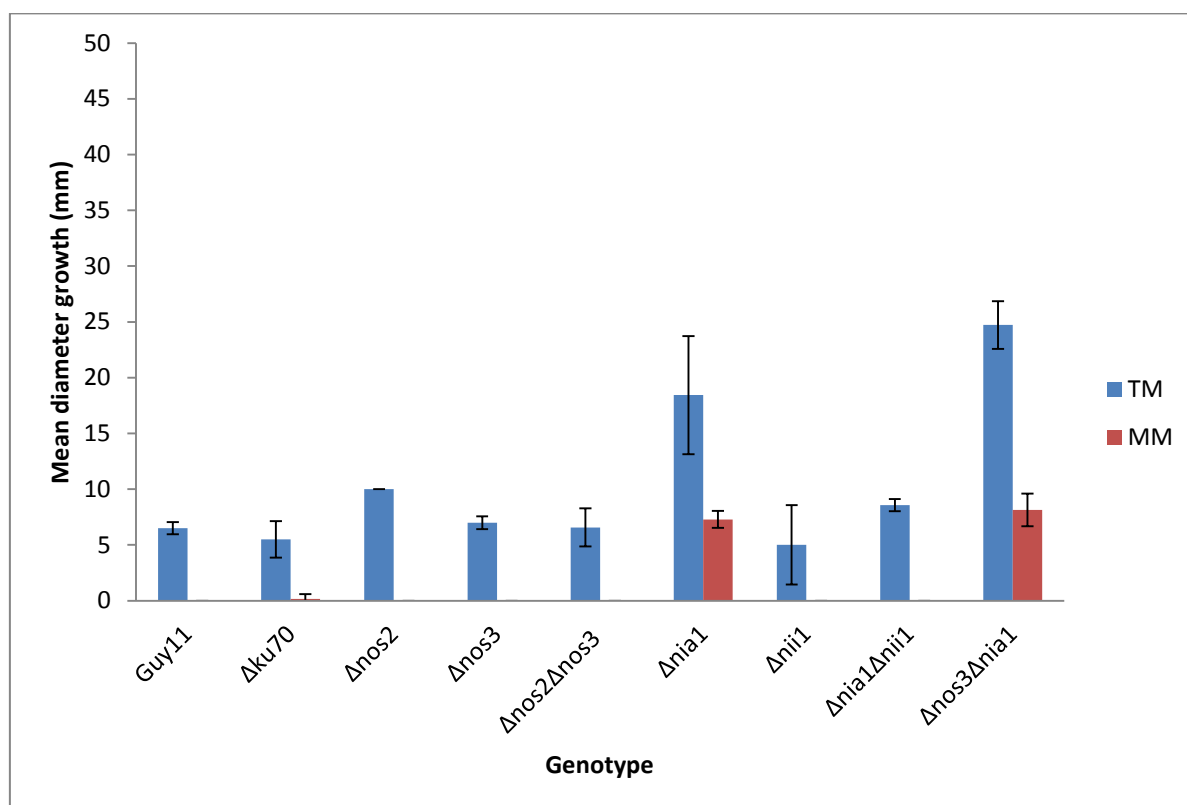


Figure 6.20. The impact of MN (nitrate salts) supplemented with 300mM potassium chlorate on TM and MM diameter growth of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated MN plates were incubated at 24°C under a 14/10 hour light/dark photoperiod for 14 days, following which, TM and MM diameter growths were recorded. Mean diameter growths were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.

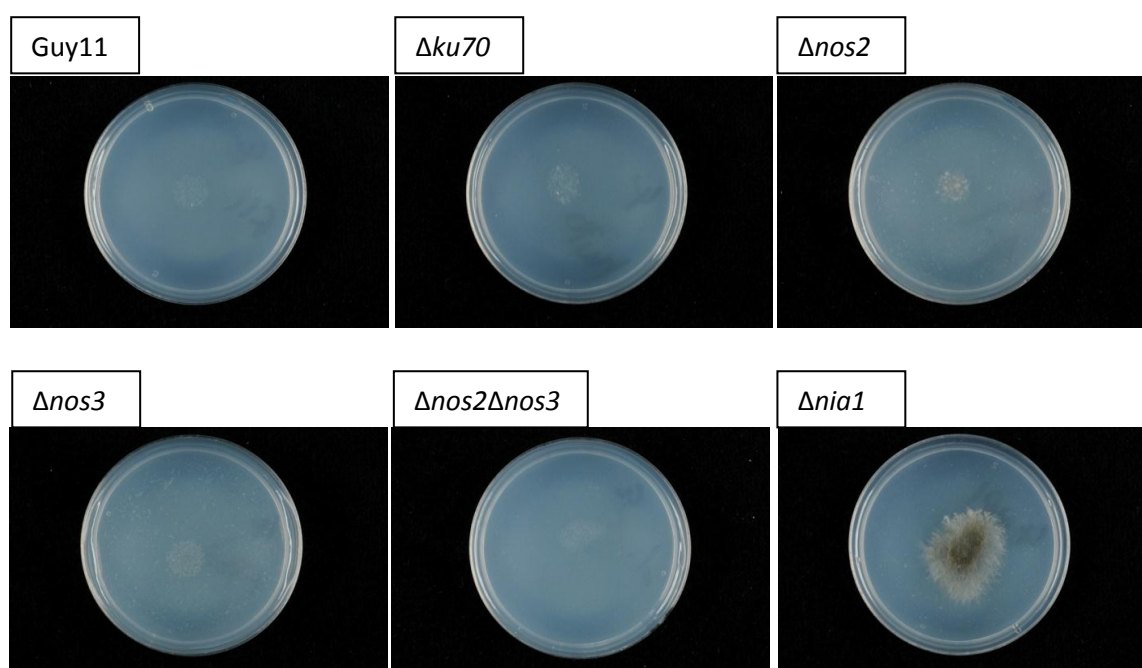




Figure 6.21. Wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ following 14 days growth on MN (nitrate salts) supplemented with 300mM potassium chlorate, incubated at 24°C.

6.3.3.8 Wildtype and mutant strains growth on minimal media (non-nitrate salts) and 300mM potassium chlorate and 3.3mM ammonium sulphate

Wildtype and mutant strains TM and MM were assessed on MN (non-nitrate salts) supplemented with 300mM potassium chlorate and 3.3mM ammonium sulphate to assess if the presence of ammonium prevents NR expression and therefore the reduction of chlorate to chlorite. No significant differences in TM or MM diameter growth were observed between Guy11 and $\Delta ku70$, (p-values: 0.294 and 0.067, respectively), Figure 6.21. Similarly, no significant TM or MM diameter growth differences were identified between $\Delta ku70$ and $\Delta nos2$, $\Delta nos2\Delta nos3$ and $\Delta nia1\Delta nii1$, (TM p-values: 0.24, 0.717 and 0.272, MM p-values: 0.2, 0.461 and 0.686, respectively). Mutant strains $\Delta nia1$, $\Delta nii1$ and $\Delta nos3\Delta nia1$ had significantly increased TM diameter growth, but wildtype equivalent MM diameter growth, (TM p-values: 1.17×10^{-5} , 1.01×10^{-8} and 1.04×10^{-7} , MM p-values: 0.461, 0.454 and 0.235, respectively). No statistical difference in TM diameter growth was observed between $\Delta nos3$ and $\Delta ku70$, however, $\Delta nos3$ exhibited a statistically reduced MM diameter growth, (p-values: 0.272 and 0.029, respectively). Images of each strain following 14 days growth on MN (non-nitrate salts) supplemented with 300mM potassium chlorate and 3.3mM ammonium sulphate are presented in Figure 6.22. Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$ and $\Delta nia1\Delta nii1$ exhibit elevated growth on 300mM

potassium chlorate and 3.3mM ammonium sulphate relative to their growth on 300mM potassium chlorate. $\Delta nia1$, $\Delta nii1$ and $\Delta nos3\Delta nia1$ demonstrate dramatically increased growth on 300mM potassium chlorate and 3.3mM ammonium sulphate relative to just 300mM potassium chlorate.

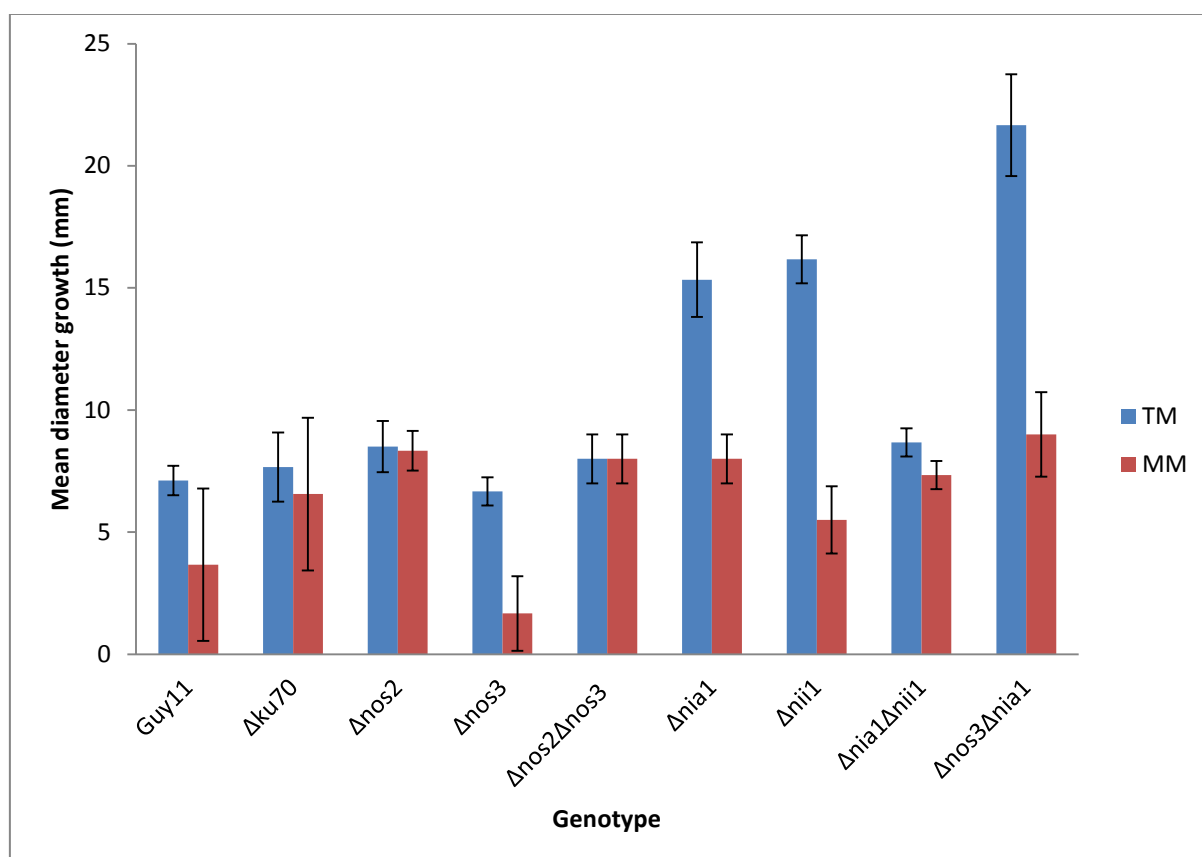


Figure 6.22. The impact of MN (non-nitrate salts) supplemented with 300mM potassium chlorate and 3.3mM ammonium sulphate on TM and MM diameter growth of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated MN plates were incubated at 24°C under a 14/10 hour light/dark photoperiod for 14 days, following which, TM and MM diameter growths were recorded. Mean diameter growths were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.

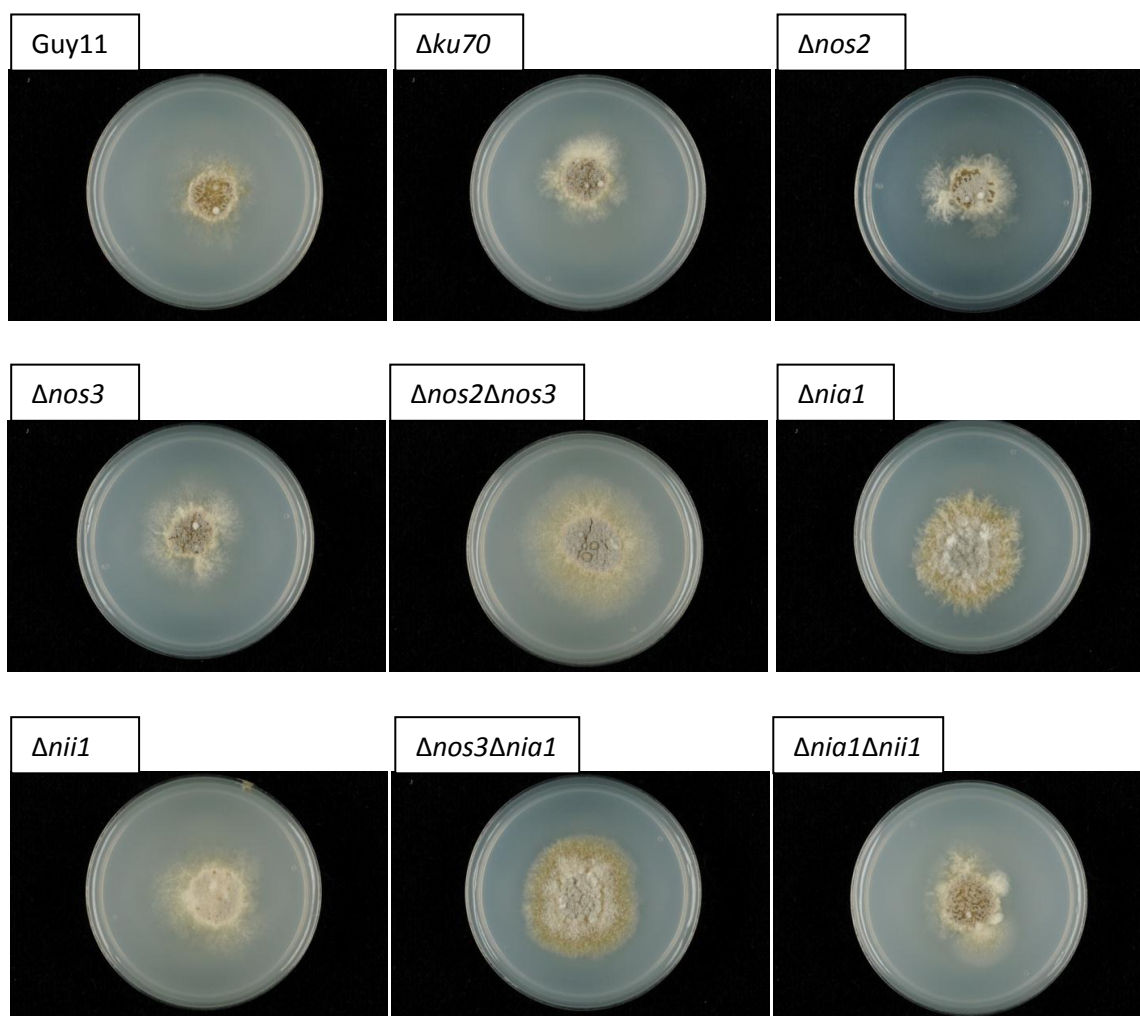


Figure 6.23. Wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ following 14 days growth on MN (non-nitrate salts) supplemented with 300mM potassium chlorate and 3.3mM ammonium sulphate, incubated at 24°C.

6.3.3.9 Wildtype and mutant strains growth on minimal media (nitrate salts) and 1mM sodium tungstate

The effect of sodium tungstate, a nitrate reductase inhibitor, on wildtype and mutant strains TM and MM diameter growths was investigated to assess the ability of the mutant strains to produce a functional nitrate reductase protein. MN plates (nitrate salts) supplemented with 1mM sodium tungstate were inoculated with wildtype and mutant strain conidia. Following 14 days incubation, Guy11 had statistically increased TM diameter growth relative to $\Delta ku70$, but

had equivalent MM diameter growth, (p-values: 0.028 and 0.209, respectively), Figure 6.23. Mutant strains $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$ and $\Delta nos3\Delta nia1$ demonstrated significantly increased TM growth and equivalent MM diameter growth to $\Delta ku70$, (TM p-values: 2.43×10^{-5} , 0.009, 0.003 and 0.042, MM p-values: 1, 0.127, 0.749 and 0.642, respectively). No significant differences in TM or MM diameter growth were observed between $\Delta ku70$ and $\Delta nos2$, $\Delta nii1$ and $\Delta nia1\Delta nii1$, (TM p-values: 0.23, 0.31 and 0.285, MM p-values: 0.57, 0.208 and 0.676, respectively). Images of each strain following 14 days growth on MN (non-nitrate salts) supplemented with 1mM sodium tungstate are presented in Figure 6.24. All strains exhibit reduced growth following the addition of 1mM sodium tungstate.

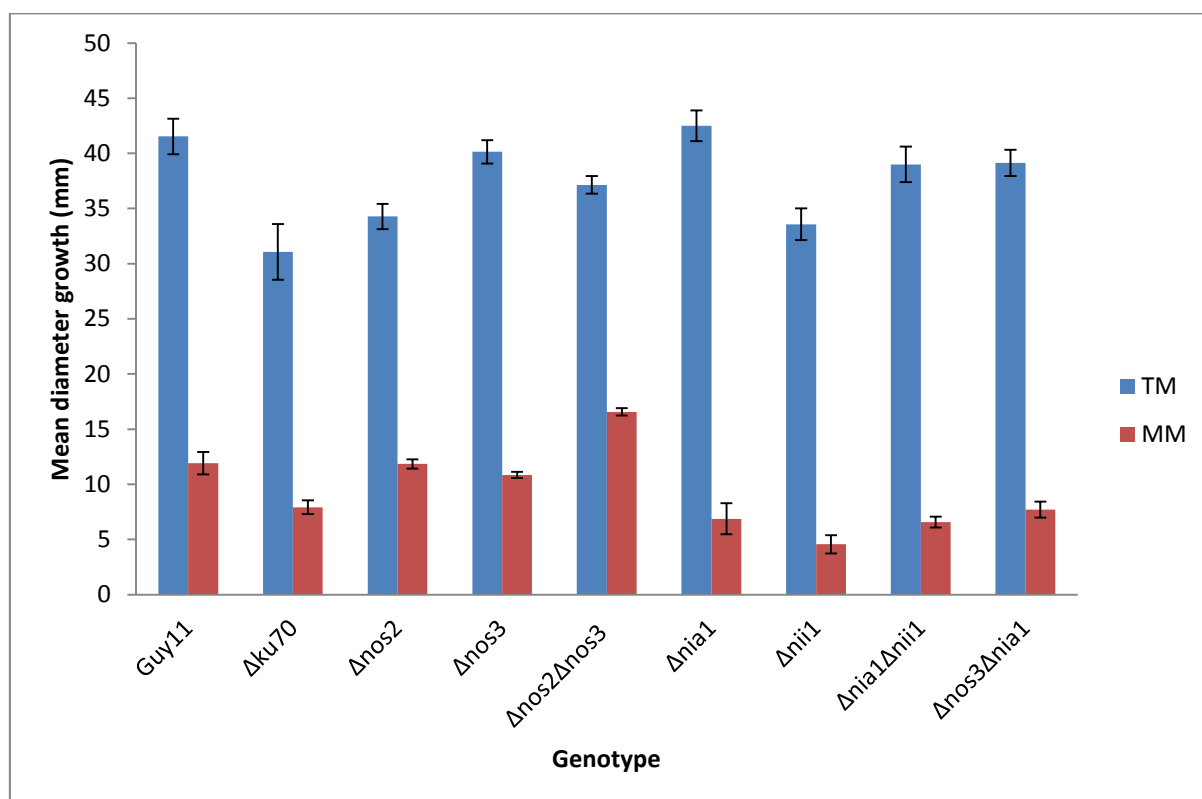
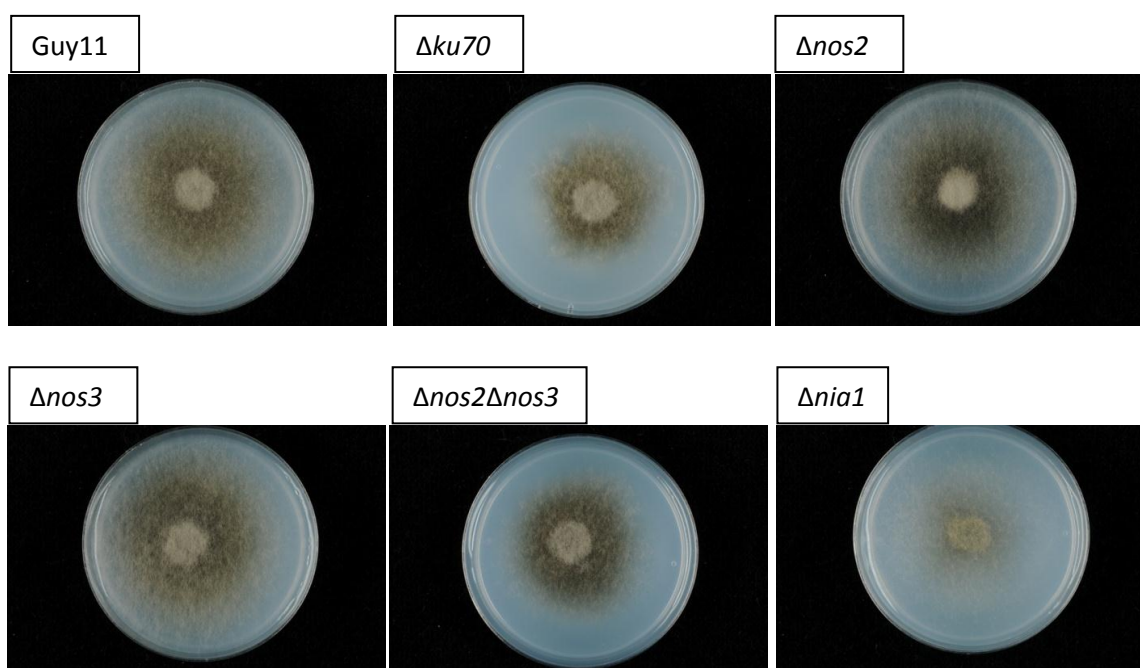


Figure 6.24. The impact of MN (nitrate salts) supplemented with 1mM sodium tungstate on TM and MM diameter growths of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated MN plates were incubated at 24°C under a 14/10 hour light/ dark photoperiod for 14 days, following which, TM and MM diameter mycelia growths were recorded. Mean diameter growths calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.



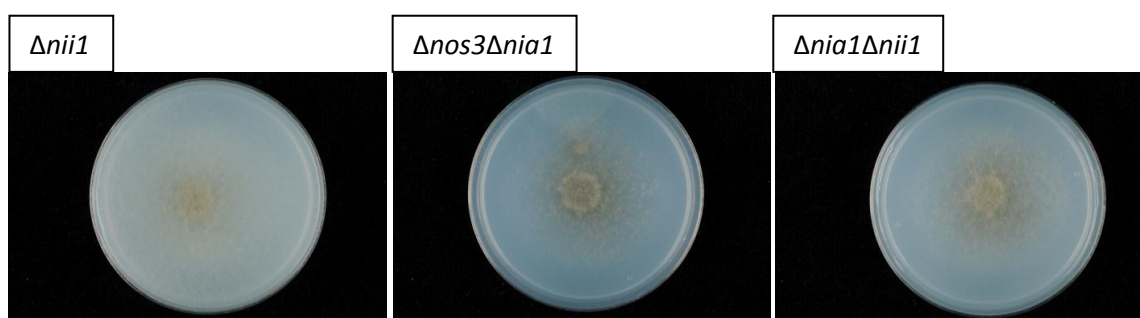


Figure 6.25. Wildtype and mutant strains Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ following 14 days growth on MN (nitrate salts) supplemented with 1mM sodium tungstate, incubated at 24°C.

6.3.4 Fungal biomass assays

6.3.4.1 Wildtype and mutant strains growth in liquid complete medium

No significant differences in biomass were recorded following the incubation of wildtype strains Guy11 and $\Delta ku70$ in liquid CM, (p-value: 0.52). No significant differences in biomass were detected between $\Delta ku70$ and $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$, (p-values: 0.317, 0.467, 0.824, 0.178, 0.612, 0.357 and 0.323, respectively), Figure 6.25. This data concurs with that found with the wildtype and mutant strains growth assays on CM, Figure 6.7.

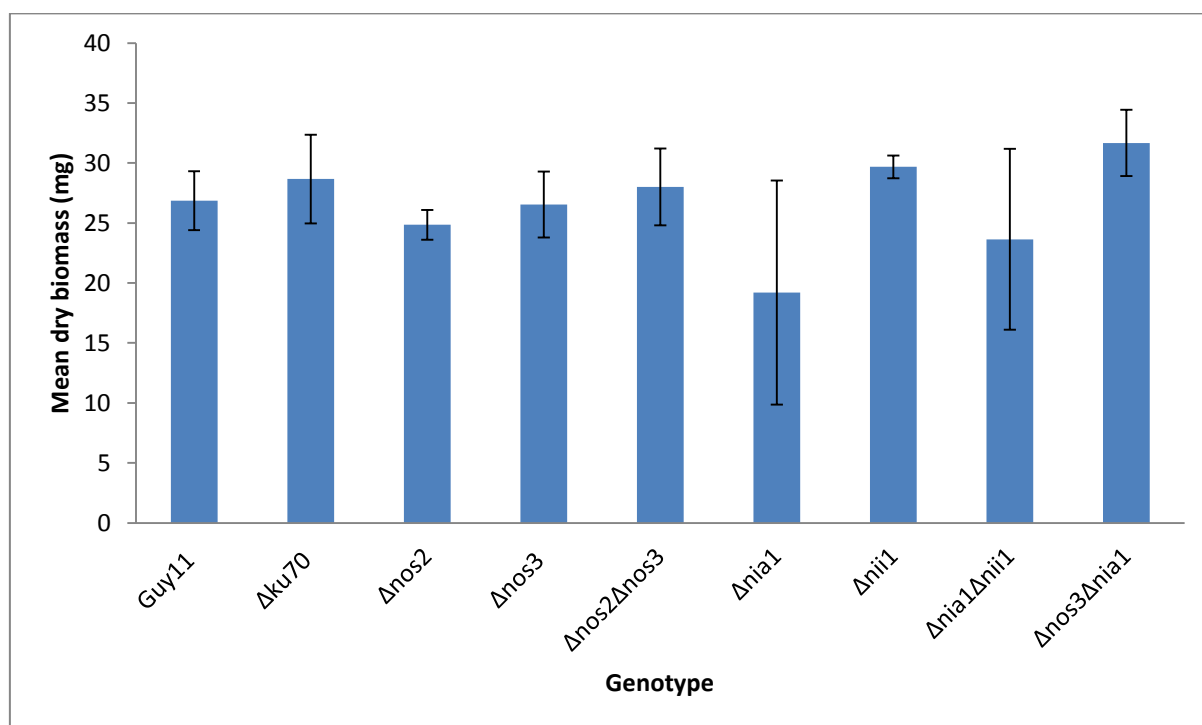


Figure 6.26. The effect of liquid CM on the growth of fungal biomass of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated solutions were shaken at 150 rpm, at 24°C, in darkness for 14 days. Mean dry biomasses were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.

6.3.4.2 Wildtype and mutant strains growth in liquid minimal media (nitrate salts)

No significant difference in biomass was recorded between wildtype strains Guy11 and $\Delta ku70$ following 14 days incubation in liquid MN (nitrate salts), (p-value: 0.311), Figure 6.26. Similarly, no significant differences were observed between $\Delta ku70$ and $\Delta nos3$ and $\Delta nos2\Delta nos3$, (p-values: 0.07 and 0.619, respectively). $\Delta nos2$ demonstrated significantly reduced biomass relative to $\Delta ku70$, (p-value 1.48×10^{-4}). However, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ showed significantly greater reductions in biomass, (p-values: 8.21×10^{-7} , 5.53×10^{-7} , 1.298×10^{-6} and 6.78×10^{-7} , respectively). This data concurs with the data for wildtype and mutant strains MM diameter growth observed during the MN (nitrate salts) growth assay, Figure 6.9.

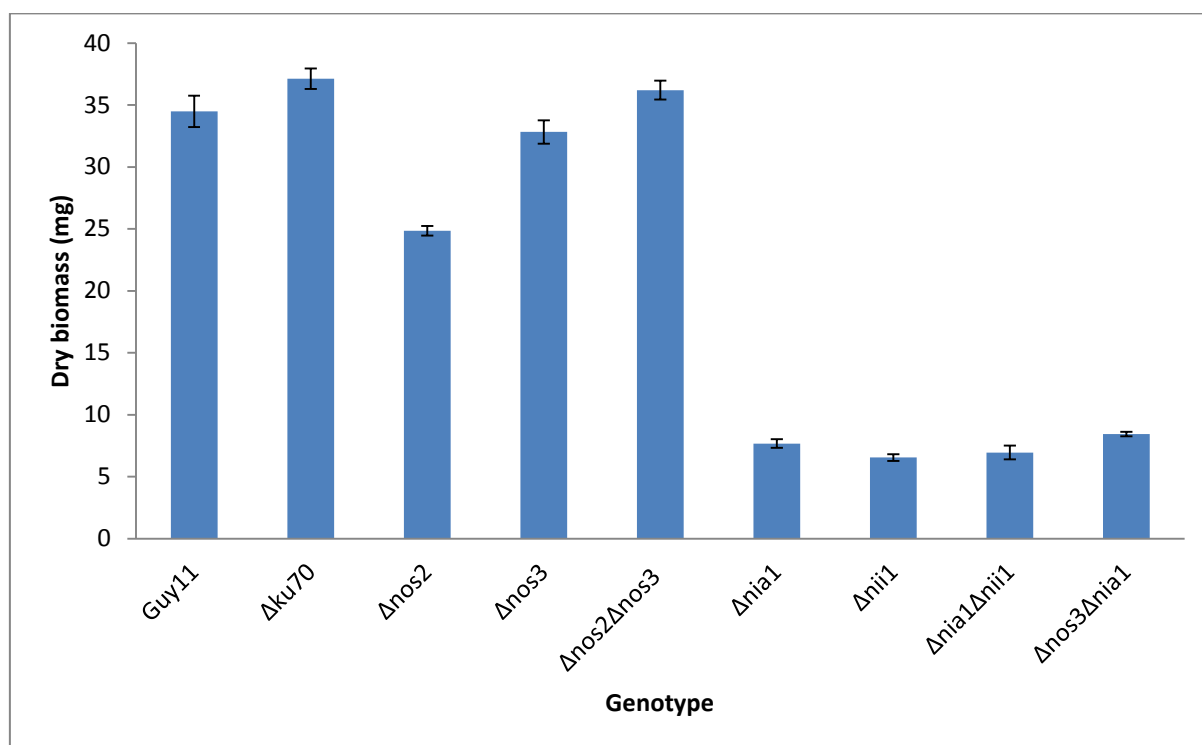


Figure 6.27. The effect of liquid MN (nitrate salts) on the growth of fungal biomass of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated solutions were shaken at 150 rpm, at 24°C, in darkness for 14 days. Mean dry biomasses were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.

