TILLING: EMS mutagenesis in *Epichloë* endophytes and mutation screening using High Resolution Melting analysis and Next Generation Sequencing

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ABSTRACT

Epichloë are fungal endophytes (family Clavicipitaceae) of pasture grasses of the sub family poöideae. These endophytes live in symbiotic association with their hosts and confer resistance to insect and animal herbivory through the production of bioactive secondary metabolites (alkaloids) that are produced *in planta*. For a number of years endophyte research has been focused at manipulating fungal genes responsible for production of alkaloids which have toxic effects on livestock. However, the techniques used to date involve genetic modification to delete genes responsible for alkaloid production and strict regulations around genetically modified organisms in New Zealand prevent commercialisation of these organisms. Traditional mutagenesis was not practical. To find mutations in secondary metabolite pathways, the mutants had to be inoculated back into plants, which would have been a laborious and time-consuming process. The aim of my research was to develop Targeting Induced Local Lesions In Genomes (TILLING) methodology in *Epichloë* to disrupt fungal secondary metabolite genes using Ethyl methanesulfonate (EMS) and screen for mutations using high throughput screening techniques such as High Resolution Melting (HRM) analysis and whole genome sequencing, MiSeq.

In order to carry out the mutagenesis, uninucleate propagules would be preferred but as most of the filamentous fungi (including *Epichloë*) are multinucleate in nature, spores were thought to be an ideal alternative for mutagenesis. However, many of the commercially used *Epichloë* strains, such as AR1 and AR37, do not readily produce spores. Therefore an alternative mutagenesis system using fungal protoplasts was investigated and employed.

EMS mutagenesis showed that the number of colonies derived from protoplasts after mutagenesis declined steadily at a reproducible rate as measured by time-course of 0, 15, 30, 45 and 60 minutes to give LD50 values. At 60 minutes there was decline in the number of colonies to the levels of 10% of the initial number. To determine the effectiveness of EMS as a mutagen positive selection, using 5-Fluoroorotic acid (5-FOA), was also performed on the mutagenized protoplasts to derive the mutation frequency of 6 mutations per 1000 mutants compared to 0.002 mutations per 1000 for non-mutagenized protoplasts. This suggests a 3000-fold EMS-induced increase in the frequency of mutations.

Having established mutation frequency from the 5-FOA, positive selection and steady decline in number of colonies from EMS mutagenesis, an EMS mutant library was screened using next generation sequencing. . However, high throughput whole genome sequencing (MiSeq) led to the detection of only three verifiable point mutations (1 in 10Mb). Microscopic observations revealed that while individual protoplasts were largely (85%) uninucleate, protoplasts typically formed clumps containing 15-30 protoplasts. In theory, multiple nuclei would lead to an overestimation of the number of mutations since each nucleus would accumulate different SNPs. However, MiSeq sequencing did not detect this, probably due to being filtered out during bioinformatics processing. Thus if methods can be devised for plating single protoplasts, EMS mutagenesis should be applicable to this system. TILLING technology can be used to reduce the time for endophyte discovery and improvement. My research demonstrated that this procedure, although very promising in terms of benefit to fungal improvement, carries certain difficulties with it that we had to address such as mutagenesis using protoplasts and subsequent mutation discovery. I succeeded in establishing TILLING methodology for mutagenesis of *E. festucae* strain Fl1 as well as optimising protocols to screen mutants.

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ABBREVIATIONS

°C	Degree Celsius
bp	Base pairs
DAPI	4'-6'- diamidino-2-phenylindole
dHPLC	Denaturing high performance liquid chromatography
DNA	Deoxyribonucleic acid
dNTPs	Dinucleotide triphosphates
ds	Double stranded
EMS	Ethyl methanesulfonate
ENU	Ethyl nitrosourea
YFP	Yellow fluorescent protein
5-FOA	5-Fluoroorotic acid
HRM	High Resolution Melting
IGV	Integrative Genomics Viewer
ltm	Lolitrem
µL	Microliter
mM	Millimolar
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
OM	Osmotic Medium
OMP	Orotidine-5'-monophosphate decarboxylase
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
perA	Peramine
Q value	Quality value
RG	Regeneration agar
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphisms
TAE	Tris-acetate-EDTA-buffer
Taq polymerase	*Thermus aquaticus* DNA polymerase
TGGE	Temperature gradient gel electrophoresis
TILLING	Targeting Induced Local Lesions In Genomes
T_m	Melting temperature