6.3.4.3 Wildtype and mutant strains growth in liquid minimal media (non-nitrate salts)

Following 14 days incubation in liquid MN (non-nitrate salts) no significant differences in biomass were detected between wildtype strains Guy11 and $\Delta ku70$, (p-value: 0.52), or between $\Delta ku70$ and $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$, (p-values: 0.529, 0.578, 0.214, 0.316, 0.501, 0.859 and 0.773, respectively), Figure 6.27. This data supports the results of wildtype and mutant strains diameter growth presented in the MN (non-nitrate salts) growth assay, Figure 6.11.

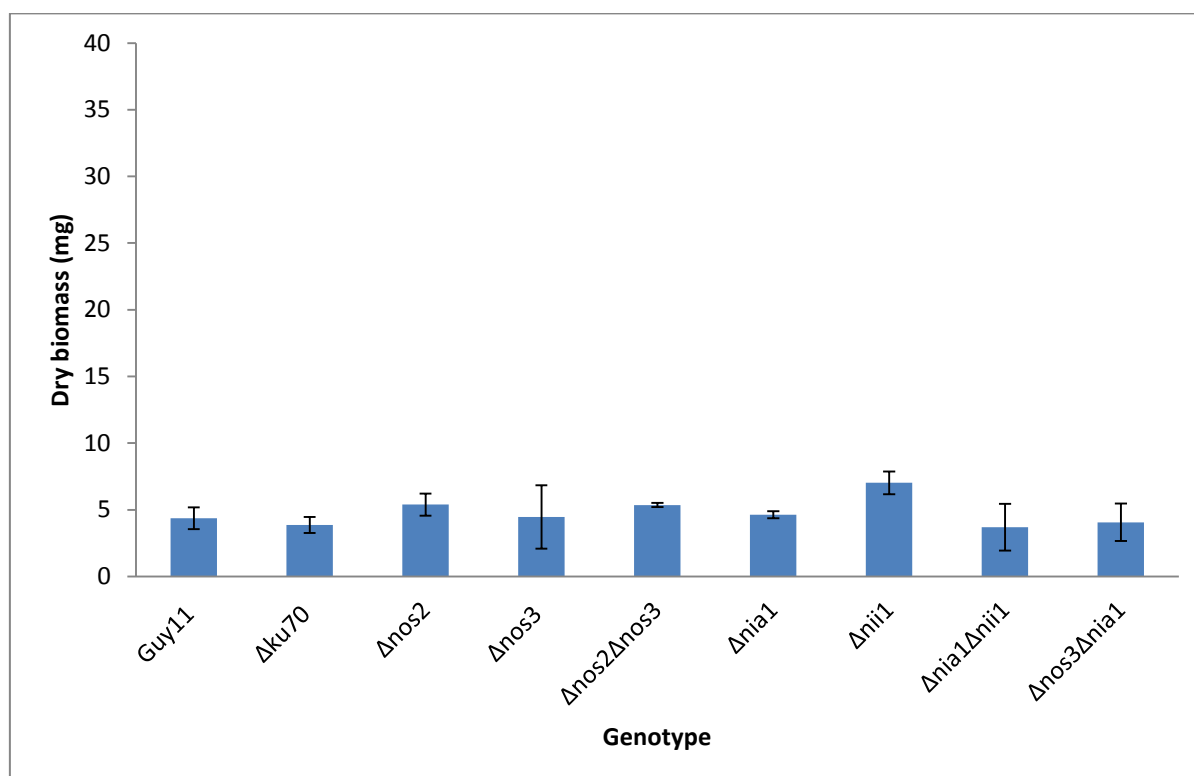


Figure 6.28. The effect of liquid MN (non-nitrate salts) on the growth of fungal biomass of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated solutions were shaken at 150 rpm, at 24°C, in darkness for 14 days. Mean dry biomasses were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.

6.3.4.4 Wildtype and mutant strains growth in liquid minimal media (non-nitrate salts) and 10mM sodium nitrate

No significant difference in biomass was observed between Guy11 and $\Delta ku70$ following 14 days incubation in liquid MN (non-nitrate salts) supplemented with 10mM sodium nitrate, (p-value 0.073) (Chapter 2.1), Figure 6.28. No significant difference was observed between $\Delta ku70$ and $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, (p-values: 0.364, 0.601 and 0.136, respectively). Mutant strains $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ all showed significantly reduced biomass relative to $\Delta ku70$ following 14 days incubation, (p-values: 4.32×10^{-4} , 2.66×10^{-4} , 0.014 and 0.004, respectively). These data concur with the MM diameter growth results presented in the MN (non-nitrate salts) 10mM sodium nitrate growth assay, Figure 6.13.

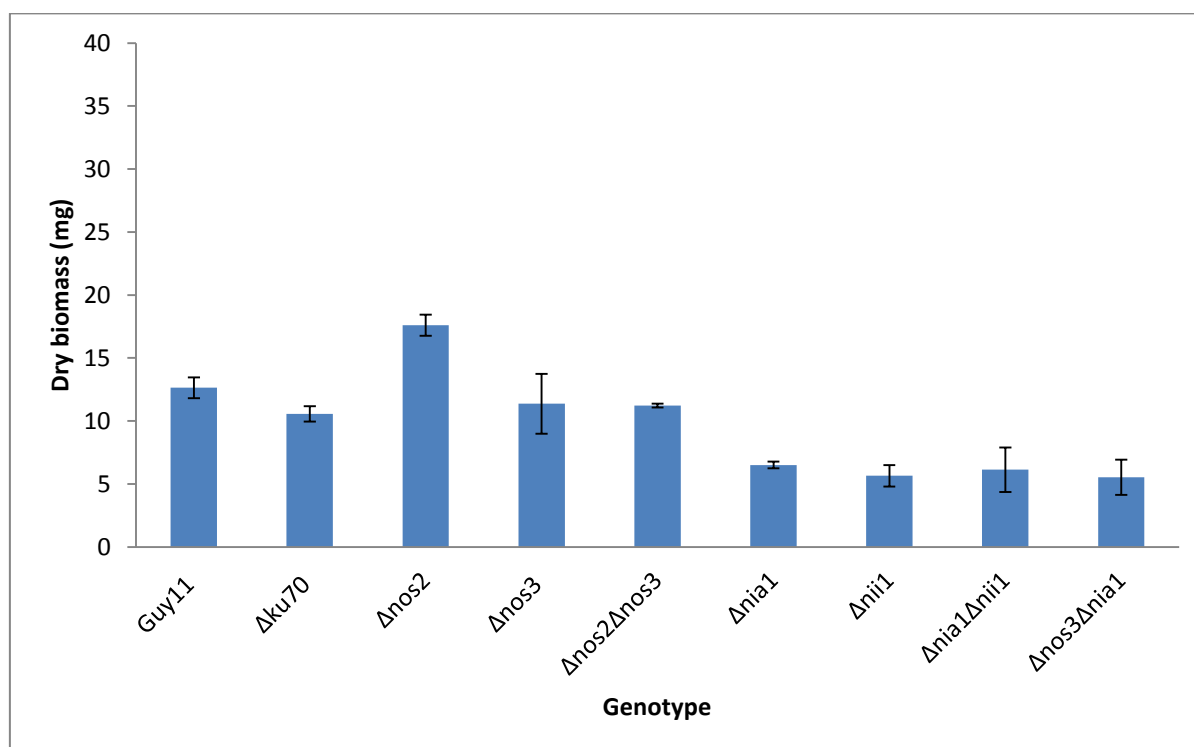


Figure 6.29. The effect of liquid MN (non-nitrate salts) supplemented with 10mM sodium nitrate on the growth of fungal biomass of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated solutions were shaken at 150 rpm, at 24°C, in darkness for 14 days. Mean dry biomasses were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.

6.3.4.5 Wildtype and mutant strains growth in liquid minimal media (non-nitrate salts) and 10mM sodium nitrite

No significant difference in biomass was recorded between Guy11 and $\Delta ku70$ following 14 days incubation in liquid MN (non-nitrate salts) supplemented with 10mM sodium nitrite, (p-value: 0.388), Figure 6.29. No significant differences in fungal biomass were observed between $\Delta ku70$ and $\Delta nos3$ and $\Delta nos2\Delta nos3$, (p-values: 0.452 and 0.556, respectively). $\Delta nos2$ exhibited a significantly increased biomass relative to $\Delta ku70$, (p-value 0.016). Conversely, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ showed significantly impaired growth when nitrite was the sole nitrogen source relative to $\Delta ku70$, (p-values: 0.01, 8.37×10^{-7} , 0.011 and 0.009, respectively).

With the exception of the $\Delta nos2$, these data correlate with the MM diameter growth data presented in MN (non-nitrate salts) 10mM sodium nitrite growth assay, Figure 6.15.

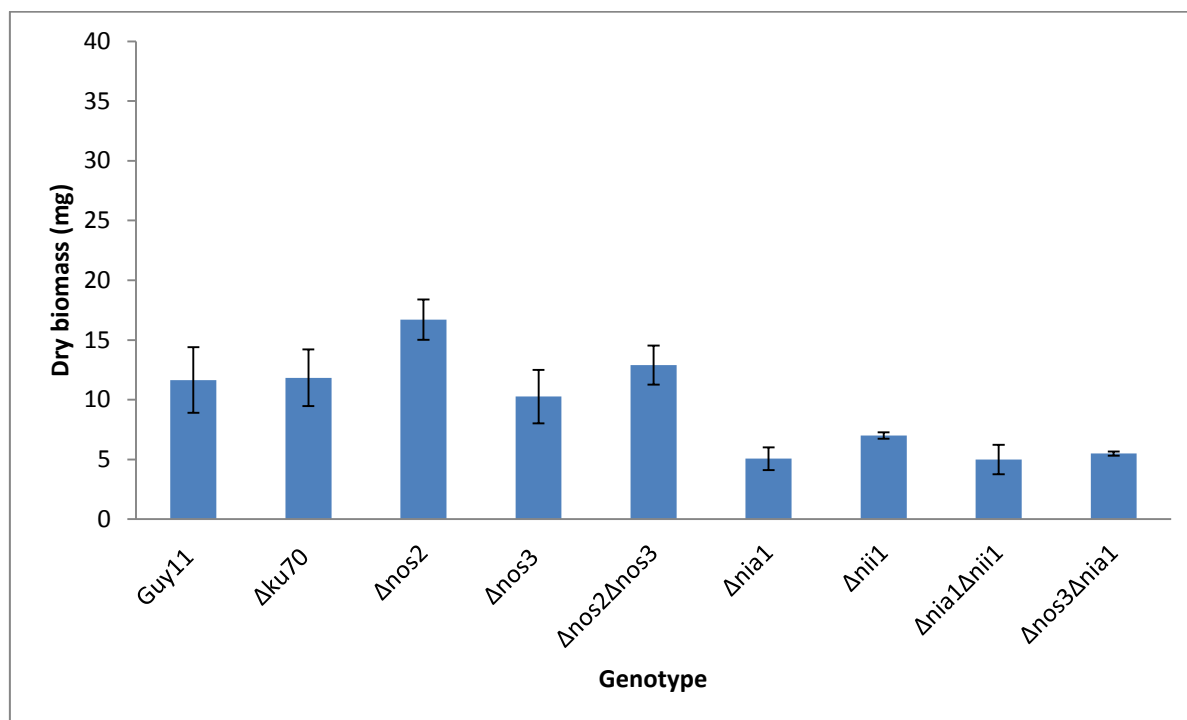


Figure 6.30. The effect of liquid MN (non-nitrate salts) supplemented with 10mM sodium nitrite on the growth of fungal biomass of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated solutions were shaken at 150 rpm, at 24°C, in darkness for 14 days. Mean dry biomasses were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.

6.3.4.6 Wildtype and mutant strains growth in liquid minimal media (non-nitrate salts) and 10mM ammonium sulphate

Growth of wildtype strains in liquid MN (non-nitrate salts) supplemented with 10mM ammonium sulphate showed no significant difference in biomass, (p-value 0.213). No significant differences between $\Delta ku70$ and $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ were recorded, (p-values: 0.06, 0.752, 0.932, 0.586, 0.104, 0.724 and 0.832,

respectively), Figure 6.30. This data concurs with the growth data presented in MN (non-nitrate salts) 10mM ammonium sulphate growth assay, Figure 6.17.

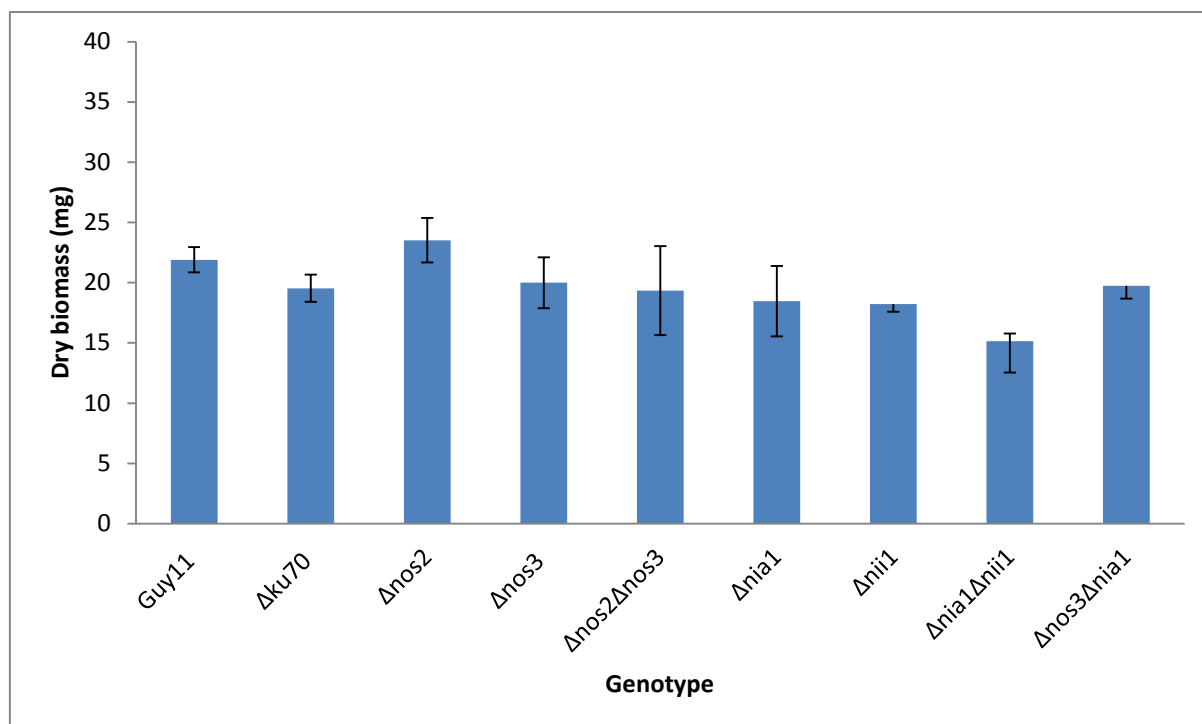


Figure 6.31. The effect of liquid MN (non-nitrate salts) supplemented with 10mM ammonium sulphate on the growth of fungal biomass of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated solutions were shaken at 150 rpm, at 24°C, in darkness for 14 days. Mean dry biomasses were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.

6.3.4.7 Wildtype and mutant strains growth in liquid minimal media (nitrate salts) and 300mM potassium chlorate

The growth of wildtype and mutant strains was reduced in liquid MN (nitrate salts) supplemented with 300mM potassium chlorate. No significant difference between wildtype strains Guy11 and $\Delta ku70$ was observed, (p-value 0.282), or between $\Delta ku70$ and $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$, (p-values: 0.331, 0.15, 0.33, 0.204, 0.927, 0.679 and 0.232, respectively), Figure 6.31. The data for Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$,

$\Delta nos2\Delta nos3$, $\Delta nii1$ and $\Delta nia1\Delta nii1$ corresponds with the data presented in MN (nitrate salts) 300mM potassium chlorate growth assay, Figure 6.19. The mutant strains $\Delta nia1$ and $\Delta nos3\Delta nia1$ exhibited significantly increased diameter growth relative to the wildtype strains on solid media. This result was not mirrored in the biomass assay.

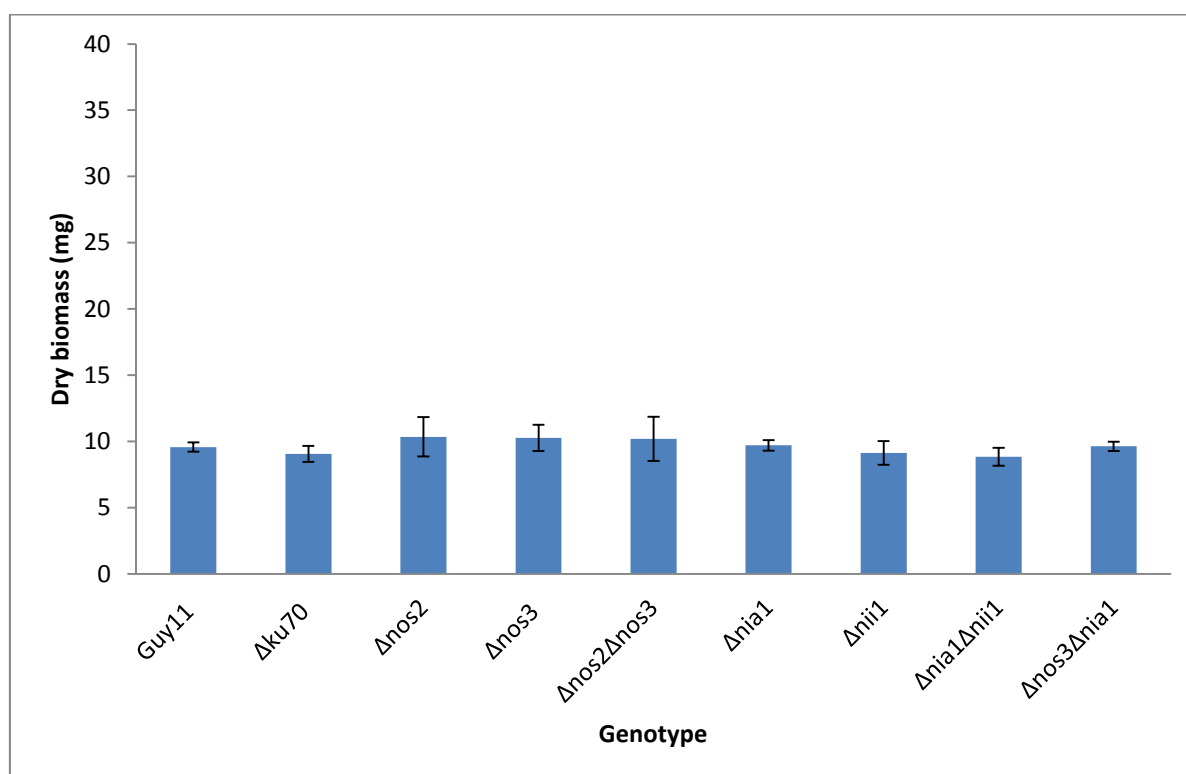


Figure 6.32. The effect of liquid MN (nitrate salts) supplemented with 300mM potassium chlorate on the growth of fungal biomass of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated solutions were shaken at 150 rpm, at 24°C, in darkness for 14 days. Mean dry biomasses were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.

6.3.4.8 Wildtype and mutant strains growth in liquid minimal media (non-nitrate salts) and 300mM potassium chlorate and 3.3mM ammonium sulphate

Wildtype and mutant strains were grown in liquid MN (non-nitrate salts) supplemented with 300mM potassium chlorate and 3.3mM ammonium sulphate. Following 14 days incubation no

significant difference was detected between Guy11 and $\Delta ku70$, (p-value 0.288), Figure 6.32. $\Delta nos2$ demonstrated a slight significant decrease in biomass relative to $\Delta ku70$, (p-value: 0.01). No significant differences in biomass were detected between $\Delta ku70$ and $\Delta nos3$, $\Delta nos2\Delta nos3$ and $\Delta nii1$, (p-values: 0.4, 0.772 and 0.548, respectively). $\Delta nia1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ exhibited significant increases in biomass relative to $\Delta ku70$ following 14 days incubation, (p-values: 0.001, 0.03 and 8.6×10^{-4} , respectively). Data from the biomass and growth assays (Figure 6.21) demonstrate only a weak correlation, with significant differences in mutant strain growth rates. However, in both assays $\Delta nia1$ and $\Delta nos3\Delta nia1$ demonstrated significantly increased levels of growth relative to $\Delta ku70$.

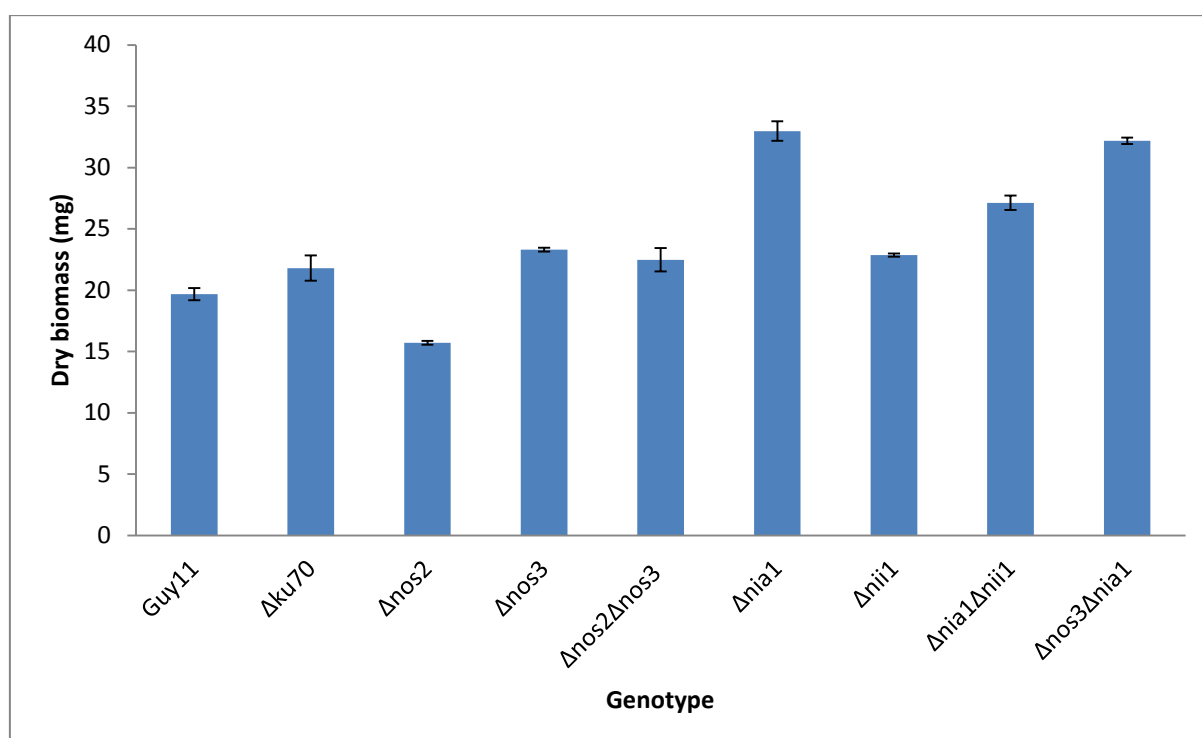


Figure 6.33. The effect of liquid MN (non-nitrate salts) supplemented with 300mM potassium chlorate and 3.3mM ammonium sulphate on the growth of fungal biomass of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated solutions were shaken at 150 rpm, at 24°C, in darkness for 14 days. Mean dry biomasses were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.

6.3.4.9 Wildtype and mutant strains growth in liquid minimal media (nitrate salts) and 1mM sodium tungstate

The effect of the nitrate reductase inhibitor sodium tungstate on wildtype and mutant strains biomass was investigated. Conidia were inoculated into liquid MN (nitrate salts) supplemented with 1mM sodium tungstate. The presence of sodium tungstate significantly suppressed fungal biomass across the wildtype and mutant strains. No significant difference in biomass was detected between wildtype strains Guy11 and $\Delta ku70$, (p-value 0.429); similarly there was no difference in biomass between $\Delta ku70$ and $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$, (p-values: 0.668, 0.97, 0.089, 0.907, 0.578, 0.096 and 0.051, respectively), Figure 6.33. This data concurs with the MM diameter growth data presented in MN (non-nitrate salts) 1mM sodium tungstate growth assay, Figure 6.23.

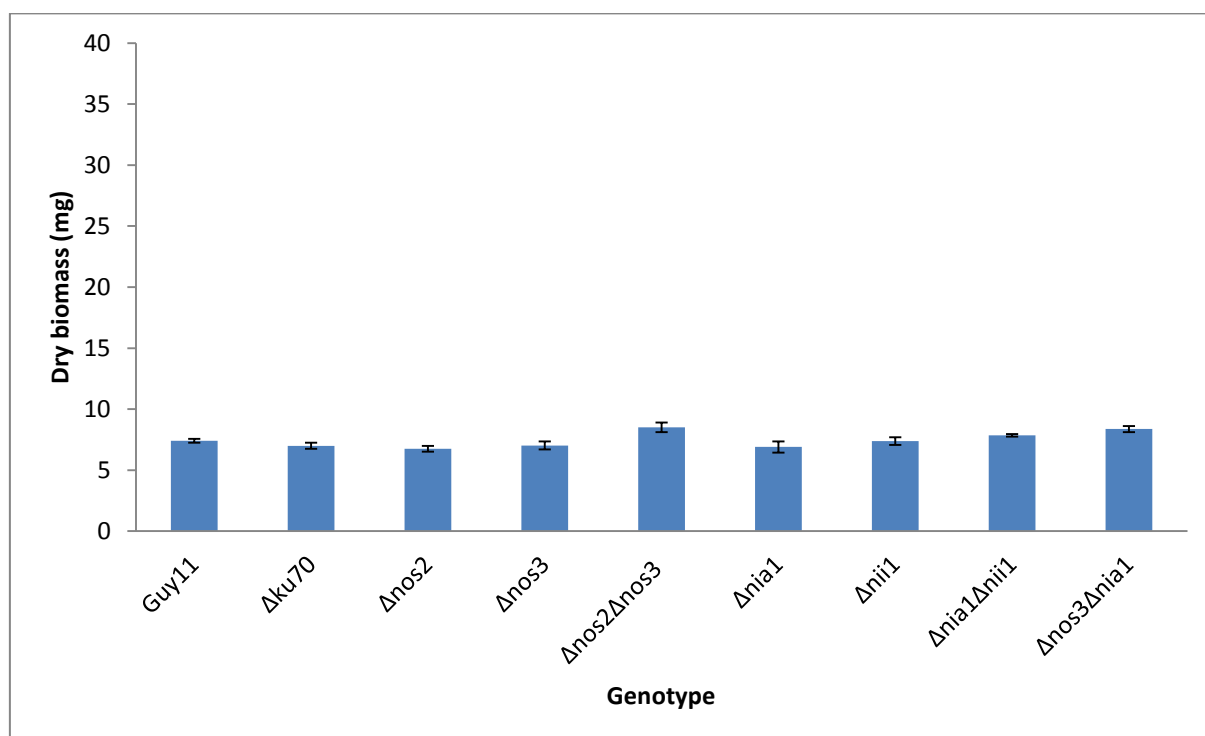


Figure 6.34. The effect of liquid MN (nitrate salts) supplemented with 1mM sodium tungstate on the growth of fungal biomass of wildtype strains Guy11 and $\Delta ku70$ and mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. Inoculated solutions were shaken at 150 rpm, at 24°C, in darkness for 14 days. Mean dry biomasses were calculated from three independent experiments, containing three biological replicates. Error bars represent the standard error of the mean.

Media	CM		MN (nitrate salts)		MN (non-nitrate salts)		MN (non-nitrate salts) 10mM sodium nitrate		MN (non-nitrate salts) 10mM sodium nitrite		MN (non-nitrate salts) 10mM ammonium sulphate		MN (nitrate salts) 300mM potassium chlorate		MN (non-nitrate salts) 300mM potassium chlorate and 3.3mM ammonium sulphate		MN (nitrate salts) 1mM sodium tungstate	
Genotype	TM	MM	TM	MM	TM	MM	TM	MM	TM	MM	TM	MM	TM	MM	TM	MM	TM	MM
Guy11	45	36	44.6	35.6	45	6.8	42.6	31.2	44	35.6	25.8	25.8	6.5	0	7.1	3.6	41.5	11.9
$\Delta ku70$	45	37.5	45	39	44.7	7	36.2	29.4	38	30.6	25.2	25.2	5.5	0.1	7.6	6.5	31	7.9
$\Delta nos2$	45	35.5	39.1	30.5	42	7.8	37.6	32.1	37.8	27.5	25.6	25.6	10	0	8.5	8.3	34.2	11.8
$\Delta nos3$	42	37.4	45	39.8	45	7.1	43	37.3	42.6	38.3	25.6	25.6	7	0	6.6	1.6	40.1	10.8
$\Delta nos2\Delta nos3$	44.1	34.1	42.5	37.4	45	7.2	37	28.3	34.6	31.6	23.3	23.3	6.5	0	8	8	37.1	16.5
$\Delta nia1$	43.8	36.8	43	10.1	45	7.8	44.3	10.3	41.6	13	21.6	21.3	18.4	7.2	15.3	8	42.5	6.8
$\Delta nii1$	44.8	38	37.3	10.1	42.71	7.8	34.3	9.1	11.5	2	27.6	27.6	5	0	16.1	5.5	33.5	4.5
$\Delta nia1\Delta nii1$	41.6	36.5	42.3	9.3	43.75	7.7	41.6	4.3	43.3	7	23.6	23	8.5	0	8.6	7.3	39	6.5
$\Delta nos3\Delta nia1$	45	35.3	40	7.1	45	9.3	40.6	7	40	10	22.3	22.3	24.7	8.1	21.6	9	39.1	7.7
Guy11 & $\Delta ku70$	1	0.056	0.299	0.186	0.377	0.801	0.072	0.271	6.2×10^{-4}	0.026	0.632	0.632	0.108	0.34	0.294	0.067	0.028	0.209
$\Delta ku70$ & $\Delta nos2$	1	0.327	0.181	0.382	0.15	0.287	0.303	0.18	0.938	0.236	0.821	0.821	0.543	0.446	0.24	0.201	0.23	0.57
$\Delta ku70$ & $\Delta nos3$	1	0.919	0.4	0.497	0.337	0.783	0.646	0.41	0.065	0.41	0.794	0.794	0.111	0.299	0.272	0.029	2.44×10^{-5}	1
$\Delta ku70$ & $\Delta nos2\Delta nos3$	0.155	0.092	0.253	0.209	0.337	0.645	0.672	0.668	0.146	0.663	0.251	0.251	0.277	0.299	0.717	0.461	0.009	0.127
$\Delta ku70$ & $\Delta nia1$	0.191	0.492	0.876	4.5×10^{-4}	0.393	0.152	5.7×10^{-4}	1.4×10^{-5}	0.144	4.4×10^{-4}	0.279	0.263	1.3×10^{-4}	3.9×10^{-10}	1.1×10^{-5}	0.461	0.003	0.749
$\Delta ku70$ & $\Delta nii1$	0.34	0.848	0.513	1.1×10^{-5}	0.114	0.187	0.245	2.0×10^{-8}	1.1×10^{-5}	1.9×10^{-7}	0.138	0.138	0.767	0.446	1.0×10^{-8}	0.454	0.31	0.208
$\Delta ku70$ & $\Delta nia1\Delta nii1$	0.125	0.389	0.09	1.1×10^{-4}	0.296	0.689	0.013	9.5×10^{-7}	0.011	1.1×10^{-5}	0.348	0.222	0.111	0.299	0.272	0.686	0.285	0.676
$\Delta ku70$ & $\Delta nos3\Delta nia1$	1	0.183	0.54	2.0×10^{-4}	0.391	0.857	0.021	3.4×10^{-6}	0.158	0.002	0.087	0.087	1.7×10^{-9}	5.7×10^{-08}	1.0×10^{-7}	0.235	0.042	0.642

Table 6.1. Summary of growth assay data, values represent the mean TM and MM diameter growths (mm), including T-test p-values.

Genotype	CM	MN (nitrate salts)	MN (non-nitrate salts)	MN (non-nitrate salts) 10mM sodium nitrate	MN (non-nitrate salts) 10mM sodium nitrite	MN (non-nitrate salts) 10mM ammonium sulphate	MN (nitrate salts) 300mM potassium chlorate	MN (non-nitrate salts) 300mM potassium chlorate and 3.3mM ammonium sulphate	MN (nitrate salts) 1mM sodium tungstate
Guy11	26.8	34.4	4.3	12.6	11.6	21.9	9.5	19.6	7.4
$\Delta ku70$	28.6	37.1	3.8	10.5	11.8	19.5	9	21.8	7
$\Delta nos2$	24.8	24.8	5.4	17.6	16.7	23.5	10.3	15.7	6.7
$\Delta nos3$	26.5	32.8	4.4	11.3	10.2	20	10.2	23.3	7
$\Delta nos2\Delta nos3$	28	36.2	5.3	11.2	12.9	19.3	10.2	22.4	8.5
$\Delta nia1$	19.2	7.6	4.6	6.5	5	18.4	9.7	32.9	6.9
$\Delta nii1$	29.6	6.5	7	5.6	7	18.2	9.1	22.8	7.3
$\Delta nia1\Delta nii1$	23.6	6.9	3.7	6.1	5	15.1	8.8	27.1	7.8
$\Delta nos3\Delta nia1$	31.6	8.4	4	5.5	5.5	19.7	9.6	32.1	8.3
Guy11 & $\Delta ku70$	0.52	0.311	0.529	0.073	0.388	0.213	0.282	0.288	0.429
$\Delta ku70$ & $\Delta nos2$	0.317	1×10^{-4}	0.529	0.655	0.016	0.064	0.331	0.01	0.668
$\Delta ku70$ & $\Delta nos3$	0.467	0.073	0.578	0.601	0.452	0.752	0.150	0.4	0.97
$\Delta ku70$ & $\Delta nos2\Delta nos3$	0.824	0.619	0.214	0.136	0.556	0.932	0.330	0.772	0.089
$\Delta ku70$ & $\Delta nia1$	0.178	8.22×10^{-7}	0.316	4.32×10^{-4}	0.010	0.586	0.204	0.001	0.907
$\Delta ku70$ & $\Delta nii1$	0.612	5.53×10^{-7}	0.5	2.66×10^{-4}	8.37×10^{-7}	0.104	0.927	0.548	0.578
$\Delta ku70$ & $\Delta nia1\Delta nii1$	0.357	1.3×10^{-6}	0.859	0.014	0.011	0.724	0.679	0.03	0.096
$\Delta ku70$ & $\Delta nos3\Delta nia1$	0.323	6.78×10^{-7}	0.773	0.004	0.009	0.832	0.232	8.6×10^{-4}	0.051

Table 6.2. Summary of biomass assay data, values represent the mean fungal biomass (mg), including T-test p-values.

6.4 Discussion

6.4.1 Conclusions

- *NOS2*, *NOS3*, *NIA1* and *NII1* are dispensable for pathogenesis on both rice and barley.
- *NOS2* and *NOS3* are not required for nitrate assimilation in *M. oryzae*.
- *NIA1* and *NII1* are required for nitrate assimilation in *M. oryzae*.

6.4.2 Pathogenicity assays

The pathogenicity assays demonstrate that *NOS2*, *NOS3*, *NIA1* and *NII1* are dispensable for the pathogenicity and virulence in *M. oryzae*. All strains developed wildtype levels of blast lesions on both barley and rice leaves, (with the exception of $\Delta nos2\Delta nos3$, which exhibited reduced lesions as a percentage area of rice leaves). Image analysis software confirmed there were no significant differences between wildtype and mutant strains in either lesion number or the percentage surface area of rice and barley leaves identified as diseased. No differences in lesion size or colouring were identified between the wildtype and mutant strains. This contrasts with the *in vitro* infection-related assay, where disruption of *NOS2*, *NOS3*, *NIA1* and *NII1* suppressed appressorium formation, by varying degrees, on inductive TAAB plastic coverslips. A potential explanation for this discrepancy is environmental cue threshold signalling, as previously discussed (Chapter 5, 5.4.1) (DeZwaan et al. 1990; Skamnioti & Gurr 2008; Takahashi et al. 2005; Xu et al. 1997; Xu & Hamer 1996). The disruption of putative genes responsible for NO generation in *M. oryzae* resulted in mutants with reduced abilities to trigger infection-related development. The mutants generate insufficient NO to overcome environmental cue thresholds and therefore generate reduced levels of appressoria. It can be assumed that mutant strain conidia inoculated onto TAAB plastic coverslips are exposed to fewer environmental cues than on their host cuticle. Therefore $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ conidia inoculated onto barley and rice cuticles are exposed to a greater number of

appressorium inducing environmental cues. The combination of multiple, host specific environmental cues (unavailable on TAAB plastic coverslips, such as cutin monomers) can be considered sufficient to overcome environmental cue thresholds triggering appressorium formation, despite each individual mutant strains deficit in NO generation.

In conclusion *NOS2*, *NOS3*, *NIA1* and *NII1* are dispensable for blast disease in rice and barley but required for infection related development on an artificial host surface.

6.4.3 Nitrogen utilization by wildtype and mutant strains

The growth of Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ in disparate nitrogen sources, in both solid and liquid media, were assessed. Disruption of *NOS2* and *NOS3* in mutant strains $\Delta nos2$, $\Delta nos3$ and $\Delta nos2\Delta nos3$ had no impact on the ability of *M. oryzae* to metabolise different nitrogen sources supplemented in solid or liquid media. No significant differences in TM or MM diameter growth were recorded between wildtype $\Delta ku70$ and $\Delta nos2$, $\Delta nos3$ and $\Delta nos2\Delta nos3$ grown on: CM, MN (nitrate salts), MN (non-nitrate salts), MN (non-nitrate salts) 11.7mM sodium nitrate, MN (non-nitrate salts) 10mM sodium nitrite and MN (non-nitrate salts) 10mM ammonium sulphate. $\Delta nos2$, $\Delta nos3$ and $\Delta nos2\Delta nos3$ exhibited high levels of MM diameter growth on media supplemented with nitrate, nitrite and ammonium; only in the total absence of nitrogen, MN (non-nitrate salts) was their MM diameter growth compromised. These data indicate that *NOS2* and *NOS3* are unlikely to be linked to nitrogen metabolism in *M. oryzae*.

By contrast, disruption of *NIA1* and *NII1* in mutant strains $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ had a significant impact on the ability of *M. oryzae* to metabolise different nitrogen sources. $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ MM diameter growths were reduced significantly relative to $\Delta ku70$, growing on MN (nitrate salts) and MN (non-nitrate salts) 10mM sodium nitrate. Disruption of the nitrate reductase gene within $\Delta nia1$, $\Delta nia1\Delta nii1$ and

Δnos3Δnia1 prevents the reduction of nitrate to nitrite and therefore inhibits nitrogen metabolism and growth. *Δnii1* carries a functional nitrate reductase gene and is able to reduce nitrate to nitrite; however, disruption of the nitrite reductase gene prevents the expression of Nii1, inhibiting the reduction of nitrite to ammonium, suppressing nitrogen metabolism and growth (Amaar & Moore 1998). *Δnia1*, *Δnii1*, *Δnia1Δnii1* and *Δnos3Δnia1* also demonstrated significantly reduced MM diameter growth on MN (non-nitrate salts) 10mM sodium nitrite compared to *Δku70*. *Δnii1* and *Δnia1Δnii1* both carry a disrupted nitrite reductase gene and are unable to reduce nitrite to ammonium. However, *Δnia1* and *Δnos3Δnia1* both carry a functional nitrite reductase gene, therefore, the addition of nitrite would be expected to recover their growth to wildtype levels. This was not observed. It has been well reported in *N. crassa* that both nitrate reductase and nitrite reductase genes are co-expressed and that disruption of the nitrate reductase gene inhibits expression of the nitrite reductase gene, preventing nitrite metabolism (Fu & Marzluf 1987). Furthermore, a key activator of nitrite reductase expression is cellular nitrate; the absence of nitrate from MN (non-nitrate salts) 10mM sodium nitrite media may prevent the expression of nitrite reductase, also suppressing nitrogen metabolism (Cr  t   et al. 1997; Cove & Pateman 1969). *Δnia1*, *Δnii1*, *Δnia1Δnii1* and *Δnos3Δnia1* TM and MM diameter growths were restored to wildtype levels on MN (non-nitrate salts) 10mM ammonium sulphate. Ammonium represents the final product of assimilatory nitrate reduction and can be utilised by *Δnia1*, *Δnii1*, *Δnia1Δnii1* and *Δnos3Δnia1* as a nitrogen source, restoring wildtype growth levels. These data demonstrate the importance of *NIA1* and *NII1* in *M. oryzae* nitrogen metabolism.

Biomass assays provide a more accurate measure of fungal growth in specific nitrogen sources relative to growth assays. The majority of biomass assay results recorded for the mutant strains *Δnos2*, *Δnos3* and *Δnos2Δnos3* concurred with the growth assay data, with no significant differences in biomass relative to the wildtype *Δku70*. Two exceptions were noted: *Δnos2* in liquid MN (nitrate salts) demonstrated significantly reduced growth, and in liquid MN 10mM

nitrite, $\Delta nos2$ demonstrated significantly increased growth relative to $\Delta ku70$. These data, combined with the growth assay results confirm that *NOS2* and *NOS3* are not required for nitrogen metabolism in *M. oryzae*.

The biomass results for $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ in liquid CM, MN (nitrate salts), MN (non-nitrate salts), MN (non-nitrate salts) 11.7mM sodium nitrate, MN (non-nitrate salts) 10mM sodium nitrite and MN (non-nitrate salts) 10mM ammonium sulphate concurred with the growth assay data, confirming both *NIA1* and *NII1* are required for nitrogen metabolism in *M. oryzae*.

6.4.4 Potassium chlorate resistance

Chlorate resistance can be used in fungi to identify genes and confirm mutants in the nitrate assimilation pathway (Lau & Hamer 1996; Zhang & Zhou 2006). Potassium chlorate is toxic to organisms containing functional nitrate reductase enzymes; chlorate is reduced by nitrate reductase to the toxic chlorite ion suppressing fungal growth (Cove 1976). The MM and TM diameter growths of mutant strains $\Delta nos2$, $\Delta nos3$ and $\Delta nos2\Delta nos3$ on MN (nitrate salts) supplemented with 300mM potassium chlorate were highly suppressed, mirroring that of the wildtype strains Guy11 and $\Delta ku70$. A similar result was observed in the biomass assay, with Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$ and $\Delta nos2\Delta nos3$ demonstrating severely suppressed growth. The addition of 3.3mM ammonium sulphate with 300mM potassium chlorate was sufficient to partially rescue TM and MM growth in Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$ and $\Delta nos2\Delta nos3$. The addition of ammonium provided a readily metabolisable nitrogen source allowing growth, its presence also suppressed the expression of nitrate reductase, preventing chlorate reduction to chlorite, and thus resulting in increased fungal growth. Again, this result was mirrored in the corresponding biomass assay. The susceptibility of Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$ and $\Delta nos2\Delta nos3$ to potassium chlorate confirms their nitrate assimilation pathway are intact.

The growth of $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ on MN (nitrate salts) supplemented with 300mM potassium chlorate differed. Mutant strains $\Delta nia1$ and $\Delta nos3\Delta nia1$ demonstrated increased TM and MM diameter growths relative to $\Delta ku70$, both mutants carry disrupted nitrate reductase genes, therefore the presence of nitrate was unable to induce *NIA1* expression and result in chlorate reduction. The $\Delta nii1$ phenotype resembles the wildtype $\Delta ku70$; $\Delta nii1$ contains a functional nitrate reductase gene, whose expression is induced in the presence of nitrate, which in turn results in the reduction of chlorate to chlorite, suppressing growth. $\Delta nia1\Delta nii1$ also demonstrated significantly reduced diameter growth in MN (nitrate salts) supplemented with 300mM potassium chlorate. $\Delta nia1\Delta nii1$ like $\Delta nia1$ and $\Delta nos3\Delta nia1$, carries a disrupted nitrate reductase and cannot reduce chlorate to chlorite. $\Delta nia1\Delta nii1$ would therefore be expected to show equivalent TM and MM diameter growth levels to $\Delta nia1$ and $\Delta nos3\Delta nia1$. By contrast to the growth assay data, the biomass assay results showed $\Delta nia1$ and $\Delta nos3\Delta nia1$ growth to be severely suppressed, equivalent to Guy11, $\Delta ku70$, $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nii1$ and $\Delta nia1\Delta nii1$. This finding raises an important issue associated with agar based growth assays. Agar itself contains nitrogen sources, this explains why both $\Delta nia1$ and $\Delta nos3\Delta nia1$ exhibit increased TM and MM diameter growths relative to $\Delta ku70$ on MN (nitrate salts) supplemented with 300mM potassium chlorate, despite lacking a suitable nitrogen source. The elevated growth of $\Delta nia1$ and $\Delta nos3\Delta nia1$ were absent in the equivalent biomass assays which contained no agar. This potentially explains why all wildtype and mutant strains were able to grow on NM (non-nitrate salts) agar. It is therefore essential that growth assay data be cross-referenced with its corresponding biomass assay.

The combination of 3.3mM ammonium sulphate with 300mM potassium chlorate increased the MM diameter growth of $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$, mirroring the wildtype strains Guy11 and $\Delta ku70$. The absence of nitrate suppressed *NIA1* expression, preventing chlorate reduction. The biomass assay data confirmed the effect of ammonium sulphate on $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ growth. $\Delta nia1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ showed

resistance to potassium chlorate, confirming the targeted replacement of *NIA1*. *Δnii1* demonstrated susceptibility to potassium chlorate along with Guy11, *Δku70*, *Δnos2*, *Δnos3*, *Δnos2Δnos3* due to their carriage of an intact *NIA1*. The *Δnia1* data is consistent with the findings of Wilson et al. (2007), who reported that disruption of *NIA1* in *M. oryzae* resulted in a mutant which was unable to metabolise nitrate but was still capable of causing wildtype levels of disease on rice (Wilson et al. 2007).

6.4.5 Sodium tungstate nitrate reductase inhibition

Sodium tungstate is a specific inhibitor of nitrate reductase during protein assembly it is selectively incorporated in favour of molybdenum, rendering the enzyme non-functional (Prins et al. 1980; Heimer et al. 1969). The effect of sodium tungstate on nitrogen metabolism in *M. oryzae* was investigated. Guy11, *Δku70*, *Δnos2*, *Δnos3* and *Δnos2Δnos3* demonstrated high levels of TM and MM diameter growth on NM (nitrate salts); however by contrast *Δnia1*, *Δnii1*, *Δnia1Δnii1* and *Δnos3Δnia1* developed equivalent TM diameter growth but significantly suppressed MM diameter growth. The addition of sodium tungstate to NM (nitrate salts) had little effect on *Δnia1*, *Δnii1*, *Δnia1Δnii1* and *Δnos3Δnia1* growth, each of which carries a disrupted nitrogen metabolism pathway. The addition of sodium tungstate to Guy11, *Δku70*, *Δnos2*, *Δnos3* and *Δnos2Δnos3* resulted in a strong reduction in MM diameter growth, the tungstate ion prevented the formation of functional *Nia1*, thus inhibiting nitrogen metabolism and growth. The partially increased MM diameter growth observed in Guy11, *Δku70*, *Δnos2*, *Δnos3* and *Δnos2Δnos3* relative to *Δnia1*, *Δnii1*, *Δnia1Δnii1* and *Δnos3Δnia1* may result from the incomplete inhibition of *Nia1* activity by tungstate, or from the extra nitrogen sources available within the agar. As this partial increase is absent from the corresponding biomass assay it suggests that the latter explanation is more likely. The biomass assay data confirms the growth assay result: the addition of sodium tungstate induces a *Δnia1* equivalent phenotype in Guy11, *Δku70*, *Δnos2*, *Δnos3* and *Δnos2Δnos3*.

The ability of *nos2*, *nos3*, *nia1* and *nii1* to generate NO remains unknown, this issue is addressed in the next chapter.

Chapter 7: Measuring Nitric Oxide in *Magnaporthe oryzae*

7.1 Measuring NO in biological samples

7.1.1 Introduction

Nitric oxide (NO) is challenging to measure in biological systems for a number of reasons. First, it rapidly reacts in cellular environments, generating a wide range of products, including NO₂, N₂O₃, N₂O₄, N₂O, HNO and peroxynitrite. Thus, in biological systems, NO exists only transiently. Its high diffusion coefficient (3300 μm²s⁻¹) and potential diffusion distance of 200 μM make NO difficult to measure accurately (Malinski et al. 1993). Second, with the possible exception of its synthesis within the mammalian immune system, the generation of NO is strictly controlled and only synthesised at low concentrations (Kone et al. 2003).

To date, the existence of NO within *M. oryzae* has yet to be definitively proved, this chapter seeks to determine if NO is generated within *M. oryzae*.

7.1.2 NO detection techniques

As the perceived importance of nitric oxide as a bioactive molecule has increased, so too has the need for quantitative techniques to measure it accurately. Currently, a wide range of techniques are available for measuring NO, that differ in their sensitivity, specificity, applicability and cost. These include: colorimetric assays (Schmidt & Kelm 1996; Tracey et al. 1990), chemiluminescent assays (Fontijn et al. 1970), electro-paramagnetic resonance (EPR) (Komarov & Lai 1995), gas chromatography and mass spectrometry (Yi et al. 2009) and fluorometric assays (Hilderbrand et al. 2005). To measure the generation of NO in living

biological samples appropriate techniques must be sensitive, specific and non-toxic. Due to these requirements many of the destructive assay techniques are unsuitable.

Colorimetric assays, such as the Griess assay, measure the conversion of arginine to citrulline in isolated protein samples, and record only the maximal activity of extracted NOS enzymes, this is difficult to relate to *in vivo* rates of NO synthesis (Lacza et al. 2005). EPR techniques are able to directly detect NO in samples, but do not allow for real-time NO monitoring and provide little or no spatial resolution (Nagano & Yoshimura 2002). Volumetric and electrochemical NO detection methods can monitor NO levels in solution; however, results are frequently hard to replicate and require calibration and corroboration by alternative techniques (Lacza et al. 2004).

Chemiluminescent assays record photons emitted following the gaseous reaction between NO and ozone. Whilst this technique is specific to NO, it is only suitable for large sample sizes (McMurtry et al. 2000). Gas chromatography and mass spectrometry, are highly specific for NO, but require expensive and specific equipment and have limited temporal and spatial resolution. In contrast, fluorometric assays meet many of the requirements needed for real-time NO monitoring in live samples. They are highly sensitive to NO, can be successfully used in single cells or small samples, are relatively non-toxic, allowing continuous data recording, and only require standard laboratory equipment for use. Therefore a fluorometric assay approach was deemed the most suitable technique to measure NO in *M. oryzae*.

7.2 Fluorescent probes

There are a wide range of different fluorescent probes available to detect NO. These probes can be split into two groups: those that directly interact with NO, and those which react with N_2O_3 (the product of NO oxidation). The advantages and disadvantages of these probes along with their modes of action are detailed below.

7.2.1 Direct NO binding probes

These probes interact physically, but not chemically, interact with NO, although binding may remove NO from the solution.

7.2.1.1 Fluorescent Nitric Oxide Cheletropic Traps (FNOCT)

FNOCTs interact and trap NO via a formal cheletropic reaction. NO is physically removed from the biological system making this method extremely specific to NO with a reduced chance of other reactive nitrogen species (RNS) influencing the results (Meineke et al. 1999). FNOCTs trap NO through the reaction with an o-quinodimethane derivative, generating a nitroxide radical adduct with an aromatic phenanthrene unit as the fluorophore. Reduction of the nitroxide radical to its corresponding hydroxylamine dramatically increases its fluorescence. Both the trap and hydroxylamine are fluorescent at different excitation and emission wavelengths, allowing three methods of NO monitoring: i), by recording the decrease in trap (o-quinodimethane derivative) fluorescence; ii) by measuring the increase in the hydroxylamine product fluorescence; iii) by measuring both the trap and hydroxylamine product fluorescence simultaneously (Meineke et al. 2000).

FNOCTs have many advantages: they are highly specific to NO, ideal for small samples, highly sensitive (detect NO at nM/L concentrations), non-cytotoxic, demonstrate homogeneous cell loading and do not react with hydrogen peroxide or superoxide (Meineke et al. 2000). However FNOCTs have several disadvantages. They react with peroxynitrite, which makes them less suitable for use in systems known to generate reactive oxygen species (ROS); they also directly remove NO from biological systems, which may potentially interfere with the organisms signalling pathways, influencing its physiology (Meineke et al. 1999). Most importantly, FNOCTs (at the time of study) are not commercially available making them unusable for monitoring NO levels within *M. oryzae*.

7.2.1.2 Acridine-TEMPO-DTCS-Fe(II)

Acridine-TEMPO-DTCS-Fe(II) is a fluorescent complex ($\lambda_{\text{excitation}} = 361 \text{ nm}$, $\lambda_{\text{emission}} = 438 \text{ nm}$) composed of 2,2,6,6-tetramethyl-piperidine-N-oxyl (TEMPO) labelled with acridine and N-dithiocarbosarcosine (DTCS)-Fe(II). Acridine-TEMPO-DTCS-Fe(II) directly interacts with NO via a spin exchange mechanism based upon the activation of guanylate cyclase through the reaction of its heme moiety and imidazole group with NO. The binding of NO to the heme iron disassociates the imidazole group causing a conformational change in the protein structure. The resulting reaction between NO and Fe(II) generates a stable non-fluorescent nitrosyl-Fe complex (Soh et al. 2001). This results in an NO concentration dependent decrease in fluorescence as the availability of fluorescent acridine-TEMPO-DTCS-Fe(II) reduces (Soh et al. 2001). EPR can additionally be used to measure the release of the stable TEMPO radical, an alternative method for monitoring NO (Katayama 2001).

The advantages of acridine-TEMPO-DTCS-Fe(II) include its direct interaction with NO and high specificity. Disadvantages include a poor detection limit of 100nM/L, making it is significantly less sensitive than other fluorescent probes, and oxygen competition with NO to bind the DTCS-Fe(II) complex, potentially underestimating *in vivo* NO levels (Katayama 2001). Similar to FNOCTs, acridine-TEMPO-DTCS-Fe(II) removes NO from biological systems potentially disrupting signalling pathways and is also currently commercially unavailable. Therefore Acridine-TEMPO-DTCS-Fe(II) was considered an impractical choice for monitoring NO within *M. oryzae*.

7.2.2 Indirect NO binding probes

A range of fluorescent probes have been developed including DAN, DAF-2 DA, DAF-FM DA, DAQ, DAR-4M AM and DAMBO-P^H which share the same basic reaction mechanism. Unlike FNOCTs and acridine-TEMPO-DTCS-Fe(II) these dyes do not interact directly with NO, but rather with the reactive nitrile species N_2O_3 , formed through the following reactions:

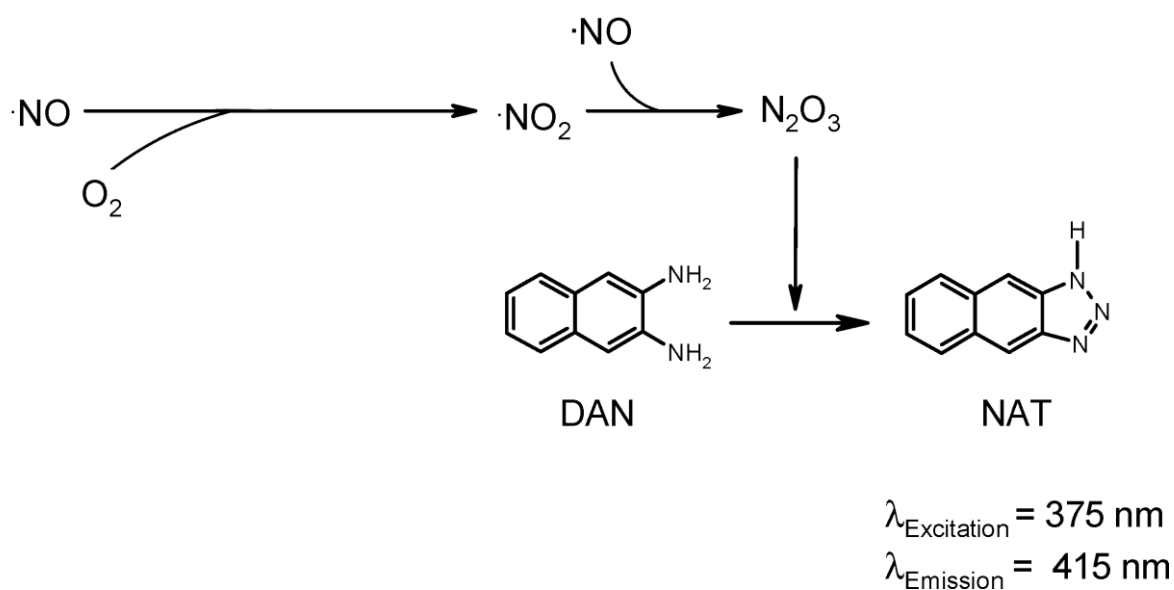
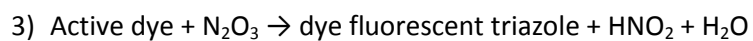
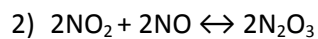
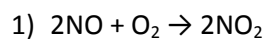


Figure 7.1. The reaction pathway detailing the formation of NAT from DAN in the presence of NO and O_2 . Mechanism adapted from Nagano (1999).

This biochemical detection pathway has the potential advantage that only a proportion of NO is removed from a biological system, reducing the impact on cellular physiology, making them more suitable for the continuous NO monitoring. However, the fact they do not directly react with NO means their reporting of its presence may be influenced or biased. For example reactions 1-3, leading to the formation of N_2O_3 from NO, are dependent on many factors, such

as O₂ levels, and not just the presence of NO. Therefore appropriate controls must be used to confirm any changes in fluorescence are due to NO dynamics.

Within this family of triazole probes, DAN (2, 3-Diaminonaphthalene), developed in 1970, was among the first fluorescent probes created specifically to detect NO (Wiersma 1970). DAN reacts with N₂O₃ forming the fluorescent triazole 2,3-naphthotriazol (NAT) $\lambda_{\text{excitation}}$ 375nm, $\lambda_{\text{emission}}$ 415nm, NAT is 90-100 fold more fluorescent than DAN. DAN offers significantly greater sensitivity (50-100 fold) to NO relative to the Griess assay which has a detection limit of 100nM/L NO (Nagano 1999). However, DAN requires UV excitation leading to high autofluorescence and cytotoxicity (Nakatsubo et al. 1998). As an alternative to DAN, diaminofluoresceins were developed by Nagano et al. (1998), to give triazoles with greatly increased quantum efficiency, relative to DAN, at longer excitation wavelengths (Gomes et al. 2006; Nagano et al. 1999). DAF-2 (DA) was originally used in conjunction with *M. oryzae* as DAF-2 (DA) represents the best studied and most widely used NO fluorescent probe in the literature (Iles M, DPhil Thesis, Oxford 2007). DAF-2 can be loaded into cells as a membrane permeable diacetate. Upon entering a viable cell, cytosolic esterases cleave the acetate groups from DAF-2 DA leaving the active dye DAF-2, which is sensitive to N₂O₃ (Kojima et al. 1998).

Advantages of DAF-2 include: a low NO detection limit of 5nM/L at less damaging excitation wavelength ($\lambda_{\text{excitation}}$ 495nm, $\lambda_{\text{emission}}$ 515nm, which leads to reduced sample autofluorescence and increased cell viability (Kojima et al. 1998). DAF-2 T exhibits a 180 fold increase in fluorescence over DAF-2. The presence of its acetate groups significantly increases cell loading efficiency and also prevent extracellular oxidation of DAF-2 DA, improving specificity for intracellular NO (Kojima et al. 1998). However, protonation of DAF-2 T OH phenolic groups at <pH7 leads to significant fluorescence quenching, resulting from a blue shift in the absorption wavelength (Kojima et al. 1998). DAF-2 also reacts with peroxynitrite and hydrogen peroxide via

the formation of a stable intermediate of DAF-2, which directly combines with NO, superseding the slower second order oxidation of NO (Jourdeuil 2002). DAF-2 also reacts with NO^- and NHO, generating higher triazole yields than with just NO (Espey et al. 2002). Furthermore Broillet et al. (2001) reported that divalent cations such as calcium interact with the dye-NO complex and favour the reaction towards the fluorescent product, potentially influencing data (Broillet et al. 2001). DAF-2 also reacts with dehydroascorbic acid (DHA) forming fluorescent DAF-2-DHAs with indistinguishable excitation and emission spectra to DAF-2 T (Zhang et al. 2002). Reducing agents such as, glutathione, dithiothreitol, catecholamines and 2-mercaptoethanol, diminish or abolish formation of DAF-2 T; through either interacting with NO, reducing the availability of NO to form N_2O_3 or by protonation of OH phenolic groups (Nagata et al. 1999, Ye et al. 2004).