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Chapter 1 INTRODUCTION

1.1 Endophyte/Grass symbiosis

Agriculture has been practised by mankind for thousands of years. During this time grasses have been the prime focus of plant improvement considering their importance in human food production and livestock feed (Vogel, 2008). Approximately 38% of global land area is currently under agricultural production (Foley et al., 2011), with 70% of this land dedicated to pastoral agriculture. Temperate forage based pastures, play a significant role within these agricultural systems, with an estimated 80% of global cow milk production derived from temperate grassland agriculture (Wilkins et al., 2003). In New Zealand cool season grass seeds were first brought from Europe in the early 19th century. The forest cover was replaced by pastures and since then the trend of cultivation of these grasses has continued (Charlton et al., 1999). The spread of ryegrass brought about a rise in sheep and cow numbers which also contributed to the economic development of New Zealand (Morris, 2013). Unfortunately this grass also caused some unwanted effects on ruminants. It had been noticed that animals grazing on these grasses would often suffer from loss of weight and fluctuating body temperatures (Cosgrove et al., 2005). As early as 1920, intercellular growth of fungi had been observed in various grass species (Latch et al., 1985). Interestingly, although aware of the presence of these fungi, scientists were oblivious of their negative effects on grazing animals until late 1970s (Stuedemann et al., 1988). At that time the fungus *Epichloë typhina* was isolated from tall fescue and it was suggested that fungal produced toxins may be responsible for reduced weight gain in cattle (Bacon et al., 1977). These fungi (family Clavicipitaceae) live in symbiotic association with cool season grasses belonging to Pooideae sub-family (Schardl et al., 2013a) and have subsequently become known as fungal endophytes (endo = within; phyte= plant) living in symbiotic association with their host plants (Mucciarelli et al., 2002).

These associations have attracted a lot of research since it has been known that the endophytes confer resistance to the host from both biotic and abiotic stresses. The host plant in return provides endophyte with nutrition, shelter and the means of transmission.

Fungi in general are known to produce a wide range of natural products, also known as secondary metabolites, and these are of enormous importance in agriculture and medical fields (Calvo et al., 2002). Generally, *Epichloë* endophytic fungi produce four classes of secondary metabolites which provide adaptive advantages to their host plants through resistance to drought (Nagabhyru et al., 2013), and protection from insects and animal herbivory (Young et al., 2013).

The secondary metabolites commonly produced by *Epichloë* are ergot alkaloids (including ergovaline), indole-diterpenoids (including lolitrem B), aminopyrrolizidine (loline) and pyrrolopyrazine (peramine) (Clay et al., 2002). Briefly, peramine is known to confer resistance against Argentine stem weevil (*Listronotus bonariensis*) (Popay, 2001) while lolines have insecticidal and feeding deterrent activities to protect their hosts (Blankenship et al., 2001). Indole-diterpenoids have activity against some insects and are also known to cause neuromuscular disorder such as ryegrass staggers in sheep (Fletcher, 2012). Ergot alkaloids confer protection against some insects but similarly to indolditerpenoids they also have anti-mammalian activity as they cause tall fescue toxicoses (Lyons et al., 1986).
Some other not so common secondary metabolites such as epoxy-janthitrems (indole-diterpene) confers resistance to various insect species (Popay et al., 2009). Secondary metabolites will be discussed at length in section 1.3.

1.1.1 Life cycle of *Epichloë* endophytes

Epichloë species have both sexual and asexual life cycles and can be either transmitted horizontally via ascospores or vertically through the seed (Figure 1) (Schardl et al., 2004). These endophytes are systemic throughout the aboveground parts of infected plants (Christensen et al., 2002). The hyphae grow longitudinally in the intercellular spaces of the host cells without breaching the host cell walls. They derive their nutrition from the adjacent host cells and apoplast (Tadych et al., 2014).

Asexual *Epichloë* endophytes have synchronised growth with their host plants (Christensen et al., 2008). Systemic colonization of the aerial parts of the seedling results from hyphae growing through apical meristem, sheaths and blades of the leaves. Asexual reproduction results in production of asexual conidiophores that are uninfective in *Epichloë* and are vertically transmitted through the seeds (Tadych et al., 2014).

During sexual reproduction a stroma develops on the host inflorescence and may result in partial or complete sterility of the host plant and is known as "choking" of the inflorescence (Craven et al., 2001). *Epichloë* are heterothallic and rely on cross fertilization of opposite mating types by transfer of the gametes from one stroma to the other. A fly of genus *Botanophila* transfers conidia produced from one stromata to another and these conidia function as spermatia to cause the fertilisation of the opposite mating types. This in turn results in formation of ascospores in the perithecia, which are forcibly ejected to infect more plants. Thus these sexual endophytes are transmitted horizontally through ascospores (Leuchtmann et al., 2014).

Sexual species in *Epichloë* are haploid whereas asexual species often arise through interspecific hybridization and are considered heteroploids (Schardl et al., 2013a).