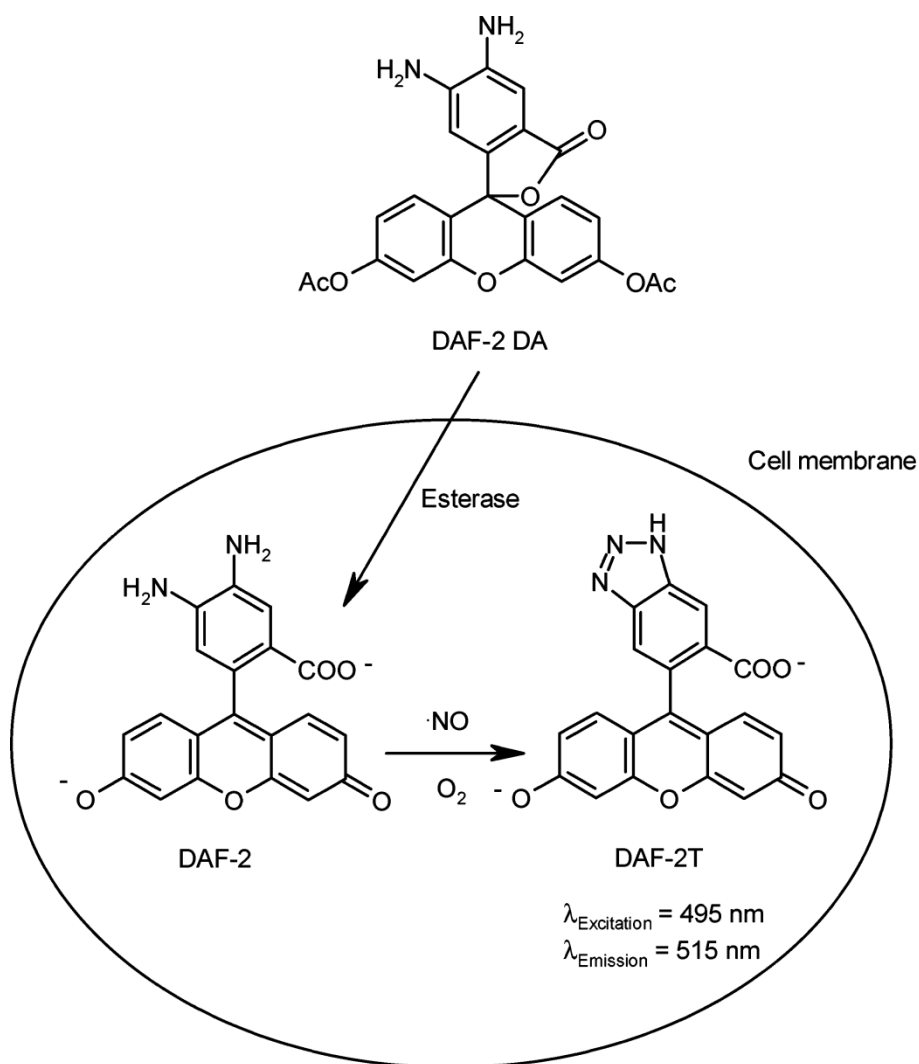


Figure 7.2. The mechanism of DAF-2 DA cell loading and conversion to DAF-2 T, through the reaction with N_2O_3 . Taken from Nakatsubo et al. (1998).

The pH sensitivity of DAF-2 was improved by replacing its chlorine atoms with fluorines, to give DAF-FM ($\lambda_{\text{excitation}} 495\text{nm}$, $\lambda_{\text{emission}} 515\text{nm}$) (Gomes et al. 2006). The fluorinated fluorescein fluorophore has greater photo-stability than chlorinated fluorescein. DAF-FM has a low NO detection limit of 3nM/L and is significantly less pH sensitive than DAF-2, allowing NO monitoring from pH 5.5 (Nagano 1999). DAF-FM is also less sensitive to peroxynitrite, and is not affected by physiological levels of nitrate or nitrite (Kojima et al. 1999). It is also less sensitive to

physiological concentrations of Ca^{2+} and Mg^{2+} and the chelating agents EDTA (Ethylenediaminetetraacetic acid), EGTA (ethyleneglycoltetraacetic acid) and DETAPA (diethyltriaminepentaacetic acid) (Nakatsubo et al. 1998; Balcerzyk et al. 2005). Nevertheless DAF-FM appears to show slow oxidation in the absence of ROS or RNS, and poor *in vivo* photostability; attributed to the light dependant disassociation of S-NO-glutathione (NO donors) and reactivity with superoxide (Balcerzyk et al. 2005; Lacza et al. 2005). In particular, susceptibility to superoxide, which is known to be generated by *M. oryzae* during appressorium maturation, suggested DAF based probes might generate ambiguous results when used with *M. oryzae* (Egan et al. 2007).

Substituting the fluorescein fluorophore with rhodamine gives a higher quantum yield and longer excitation wavelength than the DAF probe. DAR-4M (AM) has several associated advantages including enhanced pH stability; allowing it to be successfully utilised in samples as low as pH4, as DAR-4M T lacks phenolic OH groups. It demonstrates high photostability and its long excitation and emission wavelengths reduce sample cytotoxicity and autofluorescence. Like DAF-FM it has a low NO detection limit of 3nM/L (Lacza et al 2005). Lacza et al. (2005) demonstrated DAR-4M to have significant *in vitro* specificity for NO and low cross-reactivity with hydrogen peroxide and peroxynitrite (Lacza et al 2005). The only significant disadvantage associated with DAR-4M is its reactivity with high concentrations of ascorbic acid (AA) forming DAR-4M-DHAs with indistinguishable excitation and emission spectra from DAR-4M T (Ye et al. 2008).

Further modifications of the fluorophore to use the BODIPY moiety to give DAMBO- P^{H} have recently been reported, DAMBO- P^{H} has significantly improved performance over other triazole probes, but is not yet commercially available (Gabe et al. 2004; Yanga et al. 2008; Huang et al. 2007).

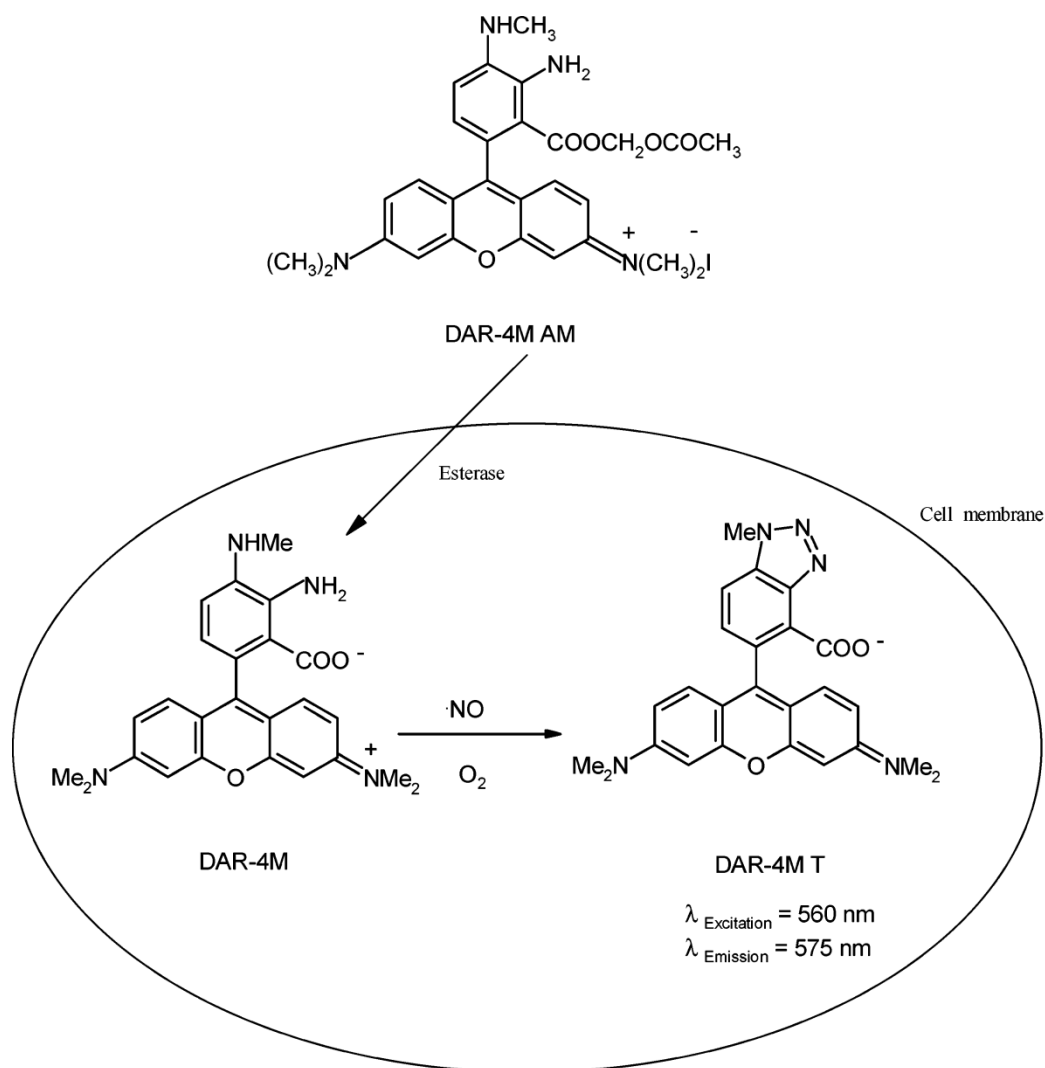


Figure 7.3. The mechanism of DAR-4M AM cell loading and its conversion to DAR-4M T following the reaction between DAR-4M and N_2O_3 . Taken from Gomes et al. (2006).

7.3 Experimental objectives

- 1) To choose a suitable fluorescent probe for monitoring NO in *M. oryzae*.
- 2) To characterise the probe of choice and develop a protocol for its use with *M. oryzae*.

- 3) To monitor NO generation within wildtype strains Guy11 and $\Delta ku70$ and confirm observed fluorescence is due the presence of intracellular NO.
- 4) To assess differences in NO generation between Guy11 and the mutant strains: $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$.
- 5) To identify the source of NO within *M. oryzae*.

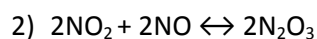
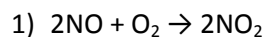
7.4 Fluorescent probe choice

Ideally, real-time monitoring of NO generation in *M. oryzae* would involve multiple NO detection methods including both fluorescent and non-fluorescent techniques. However, in reality, economic and time constraints meant only one NO measuring technique was investigated in detail. Of the fluorescent probes detailed above DAR-4M (AM) was selected as the most suitable for this purpose, based on its high NO sensitivity and specificity, coupled with its reduced cytotoxicity and commercial availability. There is extensive literature covering the use of DAR-4M (AM), although not specifically in fungi, which provide insight into the protocol development for the experimental use of DAR-4M (AM) with *M. oryzae*. A plate reader method was chosen in preference of using confocal microscopy for several reasons 1) multiple samples can be run simultaneously; 2) It provides an effective method to measure fluorescence over hours across the complete development cycle of *M. oryzae* without bleaching fluorescence or requiring significant man hours; 3) a large number of conidia can be measured as supposed to one; 4) test environmental conditions can be easily replicated.

7.4.1 DAR-4M (AM)

DAR-4M (AM) does not react directly with NO, rather DAR-4M reacts with N_2O_3 , generated through the oxidation of NO, to give the highly fluorescent triazole DAR-4M T ($\lambda_{excitation}$ 560nm, $\lambda_{emission}$ 575nm), outlined in reactions 1-3 (Kojima et al. 2001). The oxidation of NO, reaction 1,

follows a second order rate reaction pathway and is considered the rate limiting step in triazole formation (Espey et al. 2002).



7.5 Characterisation of DAR-4M (AM)

Prior to the characterisation of DAR-4M (AM) many important preliminary experiments were conducted using the viability stain fluorescein diacetate (FDA) to determine optimal conditions for utilising the FLUROstar fluorescent plate reader in conjunction with *M. oryzae*. These experiments included determining: i) the ability of the FLUROstar to measure fluorescence from conidia; ii) optimal position of the excitation and emission optics; iii) identification of a suitable 96 well plate that proved inductive to *M. oryzae* germination and development; iv) the number of wash steps required to remove contaminating extracellular esterase enzymes; v) the optimal density of conidia for fluorescence monitoring; vii) The optimal storage conditions for DAR-4M (AM) and conidia loading conditions, viii) Neither DAR-4M or DAR-4M AM affected the germination and development of Guy11 conidia inoculated into a NUNC 96 well optical bottom plate over 16 hours. These preliminary experiments are detailed in Chapter 2 (2.15).

7.5.1 *In vitro* characterisation of DAR-4M (AM)

7.5.1.1 Buffer choice

A range of pH values and types of buffer were assessed for their use with DAR-4M (AM). The ideal buffer should have no effect on conidia germination and development, or on dye stability. MES (2-(N-Morpholino)ethanesulfonic acid) and HEPES (N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) hemisodium salt) were identified as potential buffers (Good et al. 1966). Both buffers demonstrate high solubility and stability, have pKa values between pH 6-8, and are resistant to enzymatic degradation (Good et al. 1966).

The stability of DAR-4M and DAR-4M AM in MES and HEPES buffers were assessed over 16 hours. Both probes were buffered in either 10mM MES or 10mM HEPES at between pH 5-7 (MES) and pH 6.5-8 (HEPES).

DAR-4M buffered in MES pH5-6 demonstrated significant increases in fluorescence during the 16 hour test period. The observed increases in fluorescence may result from auto-oxidation of DAR-4M under acidic conditions or MES may enhance the adsorption of DAR-4M onto the NUNC 96 well plastic plates. As a result, 10mM MES was considered unsuitable for use with DAR-4M. In contrast, the esterified DAR-4M AM displayed stable fluorescence in both 10mM MES pH 5-7 and 10mM HEPES pH 6.5-8. The presence of its AM ester prevents auto-oxidation, inhibiting fluorescence generation. Thus, 10mM HEPES pH7 was selected as the most suitable buffer for use with DAR-4M and DAR-4M AM.

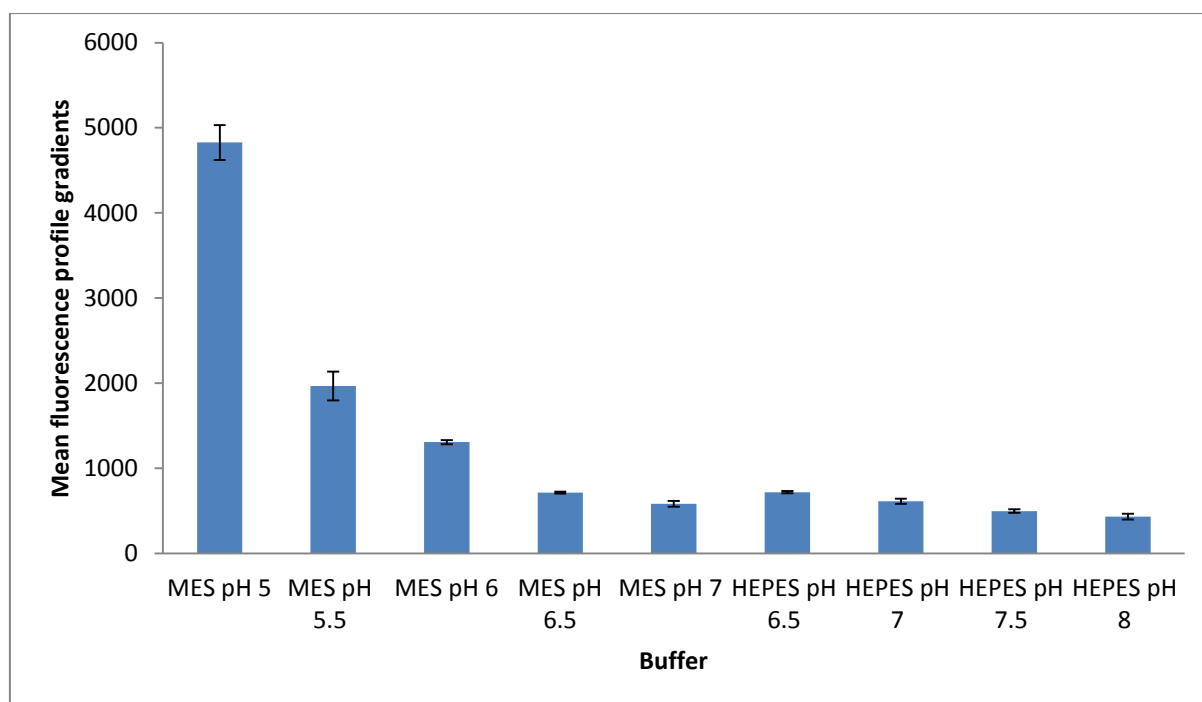


Figure 7.4. The effect of 10mM MES (pH 5-7) and 10mM HEPES (6.5-8) on 2 μ M DAR-4M fluorescence. Fluorescent profile gradients were calculated between 0 and 360 minutes. Fluorescence ($\lambda_{\text{excitation}} = 544 \text{ nm}$, $\lambda_{\text{emission}} = 590 \text{ nm}$) was measured at 20°C for 16 hours. Data represents three experimental repeats each containing three technical replicates. Error bars represent standard error of the mean.

(Fluorescent profile gradients are calculated by dividing the 360 minute fluorescence value by the 0 minute fluorescence value, the gradient is then plotted as a bar.)

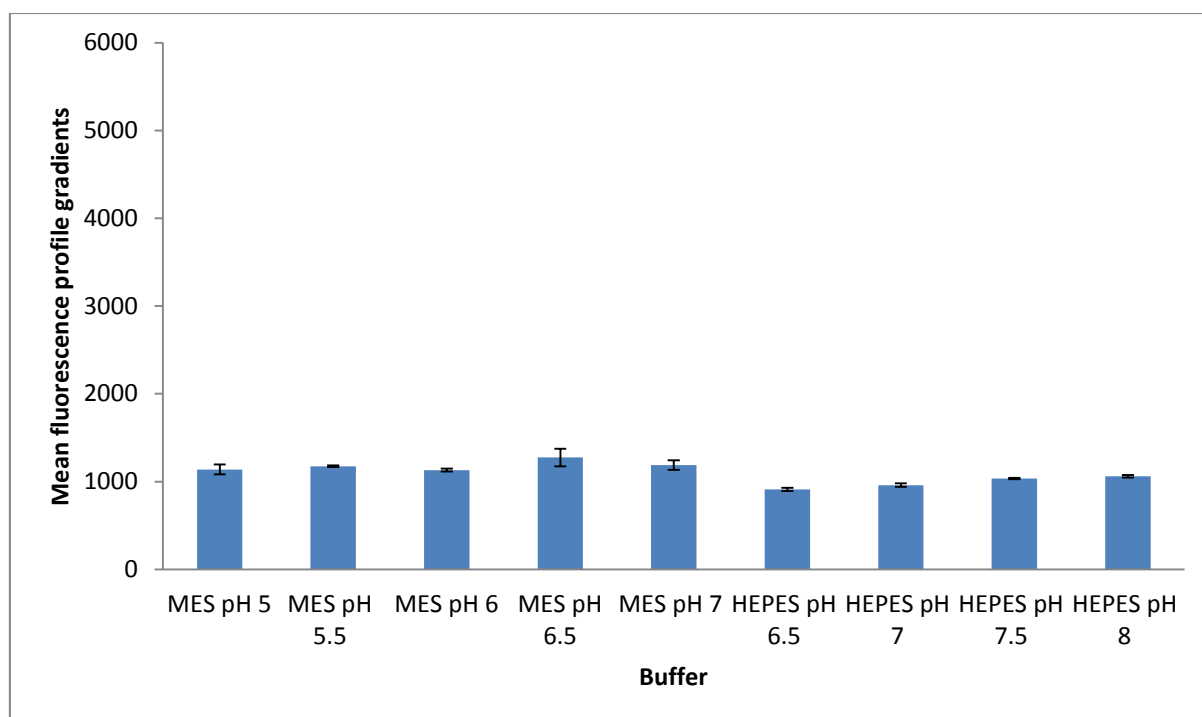


Figure 7.5. The effect of 10mM MES (pH 5-7) and 10mM HEPES (6.5-8) on 2 μ M DAR-4M AM fluorescence. Fluorescent profile gradients were calculated between 0 and 360 minutes. Fluorescence ($\lambda_{\text{excitation}} = 544 \text{ nm}$, $\lambda_{\text{emission}} = 590 \text{ nm}$) was measured at 20°C for 16 hours. Data represents three experimental repeats each containing three technical replicates. Error bars represent standard error of the mean.

The effect of 10mM HEPES pH 6.5-7 on Guy11 conidia germination and development were scored on TAAB plastic coverslips, a surface inductive for melanised appressoria formation. Guy11 conidia germination and development in 10mM HEPES were compared to conidia inoculated in demineralised H₂O. No significant differences in the percentage of melanised appressoria (MA) formation were observed between Guy11 conidia inoculated in H₂O and 10mM HEPES between pH 6.5-7.5, (p-values: 0.818, 0.51 and 0.88, respectively). However, Guy11 conidia inoculated in 10mM HEPES pH8 demonstrated a significantly reduced level of MA formation relative to Guy11 conidia inoculated in H₂O. Thus, 10mM HEPES pH7 was selected for use with *M. oryzae* and DAR-4M (AM), all further experiments were conducted using 10mM HEPES pH7. Maintaining a neutral pH is essential for MA formation in *M. oryzae*;

phytopathogenic fungi are especially sensitive to environment pH, which may regulate their infection related development (Miyara et al 2010; Davidzon et al. 2010; Yakoby et al. 2000).

Hence any assay which specifically investigates the development of infection related structures, i.e. appressoria, must be pH controlled.

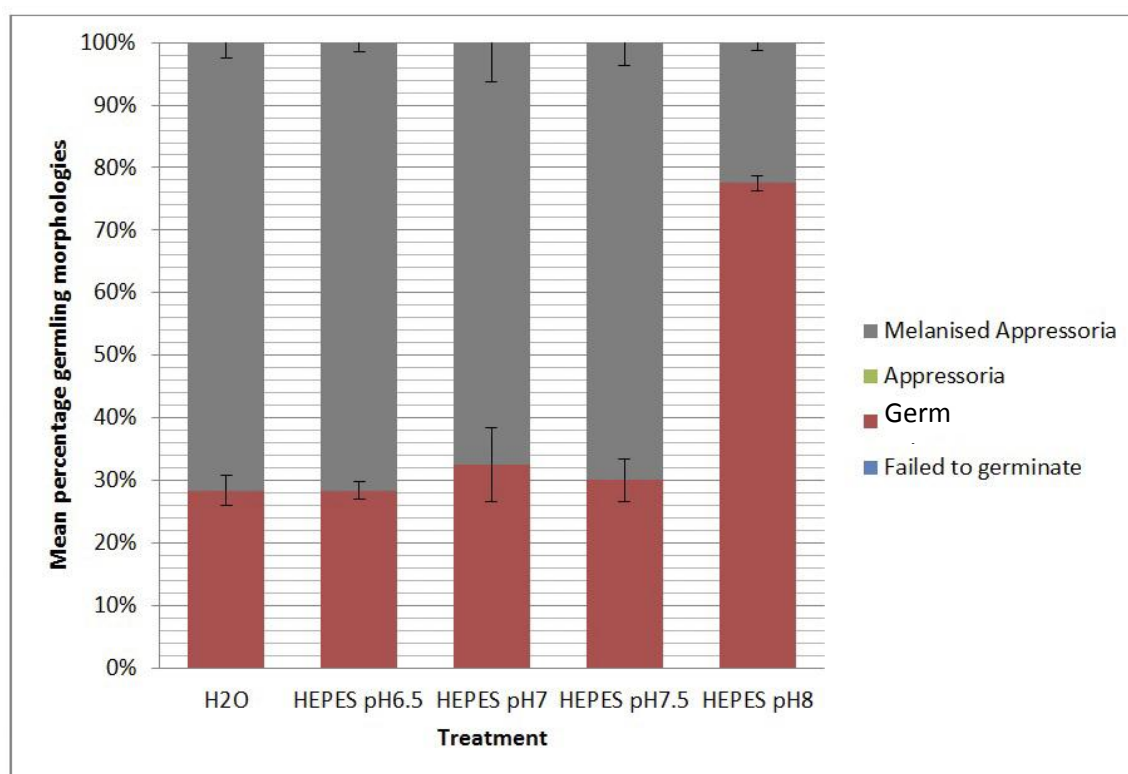


Figure 7.6. The effect of demin H₂O and 10mM HEPES pH 6.5-7 on Guy11 (1×10^4) conidia germination and development, 12hpi, on TAAB plastic coverslips, a surface inductive to MA formation. Mean percentage scores of germling morphologies were drawn from three independent experimental replicates, containing three biological replicates; for each biological replicate 120 germlings were assessed. Error bars represent the standard error of the mean.

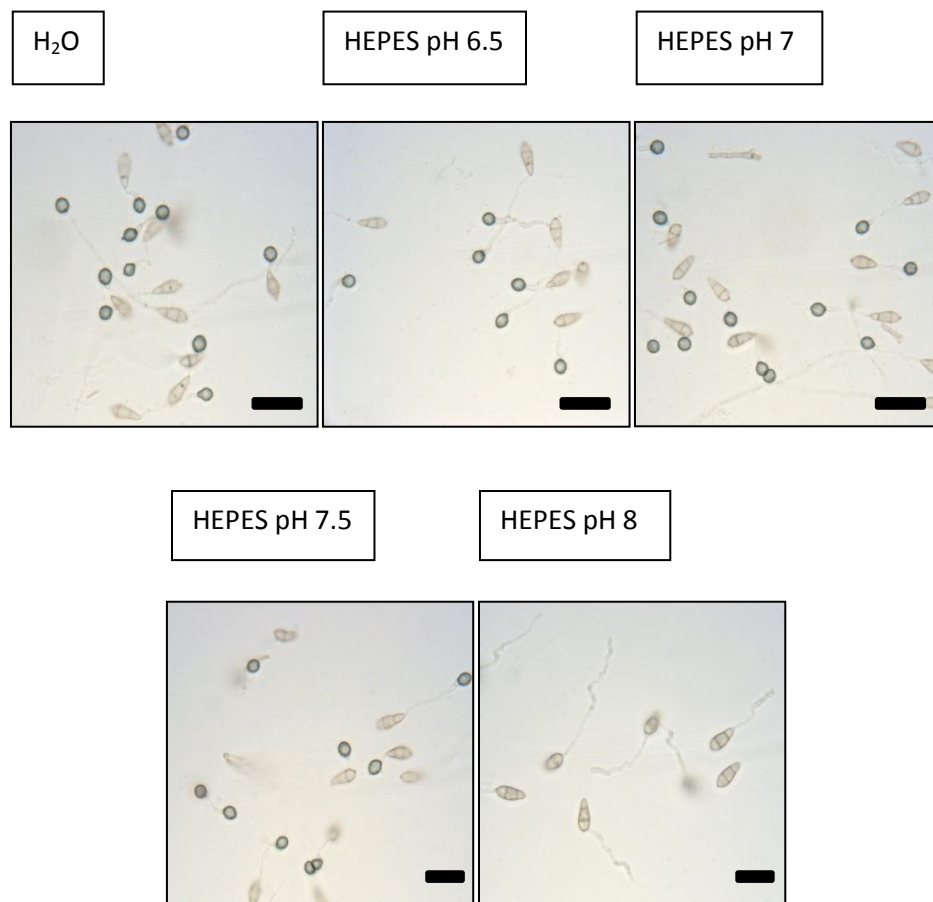
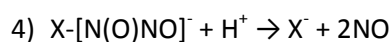


Figure 7.7. Guy11 germling morphologies following inoculation in H₂O, 10mM HEPES pH6.5, 7, 7.5 and 8, 12hpi on TAAB plastic coverslips, a surface inductive to MA formation. Scale bars = 20μM.

7.5.1.2 NO donor characterisation

To quantify the *in vitro* reaction between DAR-4M (AM) and NO, NO was exogenously supplied via one of two NO donors: DETANONOate or PAPANONOate. NONOate (1-substituted diazen-1-ium-1,2-diols) compounds are NO donors which contain the $[N(O)NO]^+$ functional group. Upon dissolution in neutral solution NONOates dissociate releasing NO, reaction 4, following a first order rate reaction pathway (Keefer et al. 1996).



The relative rate of NO release is controlled by the half-life of each NONOate; determined by their chemical structure and pH environment (Hrabie et al. 1993). NO disassociation from DETANONOate has a 57 hour half-life at pH 7.4, 20°C, resulting in a near-linear NO release over 16 hours. In contrast PAPANONOate has a half-life of 77 minute at pH 7.4 and 20°C, delivering a rapid burst of NO relative to DETANONOate (Keefer et al. 1996).

The *in vitro* response of DAR-4M and DAR-4M AM to NO were assessed; both probes were incubated with 20µM DETANONOate and 20µM PAPANONOate (buffered in 10mM HEPES pH7 with NaOH), and the fluorescence recorded over 16 hours. The control compound SULFONONOate, which does not release NO within a physiological pH range, was used to confirm that observed increases in fluorescence were due to the interaction of DAR-4M (AM) and NO/ N₂O₃ (Hrabie et al. 1993).

The kinetics of NO disassociation from DETANONOate and PAPANONOate, under non O₂ limiting environments, determines the rate of DAR-4M T formation, until all available dye has reacted. The significantly shorter half-life of PAPANONOate results in an increased concentration of NO relative to DETANONOate, culminating in a faster rate of triazole formation. The high concentration of PAPANONOate derived NO rapidly saturates and oxidises all available DAR-4M present to DAR-4M T, generating a steep initial gradient that saturates after five hours. In contrast to PAPANONOate, DETANONOate releases NO at a near-linear rate

over the equivalent time period resulting in a lower fluorescence profile gradient. The addition of SULFONONOate has no effect on fluorescence generation over the baseline auto-oxidation of DAR-4M in 10mM HEPES pH7. These data are consistent with (Kojima et al. 2001).

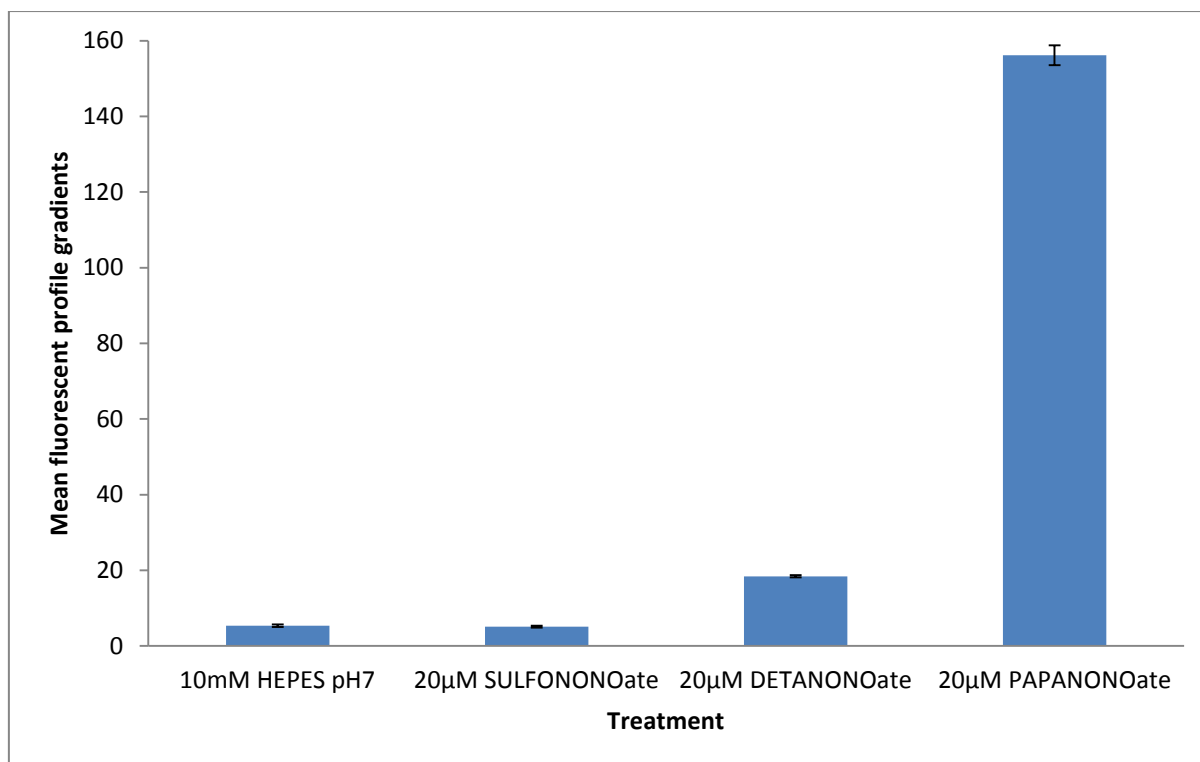


Figure 7.8. The effect of 20μM DETANONOate, 20μM PAPANONOate and 20μM SULFONONOate (25μM NaOH) on 2μM DAR-4M (10mM HEPES pH7) fluorescence. Fluorescent profile gradients were calculated between 0 and 360 minutes. Fluorescence ($\lambda_{\text{excitation}} = 544 \text{ nm}$, $\lambda_{\text{emission}} = 590 \text{ nm}$) measured for 16 hours at 20°C. Data represents three independent experiments; each containing 10 technical replicates. Error bars standard error of the mean.

The presence of the AM ester inhibits triazole fluorescence until its removal. The ester group confers cell permeability, and *in vivo* is cleaved by cytoplasmic esterases yielding DAR-4M.

However, *in vitro*, DAR-4M AM slowly degrades to its active form, DAR-4M. Thus an increase in fluorescence from DAR-4M AM was also observed with PAPANONOate in the absence of cells (Kojima et al. 1997). These data are consistent with the DAR-4M literature (Lacza et al. 2005).

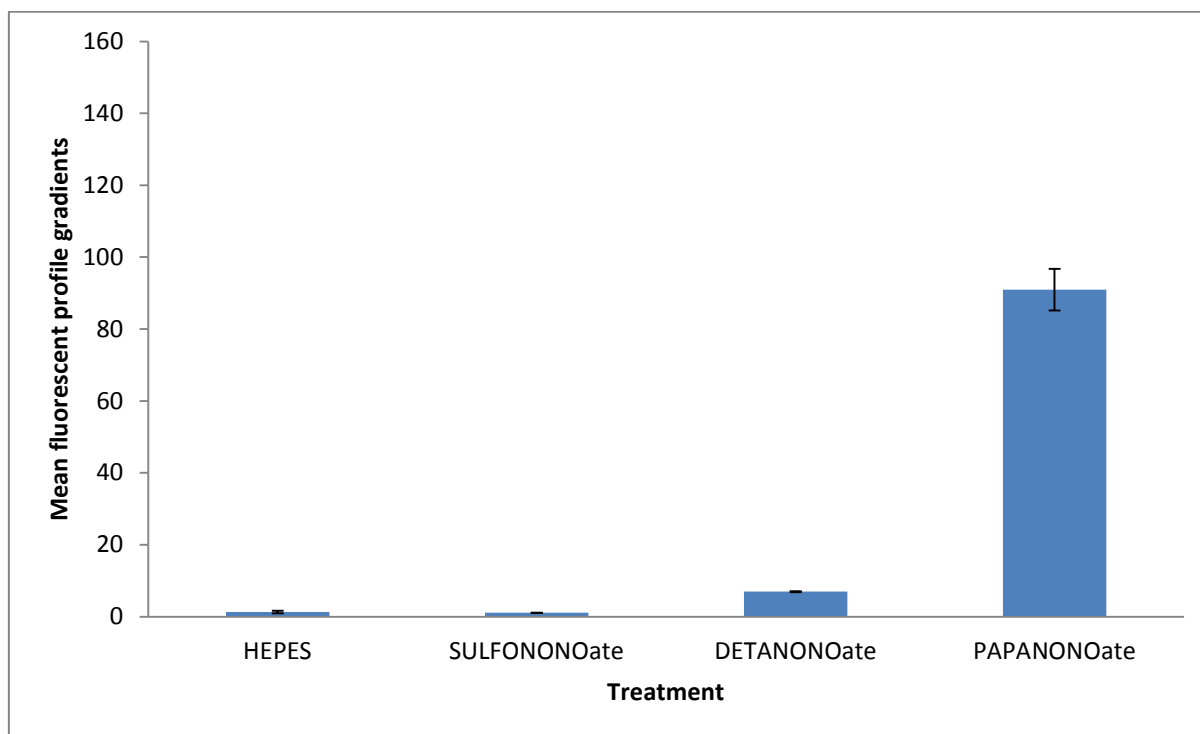


Figure 7.9. The effect of 20 μ M DETANONOate, 20 μ M PAPANONOate and 20 μ M SULFONONOate (25 μ M NaOH) on 2 μ M DAR-4M AM (10mM HEPES pH7) fluorescence. Fluorescent profile gradients were calculated between 0 and 360 minutes. Fluorescence ($\lambda_{\text{excitation}} = 544$ nm, $\lambda_{\text{emission}} = 590$ nm) measured for 16 hours at 20°C. Data represents three independent experiments; each containing 10 technical replicates. Error bars standard error of the mean.

7.5.2 *In vivo* characterisation of DAR-4M (AM)

7.5.2.1 Time course of DAR-4M fluorescence

To monitor the real time generation of NO in *M. oryzae*, Guy11 conidia were loaded in 2 μ M DAR-4M AM (10mM HEPES pH7) as outlined in Chapter 2 (2.15.2). Following the completion of dye loading, conidia were inoculated into NUNC 96 well optical bottom plates and the resulting fluorescence recorded by FLUROstar fluorescent plate reader.

Guy11 conidia loaded with DAR-4M AM showed an increase in fluorescence during the 16 hour test period. This suggests that during germination and development conidia are generating NO, leading to fluorescent triazole formation. However, there are three issues associated with this data set. Firstly, there is an initial decrease in fluorescence across all samples at the start of each experiment. Secondly, there is a significant degree of autofluorescence, both machine and cell derived. Thirdly, the observed increase in fluorescence from Guy11 conidia loaded with DAR-4M AM is not conclusive evidence that the conidia are generating NO or that DAR-4M is reacting with NO/ N₂O₃. Fluorescence may arise from non-NO specific reactions. These points must therefore be addressed when analysing the time series data.

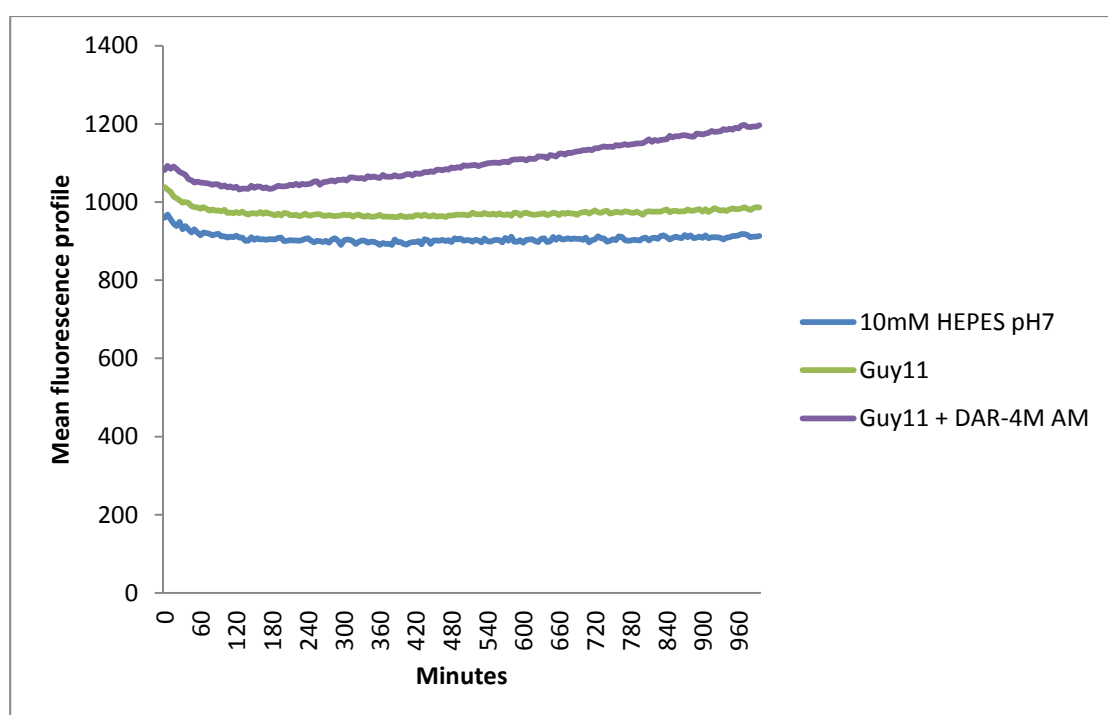


Figure 7.10. Uncorrected FLUORstar fluorescent profile data from: 10mM HEPES pH7, Guy11 germlings (1×10^4 conc.) 10mM HEPES pH7 and Guy11 (1×10^4 conc.) 10mM HEPES pH7 loaded in 2 μ M DAR-4M AM. Fluorescence ($\lambda_{\text{excitation}} = 544$ nm, $\lambda_{\text{emission}} = 590$ nm) recorded over 16 hours at 20°C. Mean fluorescence calculated from four independent experiments, each containing three biological replicates.

The initial decrease in fluorescence was found to be independent of fluorophore presence or absence and affected all samples. The magnitude of its affect was directly proportional to the starting fluorescence level, i.e. the greater the starting fluorescence the greater the initial decrease. This observation was found to be a common feature of BMG FLUORstar 96 well fluorescent plate readers across multiple machines. The manufacturer was unable to fix this phenomenon despite repeated attempts. Therefore to counteract this phenomenon, multiplicative correction factors were generated from the 10mM HEPES pH7 control sample data. The 10mM HEPES pH7 buffer control was therefore used to normalise the experimental data, Figure 7.11.

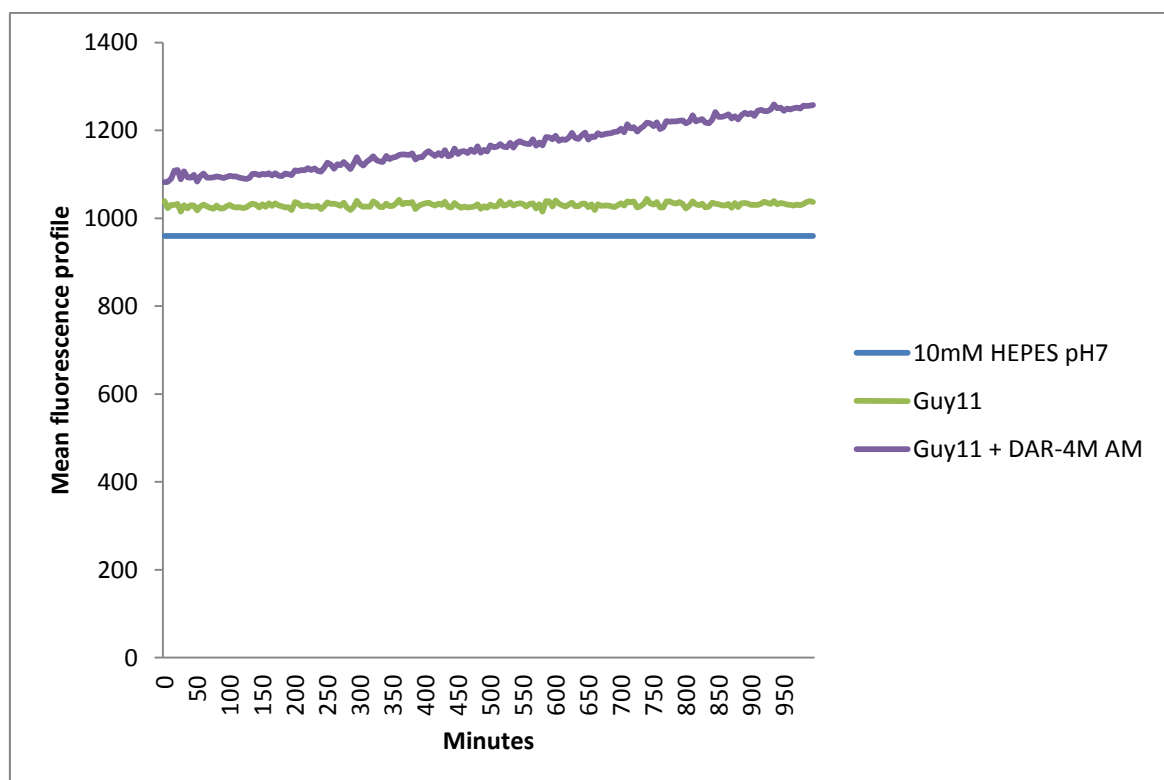


Figure 7.11. Data from Figure 7.11 corrected for the initial drop in fluorescence phenomenon. Fluorescence ($\lambda_{\text{excitation}} = 544 \text{ nm}$, $\lambda_{\text{emission}} = 590 \text{ nm}$) recorded over 16 hours at 20°C. Mean fluorescence calculated from four independent experiments, each containing three biological replicates.

Monitoring NO levels within *M. oryzae* is difficult, not only because of the transient nature of NO, but because conidia only retain a percentage of the original 2 μM DAR-4M AM loading solution. Following inoculation into NUNC 96 well plates, loaded conidia rapidly settle to the bottom of each well simplifying the optical path for excitation but yielding low levels of fluorescence. The gain setting required, also gave a high instrument dark current signal. To correct for dark current the 10mM HEPES pH7 data was subtracted from the data set to leave only the DAR-4M and conidia derived fluorescence.

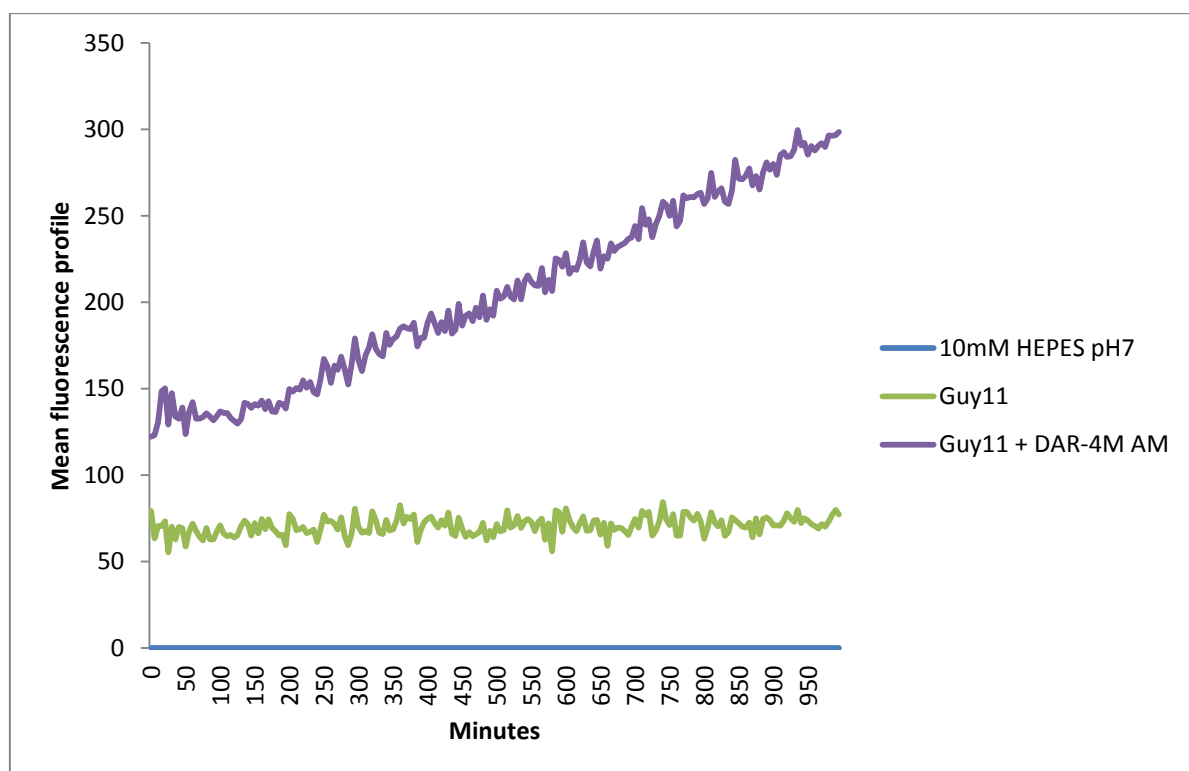


Figure 7.12. Data from Figure 7.11 corrected for the initial drop in fluorescence phenomenon and machine derived autofluorescence. Fluorescence ($\lambda_{\text{excitation}} = 544 \text{ nm}$, $\lambda_{\text{emission}} = 590 \text{ nm}$) recorded over 16 hours at 20°C. Mean fluorescence calculated from four independent experiments, each containing three biological replicates.

Finally, the increase in fluorescence from Guy11 conidia loaded with DAR-4M AM, cannot at this stage be solely assigned to intracellular NO generating DAR-4M T through reactions 1-3. DAR-4M, (as discussed in 7.2.2), may react with high concentrations of ascorbic acid (AA) forming DAR-4M-DHAs with indistinguishable excitation and emission spectra from DAR-4M T (Zhang et al. 2002; Ye et al. 2008). Currently there is no data in the literature regarding AA concentrations in *M. oryzae* or related species; however in plants it is known that AA concentrations can reach concentrations as high as 50mM (Smirnoff 1996). Therefore, it is prudent to assume AA may be present within *M. oryzae* and potentially reacting with DAR-4M to generate DAR-4M-DHAs (Linster & Clarke 2008). To confirm the findings of Zhang et al. (2002) and Ye et al. (2008) a range of buffered AA concentrations were reacted with DAR-4M and DAR-4M AM, their resulting fluorescence were recorded over 16 hours.

The addition of up to 1mM AA did not significantly increase the rate of DAR-4M derived fluorescence. However above this concentration, AA significantly increased the level of DAR-4M derived fluorescence generation in accordance with Ye et al. (2008).

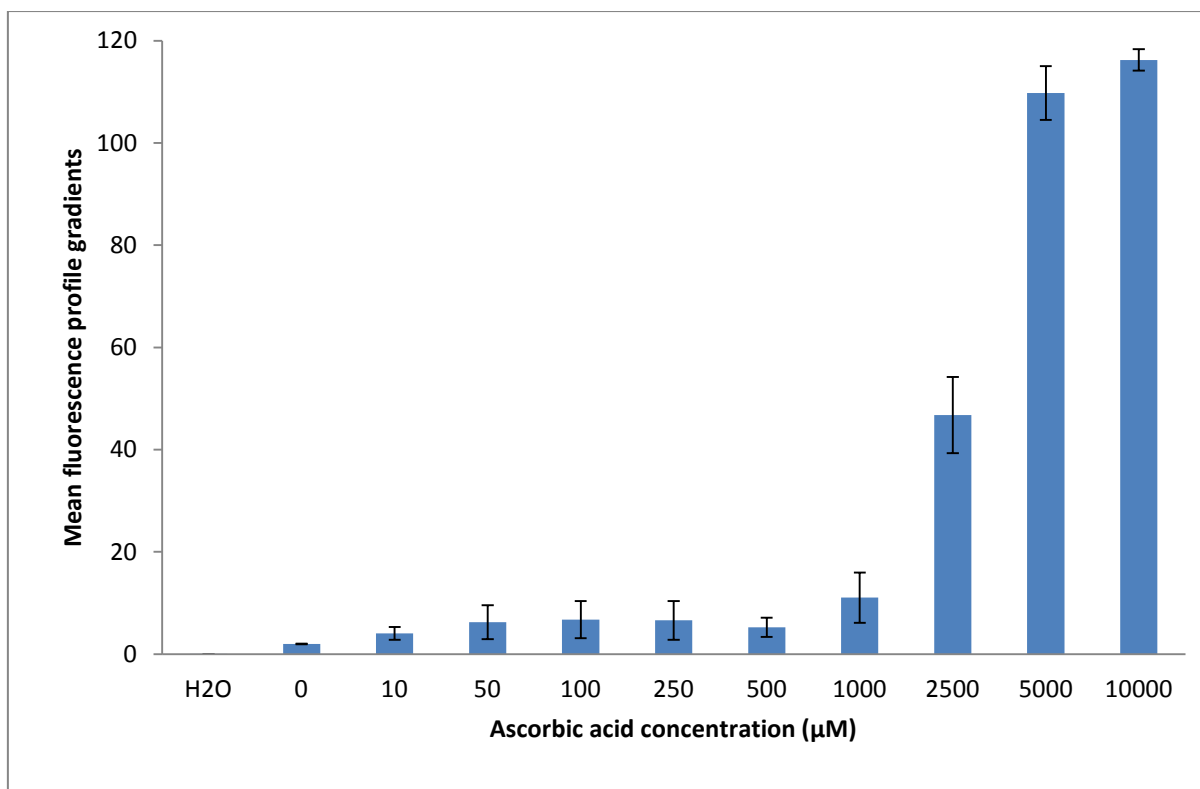


Figure 7.13. The effect of increasing concentrations of AA on 2μM DAR-4M (in buffered media) derived fluorescence. Fluorescence ($\lambda_{\text{excitation}} = 544 \text{ nm}$, $\lambda_{\text{emission}} = 590 \text{ nm}$) was recorded over 16 hours at 20°C. Gradients were calculated between 0 and 450 minutes. Mean fluorescence profile areas represent four independent experiments, each containing three biological replicates. Error bars represent the standard error of the mean.

The addition of AA had no significant effect on DAR-4M AM derived fluorescence, however, addition of 5 and 10mM AA significantly increased the level of DAR-4M AM fluorescence, p values 0.09 and 0.32 respectively). The reduced effect of AA on DAR-4M AM relative to DAR-4M is due to the presence of its AM ester group. Only DAR-4M AM which has degraded to DAR-4M (discussed in 7.5.1.2) was able to react with AA. The effect of AA on DAR-4M AM derived fluorescence was significantly less than its effect on DAR-4M. Nevertheless it is clear that high intracellular AA levels will readily react with DAR-4M.

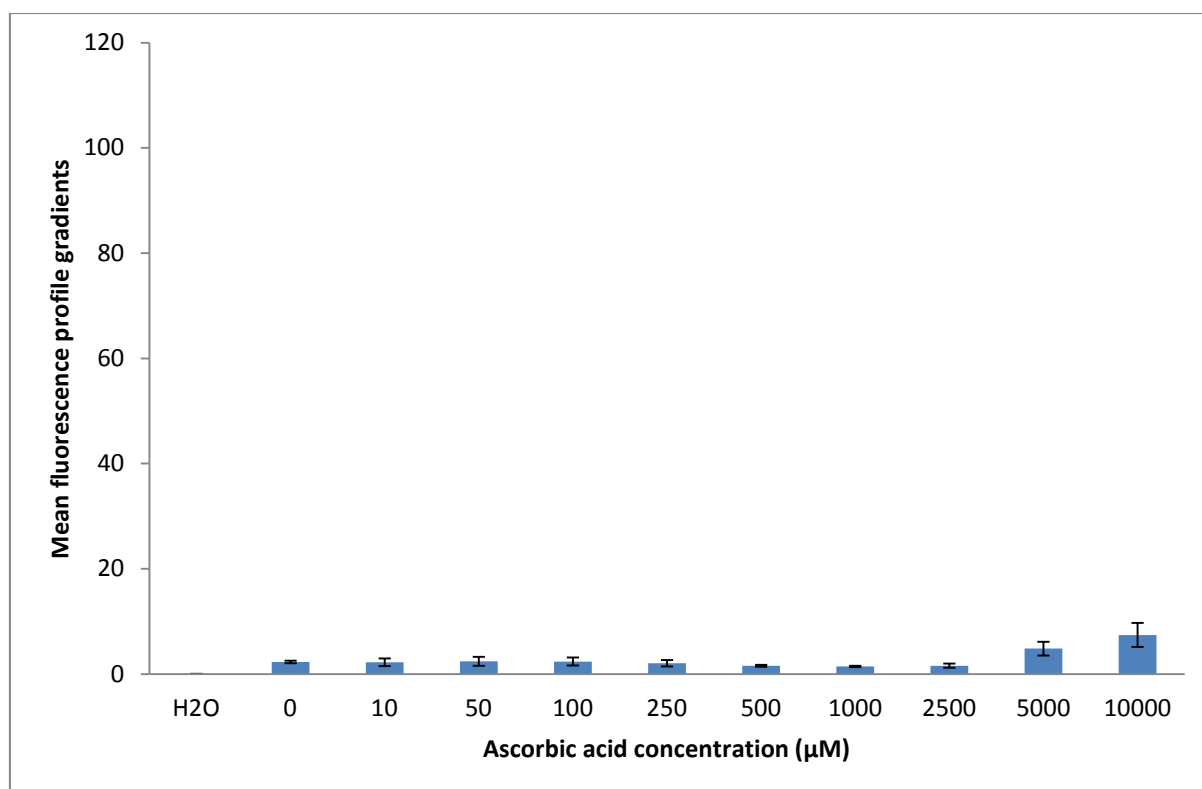
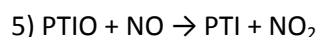


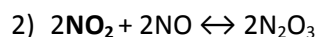
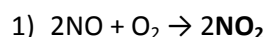
Figure 7.14. The effect of increasing concentrations of AA on 2μM DAR-4M AM (in buffered media) derived fluorescence. Fluorescence ($\lambda_{\text{excitation}} = 544 \text{ nm}$, $\lambda_{\text{emission}} = 590 \text{ nm}$) was recorded over 16 hours at 20°C. Gradients were calculated between 0 and 450 minutes. Mean fluorescence profile areas represent four independent experiments, each containing three biological replicates. Error bars represent the standard error of the mean.

7.5.2.2 The PTIO enhancement technique

Confirmation that high AA concentrations readily react with DAR-4M to form DAR-4M-DHAs presented a further problem in assigning any changes in DAR-4M AM loaded conidia fluorescence to the measure of NO. To overcome this problem, a technique using a more complex assay was developed using the NO scavenger PTIO (2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide). Unlike NOS inhibitors which interact directly with NOS enzymes to inhibit NO synthesis, PTIO scavenges NO by directly reacting with NO to form NO_2 , reaction 5 (Goldstein et al. 2003).



PTIO is widely used as an effective NO scavenger to inhibit NO responses. However, the product of PTIO mediated NO scavenging, NO₂, is an important intermediate molecule required for the formation of triazole complexes, as it helps to form N₂O₃ needed to generate fluorescent triazoles



In reaction 1, the oxidation of NO to NO₂ is a second order rate reaction, and the rate limiting step in triazole formation (Kharitonov et al. 1994). Small differences in the relative concentrations of either NO or O₂ can dramatically influence the rate of triazole formation (Espey et al. 2002). PTIO accelerates oxidative formation of NO₂ via a spin trap oxygen transfer reaction (Nakatsubo et al. 1998). At low concentrations the PTIO induced formation of NO₂ is stoichiometrically related to increased formation of N₂O₃ and hence fluorescent triazole formation (Goldstein et al. 2003). At higher PTIO concentrations formation of NO₂ predominates and NO is decreased sufficiently to avoid N₂O₃ formation. Thus a signature of NO production should be stimulation of triazole fluorescence at low PTIO concentrations and inhibition at high concentrations. This effect should be unique to NO as there is no reason to expect this bimodal effect for AA, for example.

7.5.2.3 The NO scavenger PTIO enhances *in vitro* DAR-4M fluorescence

The addition of increasing concentrations of PTIO up to 175 μM to 2 μM DAR-4M (10mM HEPES pH7) in the presence of 20 μM DETANONOate resulted in an increase in fluorescence generation relative to 2 μM buffered DAR-4M and 20 μM DETANONOate. At low concentrations PTIO accelerates the formation of NO_2 , overriding the slow O_2 dependent oxidation of NO, whilst sufficient NO is still available from DETANONOate to complete formation of N_2O_3 , and triazole formation. The addition of higher concentrations of PTIO resulted in a decrease in fluorescence generation as all the available NO is converted to NO_2 . Therefore no NO is available to react with NO_2 to complete reaction 2, resulting in decreased triazole formation, represented as a negative profile area relative to the 0 μM PTIO control. These DAR-4M data agree with results reported by Nakatsubo (1998) observed with DAN (Nakatsubo et al. 1998).

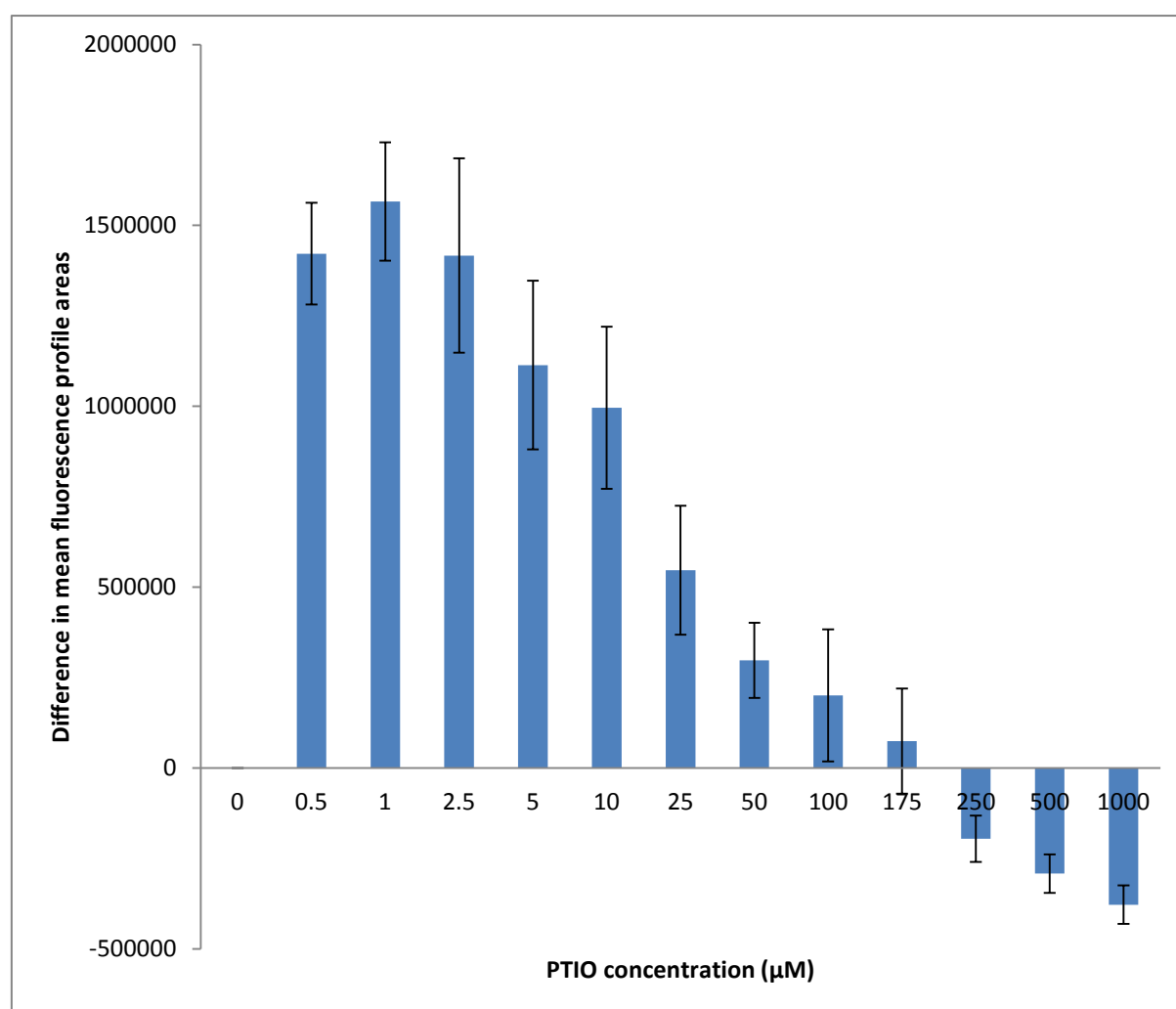


Figure 7.15. The effect of varying concentrations of PTIO on 2 μ M DAR-4M (in buffered media) and 20 μ M DETANONOate fluorescence. Data corrected as detailed in 7.5.2.6. Fluorescence ($\lambda_{\text{excitation}}=544$ nm, $\lambda_{\text{emission}}=590$ nm) was recorded over 16 hours at 20°C. Each bar represents the difference in area between the 0 μ M PTIO control and PTIO treatment. Mean fluorescence profile areas were calculated from three independent experiments, each containing three biological replicates. Error bars represent the standard error of the mean.

7.5.2.4 The NO scavenger PTIO enhances *in vivo* DAR-4M AM fluorescence

The same PTIO enhancement technique was applied to Guy11 conidia loaded in DAR-4M AM. In a similar manner the addition of 1 and 2.5 μ M PTIO significantly increased the fluorescence from Guy11 conidia loaded with DAR-4M AM relative to the 0 μ M PTIO control, (p-values: 0.002 and 0.038, respectively). The addition of 5 and 10 μ M PTIO had no significant effect, whilst, the addition of higher concentrations of PTIO significantly reduced the fluorescence. These data provide good evidence of NO generation *in vivo* in *M. oryzae*.

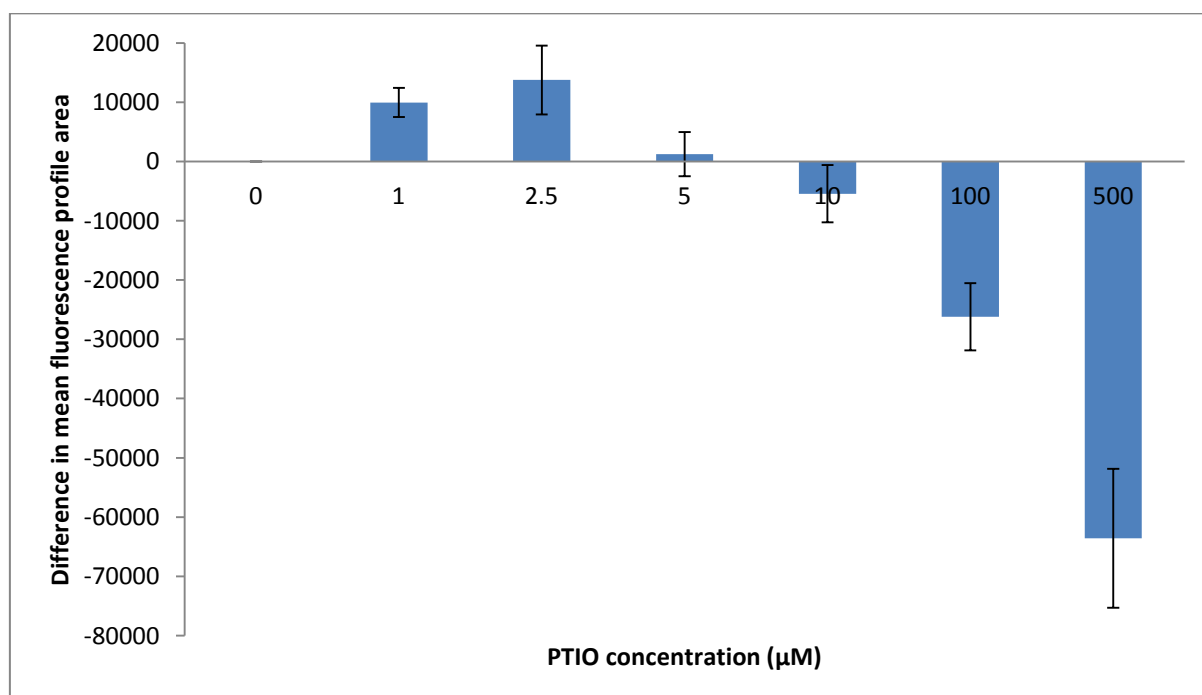


Figure 7.16. The effect of increasing concentrations of PTIO on the fluorescence of Guy11 germlings loaded in 2 μ M DAR-4M AM (in buffered media). Data were corrected as detailed in 7.5.2.6. Fluorescence ($\lambda_{\text{excitation}}=544$ nm, $\lambda_{\text{emission}}=590$ nm) was recorded over 16 hours at 20°C. Each bar represents the difference in area between the 0 μ M PTIO control profile and PTIO treatment profiles. Mean fluorescence profile areas were calculated from three independent experiments, each containing three biological replicates. Error bars represent the standard error of the mean.

7.5.2.5 Intracellular NO generation

The PTIO enhancement technique confirmed the interaction of NO/ N₂O₃ and DAR-4M AM, but provides only circumstantial evidence for NO generation within *M. oryzae*. The use of a cell permeable dye, DAR-4M AM suggests fluorescence is intracellular; however it is plausible that AM ester is hydrolysed externally and the dye is reacting with extracellular NO/ N₂O₃, forming DAR-4M T. Thus Guy11 conidia were incubated in DAR-4M (non-cell permeable) and treated with varying PTIO concentrations. If PTIO treatment of Guy11 conidia loaded in DAR-4M resulted in an increase in fluorescence relative to a non-PTIO treated control, this would indicate the NO source to be extracellular. However, if the NO source is located intracellularly, no increase in fluorescence generation should be observed following PTIO treatment of Guy11 as the stimulatory effect of low concentrations of PTIO would not be observed.

The addition of PTIO to Guy11 conidia loaded with DAR-4M (10mM HEPES pH7) resulted in a concentration dependent decrease in fluorescence profile areas between Guy11 conidia treated with PTIO and the non-PTIO treated control. With is no evidence for a PTIO dependent increase in DAR-4M derived fluorescence.

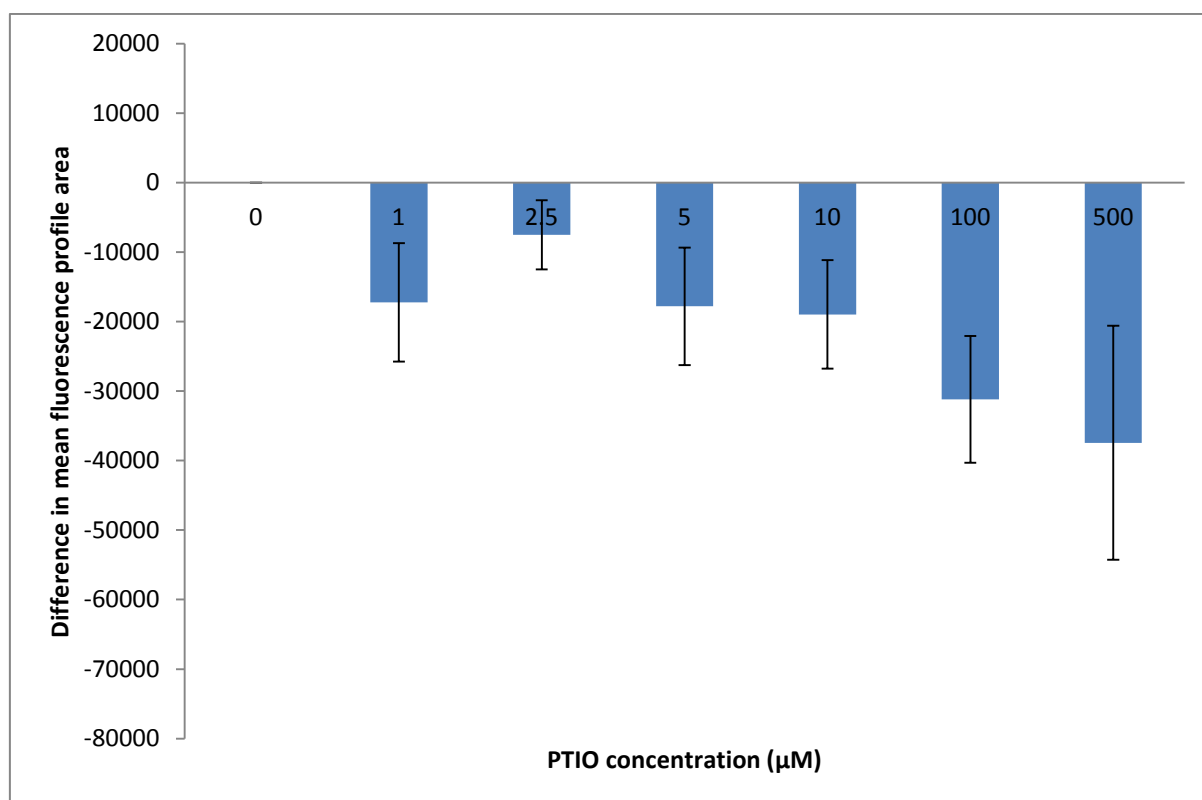


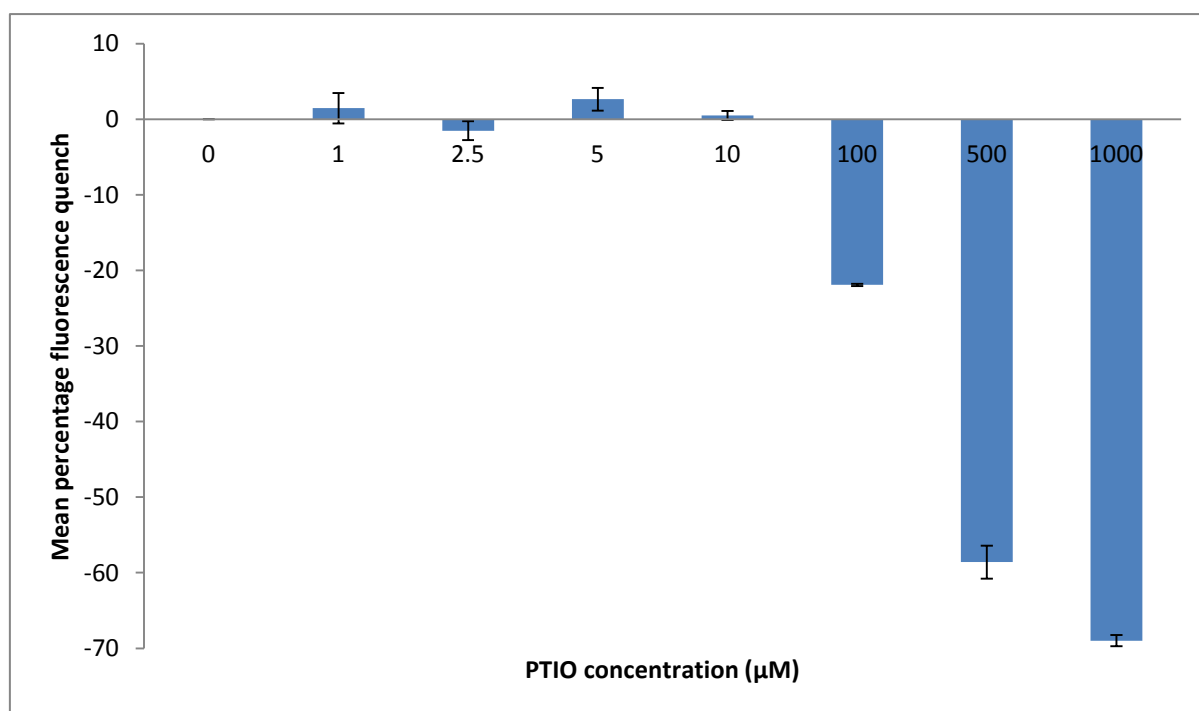
Figure 7.17. The effect of increasing concentrations of PTIO on the fluorescence of Guy11 germlings loaded with 2μM DAR-4M (in buffered media). Data were corrected as detailed in 7.5.2.6. Each bar represents the difference in area between the 0μM PTIO control profile and PTIO treatment profiles. Fluorescence ($\lambda_{\text{excitation}} = 544 \text{ nm}$, $\lambda_{\text{emission}} = 590 \text{ nm}$) was recorded over 16 hours at 20°C. Mean fluorescence was calculated from three independent experiments, each containing three biological replicates.

7.5.2.6 Compensation for PTIO quench of DAR-4M T fluorescence

PTIO has a strong concentration dependent absorption spectrum that causes a reduction in DAR-4M T fluorescence in a dose dependent manner either by absorption or by molecular quenching. The PTIO effect must be corrected for when utilising PTIO with DAR-4M in a plate reader assay, especially at high PTIO concentrations, where the quench can mask the true level of DAR-4M fluorescence. The addition of 1-10μM PTIO had no effect on DAR-4M T fluorescence.

However at 100 μ M or greater, the addition of PTIO resulted in an instantaneous quench of 25-70% of DAR-4M T fluorescence.

a)



b)

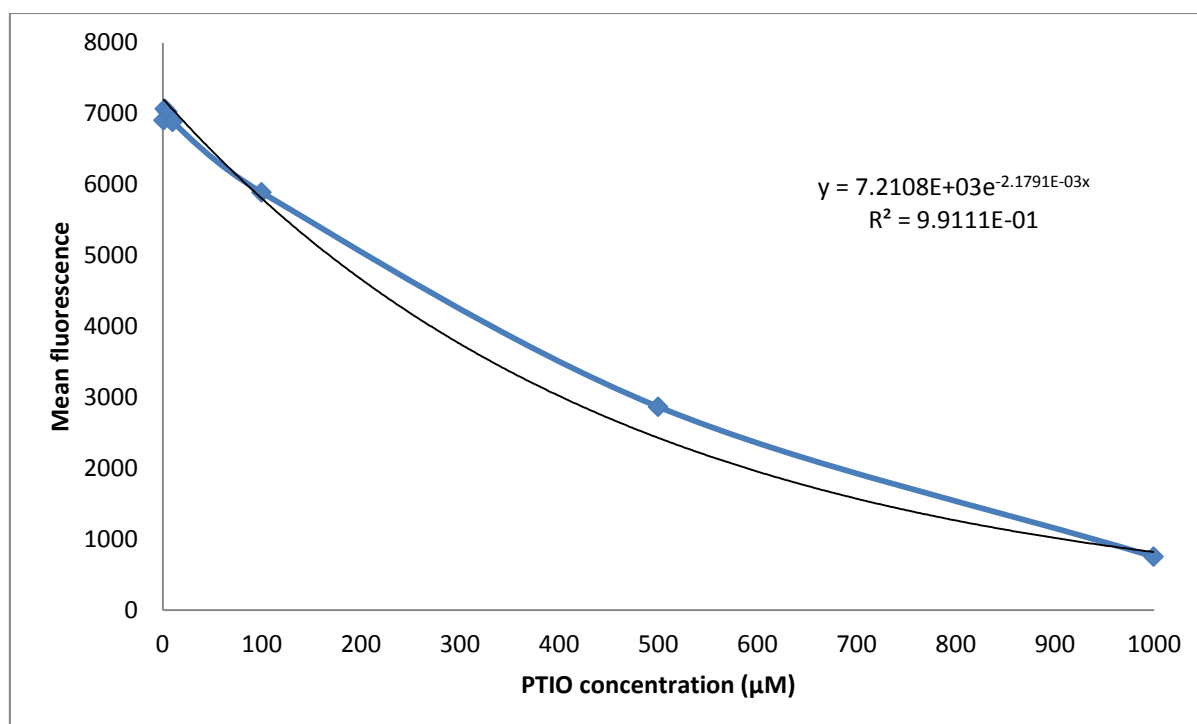


Figure 7.18. The effect of increasing concentrations of PTIO on 2μM DAR-4M T fluorescence at pH7 (a). Fluorescence ($\lambda_{\text{excitation}} = 544 \text{ nm}$, $\lambda_{\text{emission}} = 590 \text{ nm}$) was recorded at 20°C. Mean percentage fluorescence quench was calculated from three independent experiments, each containing three biological replicates. Error bars represent the standard error of the mean. (b) Fluorescence quench estimated from the first time point for varying concentrations of PTIO.

To compensate for the PTIO quench effect, individual quench correction factors were calculated for each experiment and genotype and used to normalise the quench effect. First, the multiplicative correction, (detailed in 7.5.2.1), was applied to the raw data to correct for the initial drop in fluorescence. Second, to correct the PTIO quench the initial data point for each PTIO concentration was plotted before any effect on NO production could occur (Figure 7.21). Data were fitted with an exponential trend line, manually optimised by altering the baseline subtraction value (machine and conidia autofluorescence) to generate the highest R^2 value. Finally, the resulting exponent value was used to generate normalized quench correction factors for each PTIO concentration. Each PTIO treatment data point was multiplied by its corresponding quench correction factor, generating a fully corrected data set.

The complete correction process is outlined below.

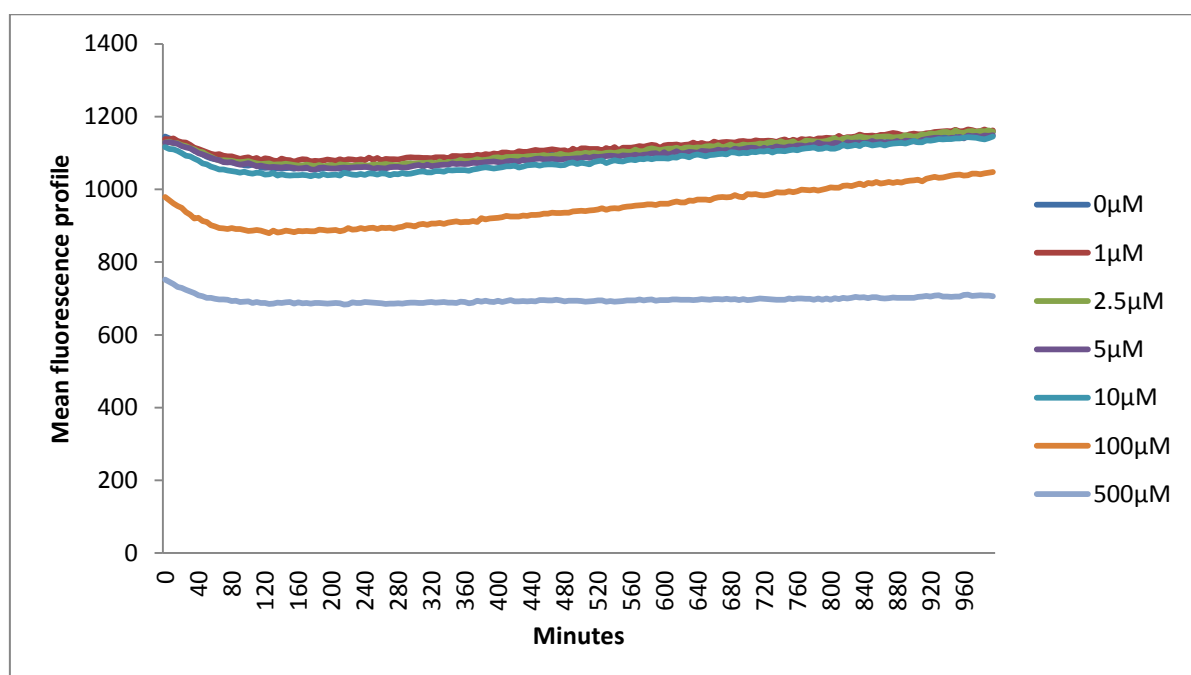


Figure 7.19. Uncorrected Guy11 fluorescent profile data. The effect of increasing concentrations of PTIO on the fluorescence of Guy11 germlings loaded with 2 μM DAR-4M AM at pH7. Fluorescence ($\lambda_{\text{excitation}}=544$ nm, $\lambda_{\text{emission}}=590$ nm) was recorded over 16 hours at 20°C. Mean fluorescence was calculated from three independent experiments, each containing three biological replicates.

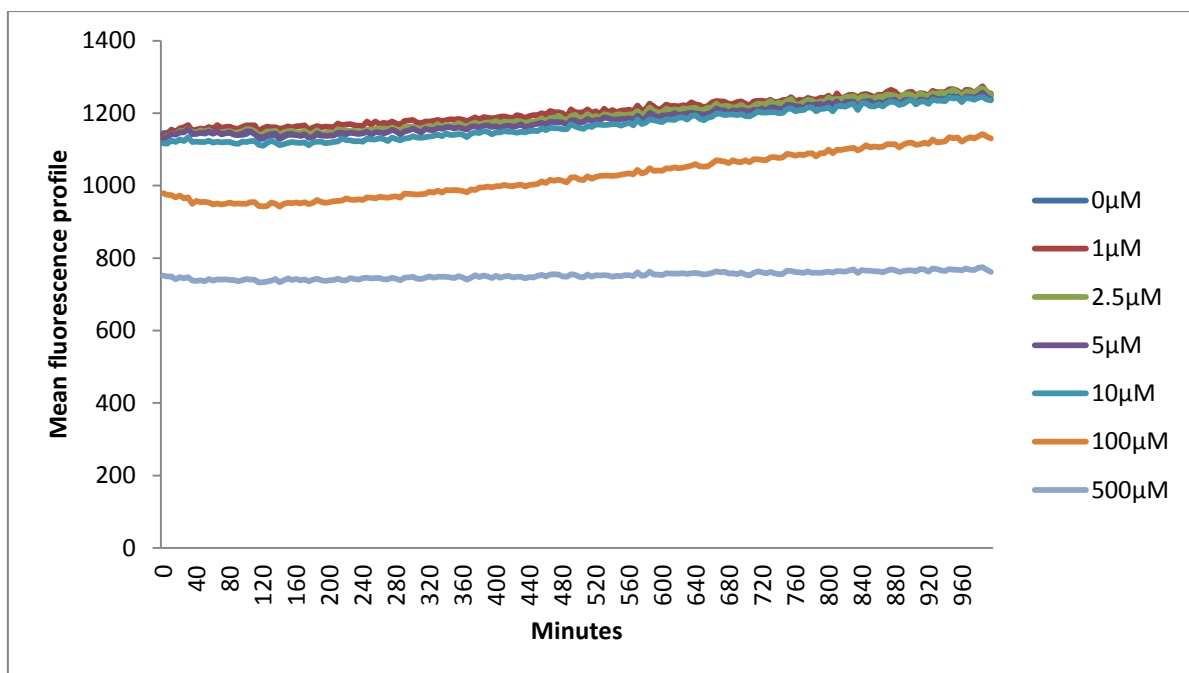


Figure 7.20. A multiplicative correction was applied to remove the initial drop in instrument sensitivity.

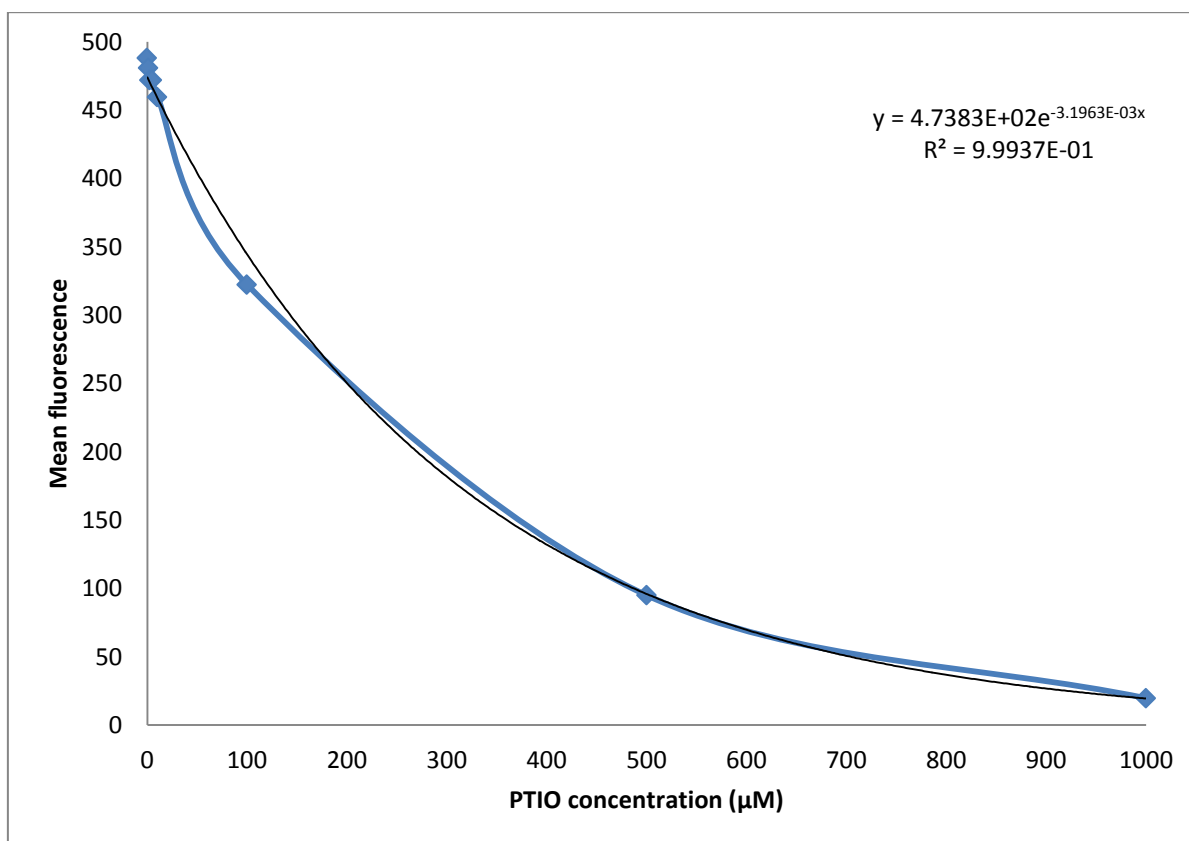


Figure 7.21. Fluorescence quench estimated from the first time point for varying concentrations of PTIO.

PTIO concentration	Correction factor
0	1
1	1.003
2.5	1.008
5	1.016
10	1.032
100	1.376
500	4.943
1000	24.441

Table 7.1. PTIO quench correction multipliers.

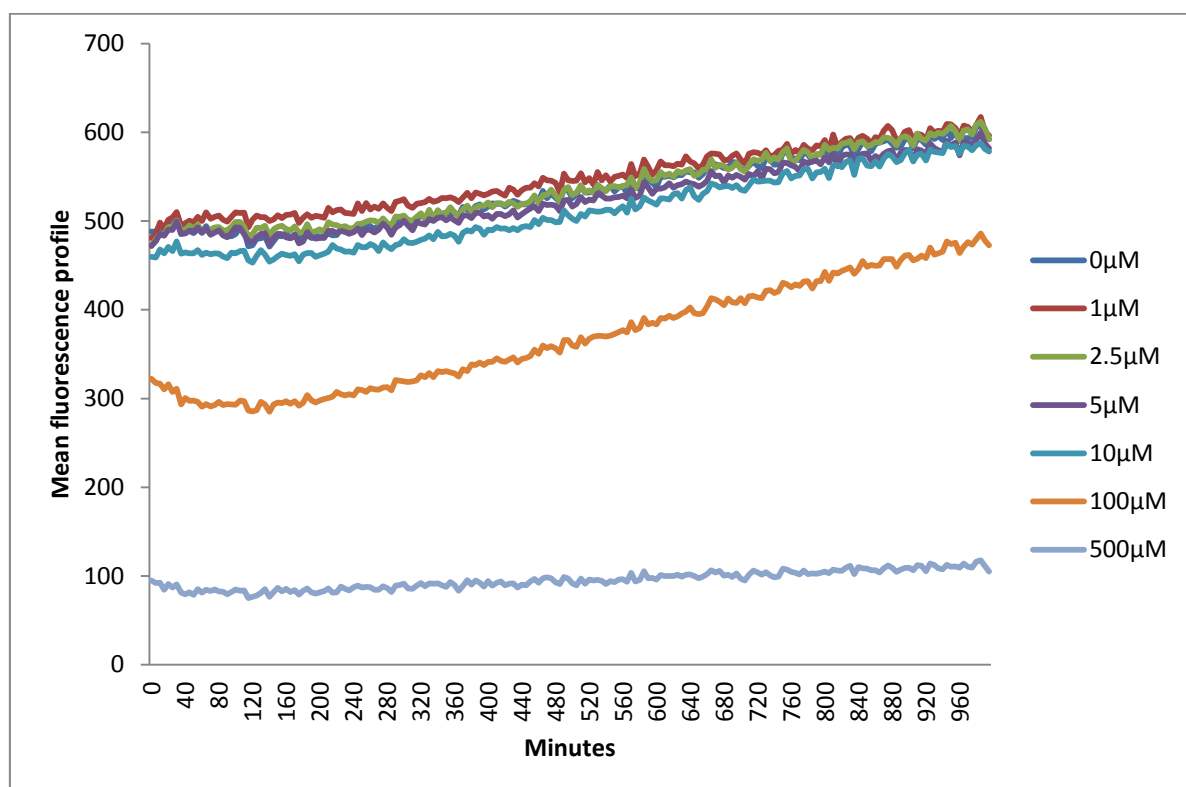


Figure 7.22. Data following baseline subtraction.

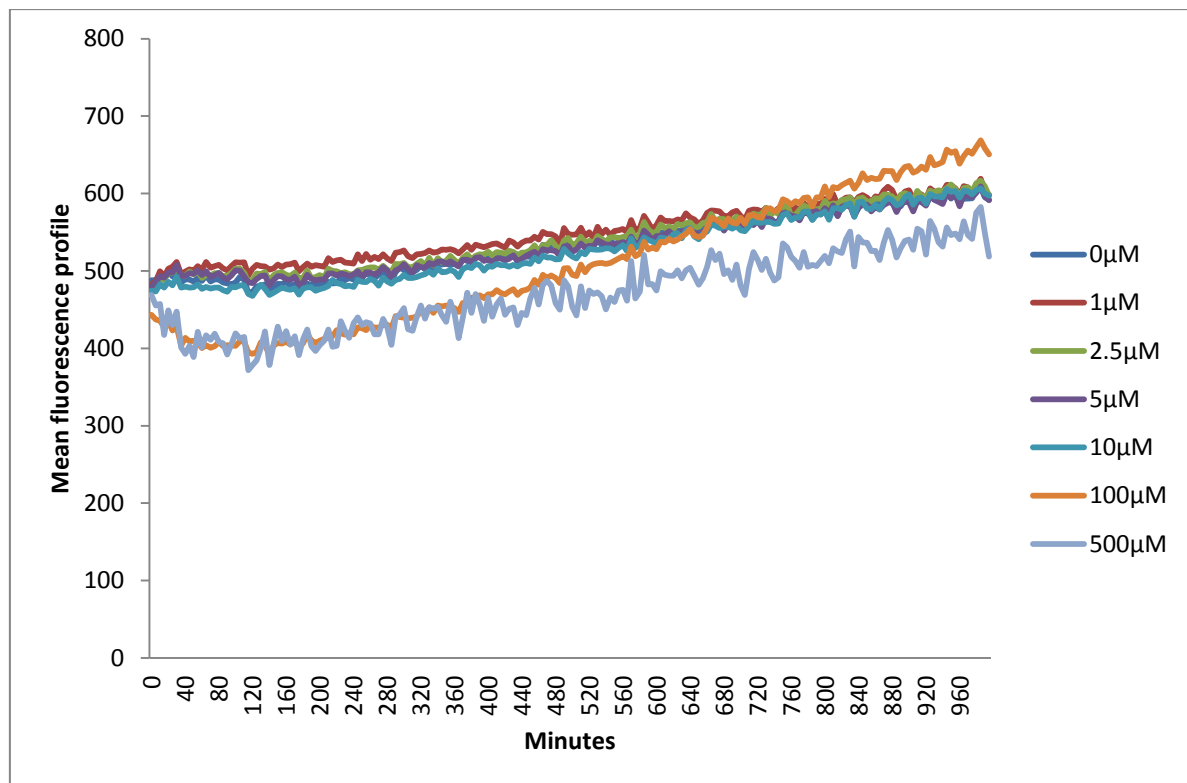


Figure 7.23. Data following quench correction.

Following the completion of the correction process the resulting data displayed several interesting features. First, an initial drop in fluorescence is still observed at high PTIO concentrations. High PTIO concentrations may induce DAR-4M T exit from the conidia resulting in the recorded drop in fluorescence. However, the application of 500 μ M PTIO is unable to abolish DAR-4M fluorescence which increased at a linear rate. This observed linear increase results from PTIO insensitive DAR-4M T formation, most likely due to the presence of intracellular ascorbic acid. The combination of these data corrections allowed the qualitative generation of NO to be monitored within Guy11, reducing errors from autofluorescence, machine error and PTIO quenching.

The final corrected data sets were presented as the average difference in fluorescence profile areas between the 0 μ M PTIO control and PTIO treatments, i.e. the 0 μ M PTIO control profile area was subtracted from each of the 1, 2.5, 5, 10, 100 and 500 μ M PTIO profile areas. 200 μ M PTIO was originally tested during preliminary experiments but was removed as a concentration for the final experiments due to the limitations of the number of wells per plate.

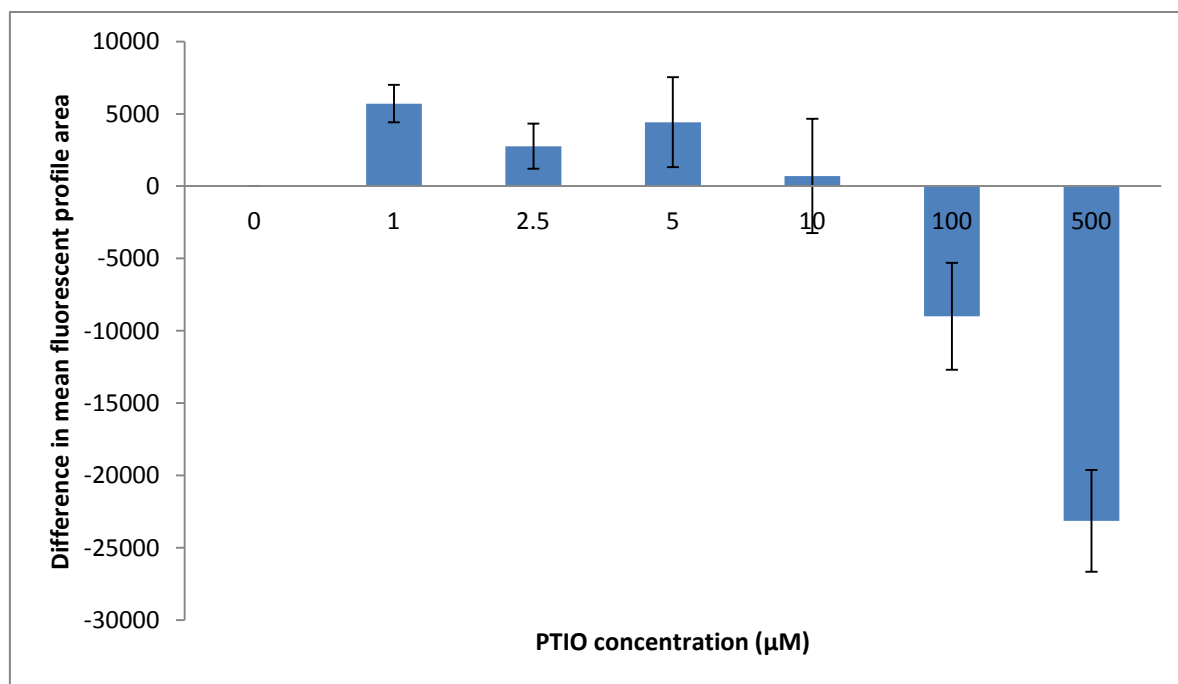


Figure 7.24. The effect of increasing PTIO on the fluorescence of Guy11 germlings loaded with 2 μ M DAR-4M AM at pH7. Multiplicative, autofluorescence and PTIO quench corrected. Each bar represents the difference in area between the 0 μ M PTIO control profile and PTIO treatment profiles. Fluorescence ($\lambda_{\text{excitation}} = 544$ nm, $\lambda_{\text{emission}} = 590$ nm) was recorded over 16 hours at 20°C. Mean fluorescence profile areas were calculated from three independent experiments, each containing three biological replicates. Error bars represent the standard error of the mean.

7.6 NO generation in *M. oryzae* wildtype strains Guy11 and $\Delta ku70$

As a result of the complex optical and biochemical effects of PTIO on fluorescence based assays, a protocol was developed to exploit the unusual signature of PTIO specifically on NO

production. A range of PTIO concentrations were selected to cover both the initial stimulation in fluorescence and also the inhibition at high concentrations. A minimum of three biological replicates per genotype per experiment were required to maintain data reliability. Therefore, the number of genotypes which could be simultaneously monitored for NO generation was limited to four per 96 well plate. Using this approach wildtype strains Guy11 and $\Delta ku70$ were assessed for their intracellular NO levels. The duration of each experiment was reduced from 16 to 12 hours to match the scoring assay time point. This also allowed a greater number of measurements to be completed during germling development.

No significant difference in mean fluorescent profile areas were observed between Guy11 and $\Delta ku70$ using the PTIO protocol, (p-values: 0.188, 0.389, 0.548, 0.612, 0.818 and 0.815, respectively). These data confirm both wildtype strains generate equivalent NO levels during the 12 hour test period. Therefore subsequent experiments used Guy11 as an internal control for comparison with three mutant strains to during each experiment.

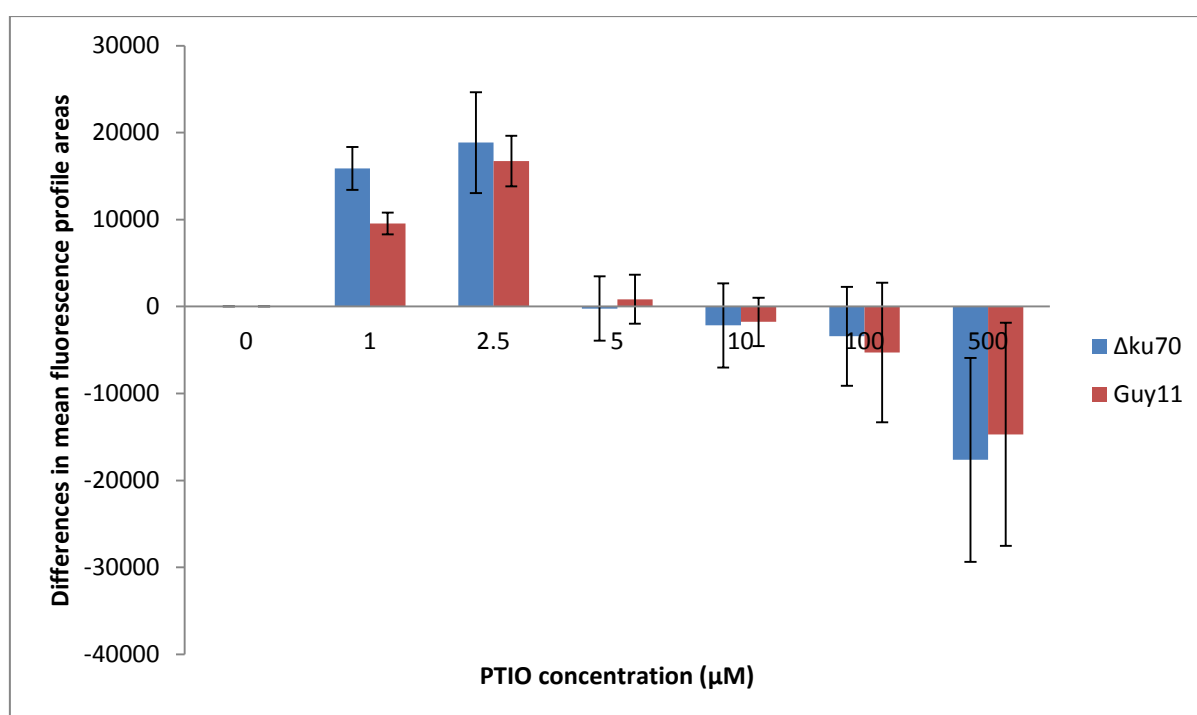


Figure 7.25. The effect of increasing PTIO concentrations on the fluorescence of Guy11 and $\Delta ku70$ germlings loaded in 2 μ M DAR-4M AM at pH7. Multiplicative, autofluorescence and PTIO quench corrected. Each data point represents the difference in area between the 0 μ M PTIO control profile and PTIO treatment profiles. Fluorescence ($\lambda_{\text{excitation}} = 544$ nm, $\lambda_{\text{emission}} = 590$ nm) was recorded over 16 hours at 20°C. Mean fluorescence profile areas were calculated from three independent experiments, each containing three biological replicates. Error bars represent the standard error of the mean.

7.7 NO generation in *M. oryzae* mutant strains

7.7.1 Assessment of $\Delta nos2$, $\Delta nos3$ and $\Delta nos2\Delta nos3$ for their ability to generate NO

The putative NOS mutants, $\Delta nos2$, $\Delta nos3$ and $\Delta nos2\Delta nos3$, were assessed for NO generation over 12 hours using the PTIO enhancement technique. All mutants showed similar or greater fluorescence stimulation at low PTIO concentrations compared to Guy11, but similar levels of fluorescence inhibition at high PTIO concentrations. T-test analysis reported no significant differences in the mean fluorescent profile areas, between Guy11 and $\Delta nos2$, $\Delta nos3$ and $\Delta nos2\Delta nos3$ at each PTIO concentration tested. From this data we infer that $\Delta nos2$, $\Delta nos3$ and $\Delta nos2\Delta nos3$ produce similar levels of NO during early development.

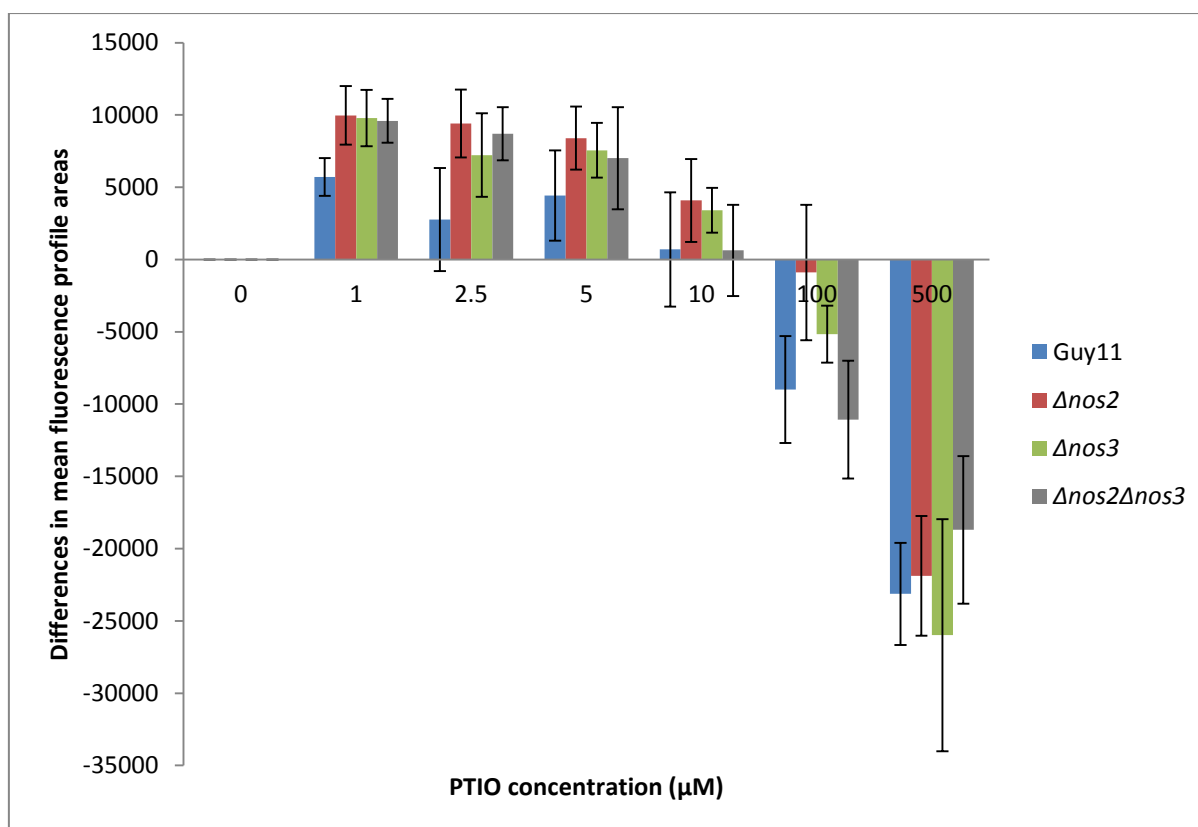


Figure 7.26. The effect of increasing concentrations of PTIO on the fluorescence of $\Delta nos2$, $\Delta nos3$ and $\Delta nos2\Delta nos3$ germlings loaded in 2μM DAR-4M AM at pH7. Multiplicative, autofluorescence and PTIO quench corrected. Each data point represents the difference in area between the 0μM PTIO control fluorescence profile and PTIO treated fluorescence profiles. Fluorescence ($\lambda_{excitation}$ =544 nm, $\lambda_{emission}$ =590 nm) was recorded over 12 hours at 20°C. Mean fluorescent profile areas were calculated from six independent experiments, each containing three biological replicates. Error bars represent the standard error of the mean.

7.7.2 Assessment of $\Delta nia1$, $\Delta nii1$ and $\Delta nia1\Delta nii1$ for their ability to produce NO

To test whether NO production was affected by disruption of nitrate reductase or nitrite reductase genes, the mutant strains *Δnia1*, *Δnii1* and *Δnia1Δnii1* were assessed for NO generation over 12 hours. However, the results were essentially identical to the Guy11 control as T-test analysis revealed no significant differences in fluorescent profile areas, between Guy11 and *Δnia1*, *Δnii1* and *Δnia1Δnii1* at each tested PTIO concentration, with the exception of *Δnia1Δnii1*, which following treatment with 500μM PTIO demonstrated a significantly reduced mean fluorescent profile area relative to Guy11 treatment with 500μM PTIO, (p-value 3.83×10^{-5}), however caution must be taken when interpreting the 500μM PTIO data as it has experienced the most extreme alteration during the correction process and therefore has the greatest error associated with it. From these data we infer that *Δnia1*, *Δnii1* and *Δnia1Δnii1* produce NO at similar rates to the Guy11 control.

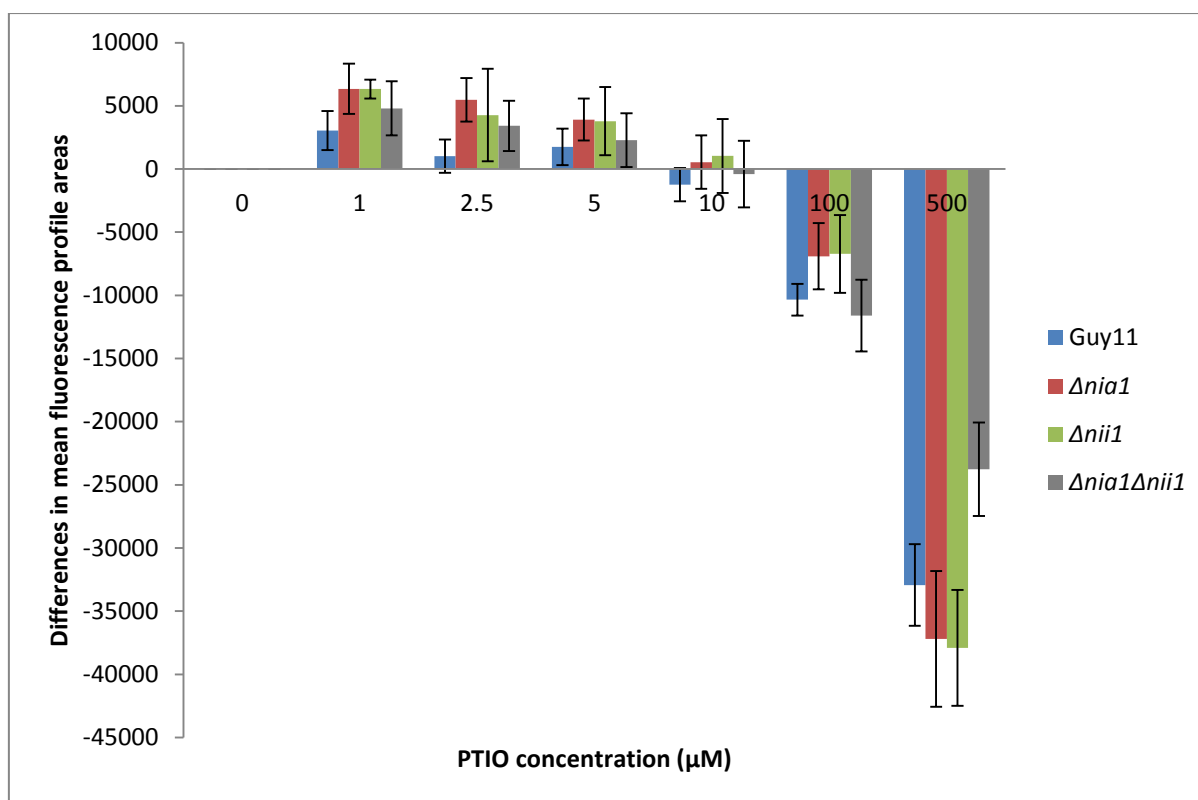


Figure 7.27. The effect of increasing PTIO concentrations on the fluorescence of $\Delta nia1$, $\Delta nii1$ and $\Delta nia1\Delta nii1$ germlings loaded with 2μM DAR-4M AM at pH7. Multiplicative, autofluorescence and PTIO quench corrected. Each data point represents the difference in area between the 0μM PTIO control fluorescence profile and PTIO treated fluorescence profiles. Fluorescence ($\lambda_{\text{excitation}}=544$ nm, $\lambda_{\text{emission}}=590$ nm) was recorded over 12 hours at 20°C. Mean fluorescent profile areas were calculated from six independent experiments, each containing three biological replicates. Error bars represent the standard error of the mean. Error bars represent the standard error of the mean.

7.7.3 Assessment of $\Delta nos3$, $\Delta nia1$ and $\Delta nos3\Delta nia1$ for their ability to produce NO

To test for any interaction between the putative NOS pathway and the nitrate and nitrite

reduction, mutant strains $\Delta nos3$, $\Delta nia1$ and $\Delta nos3\Delta nia1$ were assessed for the generation of NO

over 12 hours. No significant differences in mean fluorescent profile areas were observed between Guy11 and $\Delta nos3$, $\Delta nia1$ and $\Delta nos3\Delta nia1$ following treatment with 1 and 2.5 μM PTIO. Similarly treatment with 5 and 10 μM PTIO had no significant effect on the mean fluorescent profile areas of $\Delta nos3$ and $\Delta nia1$ relative to Guy11. The application of 5 and 10 μM PTIO to $\Delta nos3\Delta nia1$ conidia resulted in a significantly increased mean fluorescent profile areas relative to Guy11, (p-values 0.003 and 0.003, respectively). Treatment of Guy11, $\Delta nos3$, $\Delta nia1$ and $\Delta nos3\Delta nia1$ conidia with 100 and 500 μM PTIO effected a decrease in their mean fluorescent profile areas relative to their 0 μM PTIO control samples. No significant differences in fluorescent profile areas, between Guy11 and $\Delta nos3$, $\Delta nia1$ and $\Delta nos3\Delta nia1$ at 100 and 500 μM PTIO concentrations were observed.

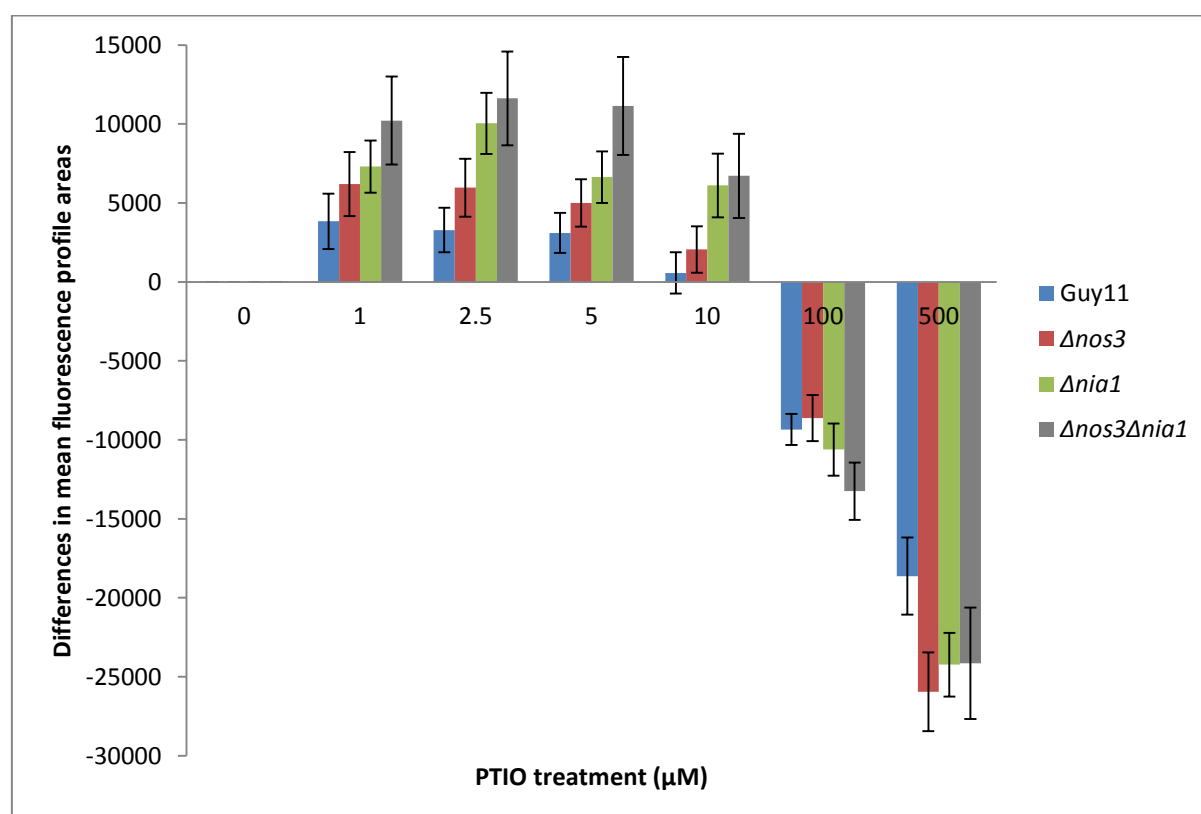


Figure 7.28, The effect of increasing PTIO concentrations on the fluorescence of $\Delta nos3$, $\Delta nia1$ and $\Delta nos3\Delta nia1$ germlings loaded with 2 μ M DAR-4M AM at pH7. Multiplicative, autofluorescence and PTIO quench corrected. Each data point represents the difference in area between the 0 μ M PTIO control fluorescence profile and PTIO treated fluorescence profiles. Fluorescence ($\lambda_{excitation}$ =544 nm, $\lambda_{emission}$ =590 nm) was recorded over 12 hours at 20°C. Mean fluorescent profile areas were calculated from six independent experiments, each containing three biological replicates. Error bars represent the standard error of the mean.

7.8 NO from a NOS-like enzyme?

To investigate the source of NO in *M. oryzae*, the mammalian nitric oxide synthase (NOS) inhibitor L-NAME (NG-Nitro-L-arginine methyl ester hydrochloride) and its inactive stereo isomer D-NAME (NG-Nitro-D-arginine methyl ester hydrochloride) were applied to Guy11 conidia loaded in 2 μ M DAR-4M AM (10mM HEPES pH7).

L-NAME a structural analogue of L-arginine, suppresses NO synthesis via competitive inhibition at the NOS arginine binding site. D-NAME the stereo isomer of L-NAME, has no effect on NOS activity (Rees et al. 1990). L and D-NAME are frequently used to inhibit NOS derived NO synthesis in mammalian studies and have also been applied in several fungal studies (Ichimori et al. 2009). Wang & Higgins (2005) explored the role NO plays in controlling germination in *Colletotrichum coccodes* using L and D-NAME (Wang & Higgins 2005). Similarly, Ninnemannt & Maier (1996) revealed the importance of NO in inhibiting light activated conidiation in *Neurospora crassa*, using L and D-NAME (Ninnemannt & Maier 1996).

The effect of L-NAME and D-NAME on the fluorescence of Guy11 conidia loaded in DAR-4M AM were assessed using several buffered (10mM HEPES pH7) L-NAME and D-NAME concentrations, up to 2.5mM. The addition of L-NAME concentrations up to 500 μ M had no significant effect on

the fluorescence of Guy11 conidia loaded in DAR-4M AM, (p-values: 0.387, 0.651, 0.260 and 0.139, respectively). However, unexpectedly L-NAME concentrations of 1.25mM and greater resulted in a significantly increased mean fluorescent profile area relative to the 0 μ M L-NAME control, (p-values: 0.006 and 1.65×10^{-4} , respectively).

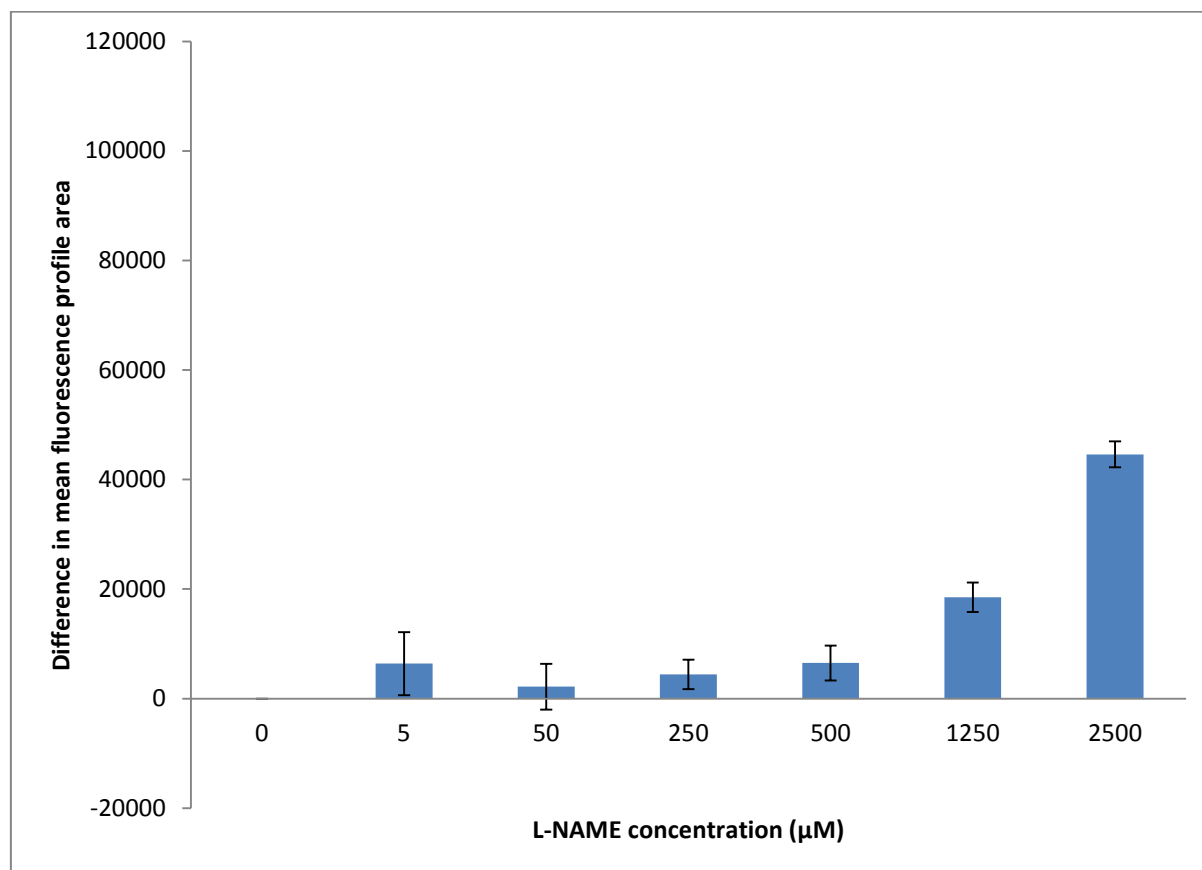


Figure 7.29. The effect of increasing L-NAME concentrations on the fluorescence of Guy11 germlings (1×10^4) loaded with 2 μ M DAR-4M AM at pH7. Multiplicative and autofluorescence corrected, fluorescence ($\lambda_{\text{excitation}} = 544$ nm, $\lambda_{\text{emission}} = 590$ nm) was recorded over 12 hours at 20°C. Each bar represents the difference in area between the 0 μ M L-NAME control fluorescence profile and L-NAME treated fluorescence profiles. Mean fluorescent profile areas were calculated from six independent experiments, each containing three biological replicates. Error bars represent the standard error of the mean.

The effect of D-NAME concentrations up to 1.25mM had no significant effect on the fluorescence of Guy11 conidia loaded in DAR-4M AM, (p-values: 0.974, 0.428 and 0.382, respectively). However, similar to L-NAME the addition of 500 and 2500 μ M D-NAME resulted in significantly increased fluorescent profile areas relative to the 0 μ M D-NAME control, (p-values: 0.004 and 6.67×10^{-6} , respectively).

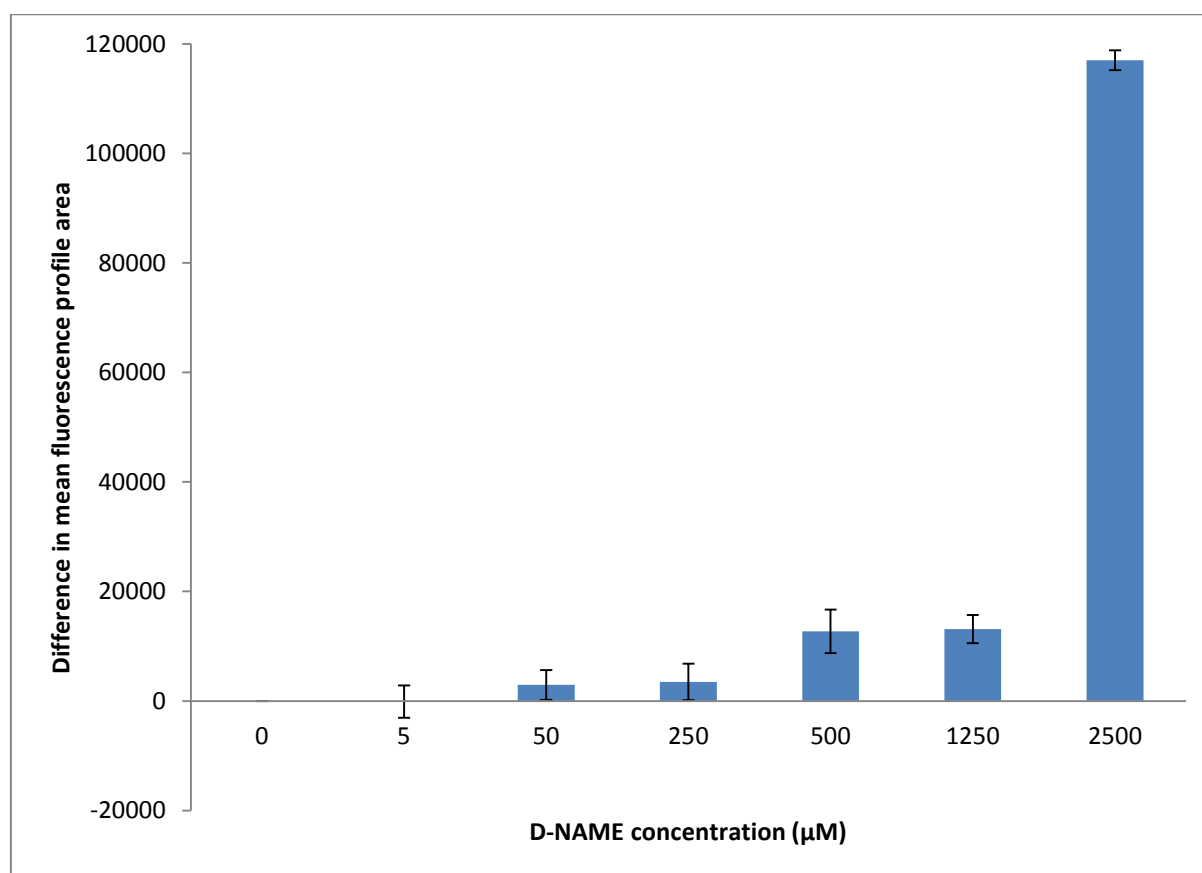


Figure 7.30. The effect of increasing D-NAME concentrations on the fluorescence of Guy11 conidia (1×10^4) loaded with 2 μ M DAR-4M AM at pH7. Multiplicative and autofluorescence corrected, fluorescence ($\lambda_{\text{excitation}} = 544$ nm, $\lambda_{\text{emission}} = 590$ nm) was recorded over 12 hours at 20°C. Each bar represents the difference in area between the 0 μ M D-NAME control fluorescence profile and L-NAME treated fluorescence profiles. Mean fluorescent profile areas were calculated from six independent experiments, each containing three biological replicates. Error bars represent the standard error of the mean.

7.9 Discussion

7.9.1 Protocol development

The data presented within this Chapter provide a detailed investigation into the use of fluorescent assays for NO detection in fungal systems. This work has identified a number of caveats related to the use of PTIO as an NO scavenger and fluorescent probes based on the formation of a triazole using N_2O_3 , which makes even routine assays quite complex.

The family of NO sensitive fluorescent probes do not directly interact with NO but with N_2O_3 , this reaction is also known to take place with other bio-molecules, such as ascorbate which may be present at high concentrations in biological specimens. Therefore the ability of these dyes to accurately report NO is affected by a greater number of potential variables than FNOCTs and Acridine-TEMPO-DTCS-Fe(II), which directly bind NO. In order to confirm the link between observed changes in fluorescence and NO, the assay was optimised for conidia concentration, conidia washing, assay temperature, assay buffer and pH and fluorescence measurement (data not shown).

The most important observation during protocol development involved a complex interaction with PTIO. PTIO is the most widely used NO scavenger in published studies (Miraglia et al. 2011; Meuchel et al. 2011; Melo et al. 2011). However, as PTIO accelerates the oxidation of NO to NO_2 it can potentially enhance NO detection by amine based fluorescent dyes. This effect has only been reported once in the literature by Nagano (1999). This study is the first to use the concentration dependence of the PTIO response, which rather than just inhibiting fluorescence as would be expected by an NO scavenger, quantitatively enhances the specificity of DAR-4M detection of NO. This technique provides a novel method for linking DAR-4M (AM) derived fluorescence with NO synthesis that minimises interference from other reactive molecules such as ascorbic acid. As the rate enhancement is dependent on the local PTIO concentration and the

rate of NO production, each assay has to titrate over a range of PTIO concentrations. In theory this technique could be applied to other amine based NO sensitive dyes, including DAFs, DAMBO-P^H improving their sensitivity and specificity for *in vivo* NO.

The third effect of PTIO on the fluorescence assay is to quench DAR-4M T fluorescence, an observation which has not previously been reported in the literature, despite multiple studies using PTIO as an NO scavenger in combination with DAR-4M (AM) (Tun et al. 2008; Tun et al. 2006). In each publication the addition of high PTIO concentrations were reported to abolish DAR-4M (AM) derived fluorescence and these observations were attributed to the NO scavenging properties of PTIO, rather than the quenching of DAR-4M T fluorescence. Indeed, the novel information regarding PTIO and fluorescence quenching extends to other dyes, meaning the results of many publications must now be viewed with a degree of caution, including those reporting NO detection in fungal systems: Gong et al. (2007), Wang & Higgins (2005), Vieira et al. (2009) and Prats et al. (2008). Understanding and compensating for the simultaneous enhancement of triazole formation at low PTIO concentrations and fluorescence quenching at high PTIO concentrations is important to give reliable results. The correction process outlined in this Chapter overcomes a number of the associated PTIO problems. However the process is not perfect with each step potentially introducing errors which are compounded by further corrections. Ideally the approach now requires cross validation against an independent method. With this in mind, the Sievers Nitric Oxide Analyser (NOA 280i) Analytix (Unit 9 Boldon Court, Burford Way, Boldon Business Park, Boldon, Tyne and Wear, NE35 9PY) which uses the chemiluminescent reaction between NO and ozone, was used to investigate differences in NO levels between the wildtype and mutant strains (M. Samalova). However, this technique was not found to be reliable or sensitive enough. Therefore an alternative NO detection technique should be employed, such as using a previously characterised NO synthesising biological system, for example mammalian immune cells, to confirm the effects of PTIO and DAR-4M.

Further information regarding NO synthesis was gained through using the non-cell permeable DAR-4M and cell permeable DAR-4M AM in combination with the PTIO enhancement technique. If the PTIO enhancement is only observed in combination with DAR-4M AM this provides strong evidence that NO is generated intracellularly, as only DAR-4M AM is able to enter into the conidia. Conversely if the PTIO enhancement is observed in combination with DAR-4M this suggests significant external NO release, as DAR-4M cannot enter the conidia and can only react with extracellular NO. This technique confirmed that NO was generated within *M. oryzae* conidia and germmlings.

In conclusion: 1) PTIO directly reacts with NO to form NO₂; 2) This reaction enhances the rate of triazole formation when used alongside NO sensitive dyes which react with N₂O₃; 3) PTIO quenches/absorbs fluorescence in a dose dependant manner and hence must be corrected for; 4) The quench correction process allows PTIO to be used in combination with NO sensitive dyes to prove the presence of NO, however it also introduces a degree of error which increases with each correction and the concentration of PTIO used.

7.9.2 NO generation within *M. oryzae*

The mutant strains $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ were assessed for their ability to generate NO using the PTIO enhancement technique. This technique demonstrated the presence of NO within each mutant strain. Guy11 conidia loaded with DAR-4M AM consistently exhibited lower fluorescence following treatment with 1, 2.5, 5 and 10 μ M PTIO than $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$. However, fluorescence behaviour was only significantly different for $\Delta nos3\Delta nia1$ treated with 5 and 10 μ M PTIO and $\Delta nia1\Delta nii1$ treated with 500 μ M PTIO, which demonstrated significantly increased fluorescence relative to Guy11.

This suggests, counterintuitively, that the disruption of *NOS2*, *NOS3*, *NIA1* or *NII1*, results if anything in an increase in NO generation. An alternative explanation, based on the DAR-4M AM conidia loading technique may explain this observation (Chapter 2, 2.15.2). Guy11 develops significantly higher numbers of conidia than $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nai1$. Thus, during dye loading the relative concentration of Guy11 conidia exceeded the mutant strains, to the point that the 2 μ M DAR-4M AM (10mM HEPES pH7) may have been insufficient concentration to successfully load all Guy11 conidia. Therefore each Guy11 conidium contained less DAR-4M AM relative to each mutant conidium. When equal conidia numbers were inoculated into a NUNC 96 well plate, a greater amount of probe would have transferred with the mutant conidia, hence giving the increased fluorescence generation observed.

These data confirm the generation of NO within *M. oryzae*, but strongly suggest that *NOS2*, *NOS3*, *NIA1* and *NII1* are not responsible for its synthesis.

Pharmacologically it appears that NO is not synthesised via mammalian NOS like enzymes in *M. oryzae* as the treatment of DAR-4M AM loaded Guy11 conidia with L or D-NAME resulted in a significant increase in recorded fluorescence relative to Guy11 conidia in the absence of L or D-NAME treatment. If NO synthesis in *M. oryzae* occurred through NOS like enzymes the addition of L-NAME would be expected to inhibit NO synthesis in a dose dependent manner; resulting in a decrease in recorded fluorescence, this result was not observed. Interestingly no isomer specificity between L-NAME and D-NAME was recorded, with the addition of both isomers eliciting an increase in fluorescence. This suggests that L and D-NAME may interact biophysically with DAR-4M T, or are possibly metabolised by *M. oryzae* as a nitrogen source leading to increased growth, metabolism and NO synthesis and recorded fluorescence. This effect appears to be specific to L/D-NAME as it was not observed with other amines which share similar chemical structures, such as spermine, spermidine, arginine or citrulline (data not shown).

In summary, the application of varying PTIO concentrations with DAR-4M (AM) represents an improved technique to quantitatively demonstrate NO synthesis *in vivo*. This technique provides evidence that *M. oryzae* generates NO, but that *NOS2*, *NOS3*, *NIA1* and *NII1* are dispensable for NO synthesis. Finally, NO in *M. oryzae* is not synthesised by mammalian like NOS enzymes, and therefore the source of NO within *M. oryzae* remains undetermined.

Chapter 8: Discussion

8.1 Main thesis conclusions

- NO is generated by *M. oryzae*, as evidenced by the plate reader assay and the PTIO enhancement technique.
- Nos2, Nos3, Nia1 and Nii1 are not responsible for NO generation recorded in *M. oryzae*, as evidenced by the plate reader assay.
- NO is not generated by animal-like NOS, as demonstrated through the application of the mammalian NOS inhibitor L-NAME.
- The source of NO remains obscure.
- $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ exhibit differing phenotypes relative to the wildtype strains on a surface inductive to melanised appressoria formation in the wildtype strains Guy11 and $\Delta ku70$.
- Application of PTIO to wildtype *M. oryzae* indicates NO is involved in germination and appressorium formation.
- *NOS2*, *NOS3*, *NIA1* and *NII1* are dispensable for pathogenicity.
- *NOS2* and *NOS3* are dispensable for nitrate assimilation.
- *NIA1* and *NII1* are essential for nitrate assimilation.

8.2 NO generation in *M. oryzae*

8.2.1 Cytochrome P450; a NOS in disguise?

The combination of the plate reader assay and PTIO enhancement technique confirmed that NO generation in *M. oryzae* is not via Nos2, Nos3, Nia1 or Nii1. It is therefore logical to assume that

NO generation in *M. oryzae* may be produced by enzymes other than NOS, NR or NIR. A class of enzymes potentially capable of NO generation are cytochrome P450(s). The Broad Institute database lists over 200 annotated P450 genes within the *M. oryzae* genome. The cytochrome P450 family encompasses a large number of cysteine-heme enzymes which are found across the Domains of Life. The term cytochrome P450 is frequently used in a broad sense, including proteins which contain a thiolate-ligated heme, but which demonstrate no structural similarity or absorption maximum at 450nm in the presence of carbon monoxide (Omura 2005). In this broad sense, NOS may be considered part of the cytochrome P450 family. There are several key similarities between NOS and P450s in that they; i) contain thiolate-ligated heme domains, and share up to 60% sequence homology (Masters 2005); ii) can catalyse the formation of citrulline and NO from NG-hydroxy-L-arginine (NOHA), (the intermediate formed during the synthesis of NO by mammalian NOS from L-arginine) (Masters 2005); iii) can accept electrons from NADPH (Mansuy et al. 1995); iv) have similar spectroscopic properties between their prosthetic heme groups (Rousseau et al. 2005); v) share similar catalytic intermediates (Gorren & Mayer 2006); vi) exhibit heme spin which is modulation by substrate binding (Meunier et al. 2004). There are however significant differences between NOS and cytochrome p450s, the most important being the dependence of mammalian NOS on H_4B , for NO synthesis.

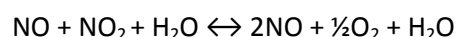
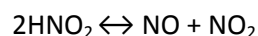
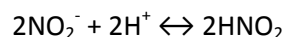
Andronik-Lion et al. (1992) demonstrated that liver microsomal cytochrome p450 synthesises NO through the oxidation of arylamidoxime, an analogue of NOHA (Andronik-Lion et al. 1992). These researchers also reported that liver microsomal cytochrome p450s catalyse oxygen and NADPH dependent oxidative denitration of NOHA, forming citrulline and NO (Boucher et al. 1992). The addition of NOS inhibitors failed to suppress NO synthesis, but the addition of cytochrome p450 inhibitors miconazole and dihydroergotamine inhibited NO synthesis. Boucher et al. (1992) proposed a two step mechanism for NO synthesis by; i), classical NOS catalysis of the oxidation of L-arginine to NOAH; ii), cytochrome P450 catalysis of the second oxidation step, converting NOAH to NO and citrulline. Both reactions require oxygen and

NADPH (Boucher et al. 1992). There is much evidence within the literature that cytochrome p450s are able to synthesise NO from a number of precursor molecules, including NOAH, but there is no evidence for P450s catalysing the conversion of L-arginine to NOAH (Andronik-Lion et al. 1992; Meunier et al. 2004). Therefore, cytochrome P450s may provide an alternative or combined source of NO. However, the lack of mammalian NOS homologues within the *M. oryzae* genome obviates the notion of P450 mediated NO synthesis, as proposed by Boucher et al. (1992). However, it remains feasible that *M. oryzae* P450s synthesises NO using analogues of NOHA. This would also explain why the application of mammalian NOS inhibitors did not suppress DAR-4M (AM) derived fluorescence in Guy11 germlings.

8.2.2 Non-enzymatic NO synthesis?

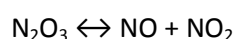
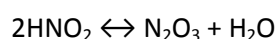
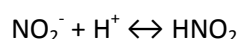
Alternatively, NO can be generated through multiple non-enzymatic pathways, several of which are outlined below. Could these be the source of NO in *M. oryzae*?

Nitrite, in acidic conditions, can be reduced to NO via the following reaction.



Bethke et al. (2004) observed this process in the apoplastic space of barley. They also found that the addition of the phenol catechin increased the rate of NO formation (Bethke et al. 2004). A similar result has been observed in connection with the reductant ascorbate. Ascorbate-mediated reduction of nitrite to NO occurs in low pH <4 environments, such as the apoplastic space and chloroplasts, where ascorbate is known to accumulate (Lamattina et al. 2004).

NO generation through the light dependent conversion of NO₂ and carotenoids have been reported in plants. Cooney et al. (1994) demonstrated that the addition of NO₂ to plants in darkness results in nitrite accumulation and cellular damage. However this effect is suppressed in light (Cooney et al. 1994). Wood et al. (1996) reported NO release from S-nitroso glutathione, following light dependent homolysis of the S-N bond (Wood et al. 1996).



As discussed in Chapter 1 1.3.2.4 and 1.3.3.3, both haemoglobin and myoglobin are capable of reducing nitrite to NO under hypoxic conditions and are thought to regulate cardiac processes (Shiva et al. 2007; Lundberg et al. 2008).

Two of these non-enzymatic processes, the light-dependent conversion of NO₂ and carotenoids, and the homolysis of the S-N bonds, can be discounted as the observed source of *M. oryzae* NO, as the plate reader assays were conducted in darkness. Nitrite, generated through the nitrogen assimilatory pathway, is present within *M. oryzae*. *in silico* analyses of *M. oryzae* nitrate reductase gene (Chapter 3, 3.3.3) suggests it is localised outside of the mitochondria. Burn et al. (1974) reported a similar subcellular localisation for *Candida utilis* nitrate reductase (Burn et al. 1974). It is therefore possible that nitrite formation occurs in environments with sufficiently low pH for nitrite to be reduced to NO. However, disruption of *NIA1* would be expected to decrease DAR-4M (AM) derived fluorescence, as the availability of nitrite is suppressed. Alternatively, disruption of *NII1* should enhance DAR-4M (AM) derived fluorescence, due to the increased intracellular nitrite abundance. Neither of these predictions was observed, thus the acidic reduction of nitrite to NO is unlikely to be the source of NO in *M. oryzae*.

The presence of ascorbate in *M. oryzae* has yet to be verified conclusively, however the presence of ascorbate is suggested by the non-PTIO quenchable increase in DAR-4M (AM) derived fluorescence, observed following the treatment of the wildtype Guy11 with high concentrations of PTIO, (Chapter 7, Figure 7.23). Therefore, the reducing of nitrite to NO by ascorbate does suggest a possible pathway for the generation of NO in *M. oryzae*, but it merits more robust investigation.

The reduction of nitrite to NO by haemoglobin in *M. oryzae* is also possible as *M. oryzae* carries a single haemoglobin gene, MGG_00198, as annotated by the Broad Institute. Haemoglobin has been isolated from *Neurospora crassa*, *Candida norvegensis*, *Candida mycoderma*, *Penicillium notatum* and *Saccharomyces* spp. (Oshino et al. 1972; Iwaasa et al. 1992). However, the role of haemoglobin in fungi has received little attention, therefore the potential of the protein encoded by MGG_00198 to generate NO is little more than speculation.

In summary, there are potentially several non-enzymatic pathways which may be able to generate NO with *M. oryzae*. However, their presence within *M. oryzae* remains to be substantiated.

8.2.3 Environmental regulation of nitrate reductase and nitrite reductase

Whilst, Nia1 and Nii1 do not contribute to the generation of NO within *M. oryzae*, as observed during the plate reader assay, Nia1 and Nii1 may still contribute to NO generation under specific environmental conditions. For example, it has been reported that NR and NIR genes in *Fusarium oxysporum*, *Cylindrocarpon tonkinense*, *Aspergillus nidulans* and *Fellomyces fuzhouensis*, required for dissimilatory nitrate reduction, are expressed only under hypoxic or anoxic conditions (Zhou et al. 2002; Kobayashi et al. 1996; Morozkina & Kurakov 2007; Kurakov et al. 2000; Shimizu et al. 2009). The existence of a dissimilatory nitrate reduction pathway within *M. oryzae* remains to be confirmed. However, if present then under the normoxic conditions,

which the plate reader assay was conducted, *NIA1* and *NII1* would not be expressed and the impact of their disruption on NO generation in *M. oryzae* would be overlooked. The recorded generation of NO in $\Delta nia1$, $\Delta nii1$ and $\Delta nia1\Delta nii1$ under normoxic conditions means there is a pathway independent of dissimilatory nitrate reduction which generates NO in *M. oryzae*.

Nitrate and nitrite reductase gene expression and protein activity in plants have been reported to be regulated by light. Vincentz & Caboche (1991) reported *Nicotiana plumbaginifolia* NR mRNA expression to be light-regulated. Wildtype plants, placed in darkness, showed no detectable NR mRNA, however, after four hours, following the transition from dark to light; NR mRNA was rapidly detected (Vincentz & Caboche 1991; Jolma et al. 2010). Moreover, Sharma & Sopory (1984) demonstrated that NR and NIR activity in maize is regulated by phytochrome. Following treatment with far red light, their activities were increased significantly (Sharma & Sopory 1984). To a lesser extent, light has been shown to influence nitrogen metabolism within fungi, specifically in *N. crassa* (Innocenti et al 1983; Sommer et al. 1989; Chen et al. 2010; Chen et al. 2009). Klemm and Ninnemann (1979) reported nitrate reductase activity in *N. crassa* is regulated by light. By inference, *M. oryzae* Nia1 activity may also be light regulated. As the plate reader assays were conducted in darkness, the expression/regulation of *NIA1*/Nia1 may have been suppressed and the impact of its disruption on NO synthesis overlooked. However, as NO generation was still observed during the plate reader assays there is likely to be a Nia1 independent NO synthesis pathway in *M. oryzae*, which is unaffected by the presence or absence of light.

In summary, the ability of Nia1 and Nii1 to generate NO within *M. oryzae* may have been underestimated due to the dark conditions of the plate reader assay. However, the observed NO generation in mutants means a pathway exists which is independent of low oxygen and light.

8.3 NO and infection development

8.3.1 Germling morphogenesis on an inductive artificial surface

The various mutant germling phenotypes observed on inductive TAAB plastic coverslips are not due to differences in their abilities to generate NO. A more plausible explanation is the notion that replacement of *NOS2*, *NOS3*, *NIA1* and *NII1* has disrupted physiological pathways which result in the observed mutant phenotypes, similar to *AtNOS1* which was originally thought to be a NOS homologue in *A. thaliana*, however further investigation revealed that it is part of the GTPase family involved in mitochondrial ribosome biogenesis (Zemojtel et al. 2006; Moreau et al. 2008). Alternatively, variation between TAAB coverslip surfaces may influence the mutant phenotypes. The inductivity for melanised appressoria formation on TAAB coverslips is abolished by their submergence in 100% ethanol for 24 hours (data not shown). Intriguingly, when the ethanol (used to submerge the coverslips) is evaporated on non-inductive glass microscope slides and inoculated with Guy11 conidia, 100% melanised appressoria formation rates were observed 12hpi (data not shown). This strongly indicates the presence of an ethanol soluble molecule, possibly a plasticiser (phthalates added to plastic, which function by embedding themselves between the plastic polymer chain, lowering the glass transition temperature for the plastic, increasing their flexibility (Ali et al. 2011), which induces melanised appressoria formation in *M. oryzae*. Therefore differences between mutant and wildtype phenotypes may result from differences in the abundance of this molecule, hence the differences in Guy11 MA formation observed between batches of TAAB coverslips.

8.3.2 Germling morphogenesis on an inductive artificial surface following treatment with the NO scavenger PTIO

The application of the NO scavenger PTIO to wildtype and mutant germlings on inductive TAAB coverslips decreases the percentage of germlings which form melanised appressoria. This

indicates that NO may be involved in appressorium formation. PTIO also inhibits wildtype germination in a concentration-dependant manner, so suggesting a role for NO in *M. oryzae* germination. Wang & Higgins (2005) reported such a finding in germination in *Colletotrichum coccodes*, but through the application of mammalian NOS inhibitors (Wang & Higgins 2005). Intriguingly, even at high concentrations PTIO could not completely abolish wildtype Guy11 germination and appressorium formation; this suggests the existence of NO independent pathways controlling both germination and appressorium formation in *M. oryzae*.

8.3.3 Germling morphogenesis on an inductive artificial surface following treatment with the NO donor DETANONOate

Due to the observation that the wildtype and mutant strains generate equivalent levels of NO, it is unsurprising that exogenous NO application, through the NO donor DETANONOate, fails to restore a wildtype level of appressoria formation in the mutant strains. The continuous release of exogenous NO by DETANONOate may also function as a toxin and cause nitrosative stress to the germlings hence preventing their growth. The fact that *NOS2*, *NOS3*, *NIA1* and *NII1* are not responsible for NO generation within *M. oryzae* means that the method of NO delivery is not a factor in restoring the mutant phenotypes to a wildtype equivalent. If this was the case then small independent doses of NO would be expected to restore the mutant phenotypes to wildtype rather than continuous exposure to NO, analogous to NO generation by mammalian eNOS and nNOS.

8.3.4 Pathogenicity

The question as to what role NO plays in *M. oryzae* pathogenesis remains unanswered. The combination of the plate reader and the PTIO enhancement technique provides evidence for NO generation, but as there is no difference in NO generation or indeed pathogenicity between the wildtype and mutant strains it is therefore not possible to determine whether NO is or is not

involved, only that *NOS2*, *NOS3*, *NIA1* and *NII1* are dispensable for pathogenesis. The differences observed between the mutant and wildtype strains on TAAB plastic coverslips is likely due to the surface variation specifically regarding plasticizers. The hypothesis regarding environmental threshold signalling is no longer relevant as *NOS2*, *NOS3*, *NIA1* and *NII1* are not responsible for NO generation.

8.4 Nitrogen metabolism

The growth and biomass assay data support the roles of *Nia1* and *Nii1* in nitrate assimilation pathway in *M. oryzae* (Wilson et al. 2007). The observation that $\Delta nia1$, $\Delta nii1$ and $\Delta nia1\Delta nii1$ are equally pathogenic to the wildtype strains, suggests that the nitrate assimilation pathway in *M. oryzae* is not linked to pathogenesis. Interestingly however, Lau & Hamer (1996) demonstrated that two genes *NPR1* and *NPR2* (nitrogen pathogenicity regulation), are regulators of pathogenicity in *M. oryzae* and may regulate nitrogen metabolism (Lau & Hamer 1996). Therefore, whilst the disruption of the nitrate assimilation pathway does not affect pathogenesis, disruption of pathogenesis prevents nitrate assimilation.

The growth and biomass assay data confirms that *Nos2* and *Nos3* are dispensable for nitrate assimilation. When, coupled with the plate reader data, this lends weight to the notion that *NOS2* and *NOS3* are more likely to be bifunctional P450s, and not NOSs.

8.5 Future directions

Following extensive research, at Oxford, a large number of important questions remain unanswered regarding the generation of NO in *M. oryzae*. To further our understanding these questions must be addressed.

8.5.1 Complementation and GFP tagging of Nos2, Nos3, Nia1 and Nii1

The re-introduction of the *NOS2*, *NOS3*, *NIA1* and *NII1* into $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ is required to confirm the observed phenotypes are the result of the disruption of *NOS2*, *NOS3*, *NIA1* and *NII1* genes. However, as the plate reader assay reported $\Delta nos2$, $\Delta nos3$, $\Delta nos2\Delta nos3$, $\Delta nia1$, $\Delta nii1$, $\Delta nia1\Delta nii1$ and $\Delta nos3\Delta nia1$ to generate equivalent levels of NO to the wildtype strains Guy11 and $\Delta ku70$, the re-complementation of each mutant was considered less critical. Re-complementation of the mutant strains with their corresponding gene cassettes fused with a GFP marker would however provide information regarding the sub-cellular localisation of Nos2, Nos3, Nia1 and Nii1.

8.5.2 Expression profiling of NIA1 and NII1 under varying environmental conditions

As discussed previously, NIR and NR expression and protein activities are regulated by certain environmental conditions, such as oxygen levels and light availability. It is important to determine the relative levels of NR and NIR expression in light versus dark conditions and normoxic versus hypoxic/anoxic conditions. This may explain why the disruption of these genes has no impact on NO generation, as recorded by the plate reader assay. Expression profiling of *NII1* in Guy11 and $\Delta nia1$ would determine if *NII1* expression is regulated by Nia1. If this is the case the disruption of *NIA1* would be expected to prevent the expression or activity of Nii1, perhaps explaining why the disruption of *NII1* does not alter NO generation, and also furthering our understanding of the nitrate assimilation pathway within *M. oryzae*.

8.5.3 Investigating alternative NO generating pathways in *M. oryzae*

To investigate the potential generation of NO by cytochrome P450 in *M. oryzae*, the plate reader assay and PTIO enhancement technique should be combined with cytochrome P450 inhibitors. However, care must be taken, *M. oryzae* carries over 200 annotated cytochrome P450s and the application of an inhibitor may indirectly alter DAR-4M (AM) derived fluorescence through the disruption of a separate physiological pathway. Equally, finding a suitable inhibitor may prove challenging due to the broad diversity amongst cytochrome P450s. Documented mammalian cytochrome P450 inhibitors include such chemistries as 8-Methoxypsoralen, Tryptamine, 2-Phenyl-2-(1-piperdiny)propane (PPP), Ticlopidine and N,N'N'-Triethylenethiophosphoramidate (ThioTEPA), but there is no guarantee any of these inhibitors will perturb *M. oryzae* cytochrome P450s activities (Khojasteh et al. 2011).

Non-enzymatic pathways merit investigation as a potential source of NO within *M. oryzae*. Although this will be difficult to investigate in *M. oryzae*.

8.5.4 Validation of the plate reader assay and PTIO enhancement technique

Validation of these techniques would provide irrefutable evidence of NO generation in *M. oryzae* and would represent the most robust study to-date regarding NO generation and fungi. These could be validated by two separate approaches. Firstly, an alternative NO sensitive triazole forming dye such as DAF-FM DA or DAMBO-P^H should be used. Both dyes report NO through the binding of N₂O₃ and therefore, whilst untested, should be compatible with the PTIO enhancement technique. If DAF-FM DA or DAMBO-P^H generates data similar to the DAR-4M (AM) observations, this gives evidence for NO synthesis in *M. oryzae*. Secondly, a positive control test sample, such as mammalian white blood cells, should be loaded with DAR-4M AM and their fluorescence measured using the plate reader assay and PTIO enhancement technique. If the cells fluorescence matches the pattern recorded with *M. oryzae* this will provide categorical evidence that the combination of the plate reader assay and PTIO enhancement technique measure NO and that *M. oryzae* synthesises NO.

Until these validation experiments are completed we cannot be 100% certain the fluorescence recorded from DAR-4M AM loaded *M .oryzae* germlings is due to NO. Hence, there is still a significant amount of work remaining regarding NO and fungi.

To date this work provides the most robust evidence for NO within fungi and as such should be considered a major advancement in the investigation of NO and fungi.

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