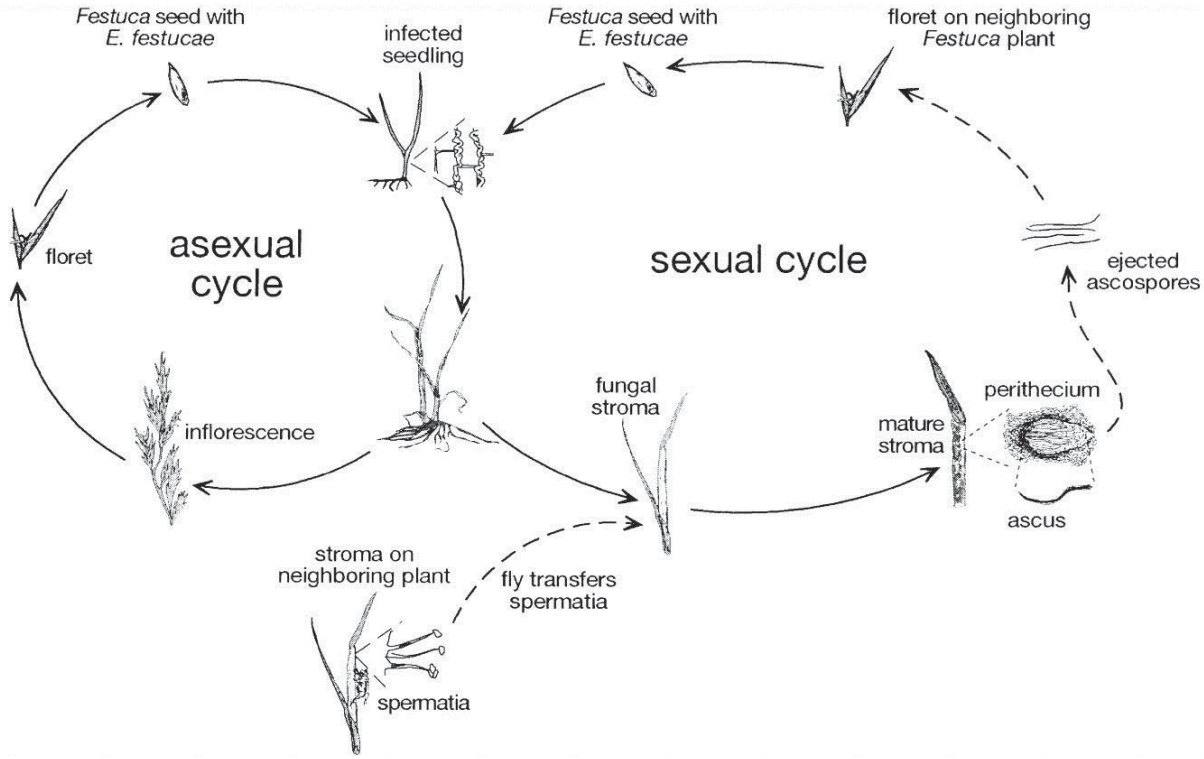


Figure 1: Life cycle of *Epichloë* endophytes

Epichloë endophytes are transmitted vertically (asexual) through seed and horizontally (sexual) through ascospores. Image from Clay et al., (2002).

1.2 Taxonomy of *Epichloë* endophytes

According to the previous botanical code of nomenclature and the dual naming system for fungi, the sexual species were classified in the genus *Epichloë* while asexual species formed the genus *Neotyphodium*. Recent phylogenetic studies indicated the need for changes in the nomenclature for both *Epichloë* and *Neotyphodium* species which would group them into one monophyletic clade (Schardl et al., 2013a). According to the recent taxonomy, 34 *Epichloë* species have been recognized based on their mating compatibility and phylogeny (Leuchtmann et al., 2014). Based on the principle of "one fungus = one name", *Neotyphodium* species have been synonymized under genus *Epichloë* (Leuchtmann et al., 2014).

The majority of asexual endophytes have been proposed to arise from their sexual counterparts through interspecific hybridisation (Moon et al., 2000). Microsatellite analysis has shown that hybrid asexual endophytes have multiple loci for genes whereas sexual endophytes have only single loci (Moon et al., 1999).

A heteroploid genome may reduce the chances of a deleterious mutation disrupting a gene thus increasing the fitness of the endophyte. Genetic evidence also suggests that *Epichloë* endophytes coevolved with their host grasses (Schardl et al., 2004). Attempts to artificially inoculate identical strains of *Epichloë* to different host species may result in varying degrees of incompatibility (Christensen, 1995).

1.3 Secondary metabolites and their agricultural implications

Secondary metabolites are compounds that are not essential for the normal growth and development of an organism but are beneficial in terms of long term survivability. Endophytes are known to produce secondary metabolites that benefit their host plants in a number of ways. The level of production of these alkaloids is influenced by tissue type, plant and endophyte genotype and environmental conditions (Thom et al., 2012). During last three decades of endophyte research in New Zealand the emphasis has been on secondary metabolite screening and selection of endophyte strains based on the alkaloids produced.

There are four characterised major alkaloids produced by the *Epichloë* endophytes.

Aminopyrrolizidine (lolines)

The most abundant pyrrolizidines are N-formylloline and N-acetylloline. These lolines and other derivatives are found in high concentrations, often exceeding 2% of dry plant mass (Craven et al., 2001). N-formylloline has been reported to have insecticidal properties (Blankenship et al., 2001), the activities on insects vary from being a metabolic toxin to a feeding deterrent. Although the concentration of lolines is lower in roots as compared to shoots, it still confers protection against insects (Patchett et al., 2011). For loline alkaloid pathway, 10 genes have been identified in the gene cluster (Schardl et al., 2013b).

Pyrrolopyrazine (peramine)

Peramine is the only pyrrolopyrazine that has so far been detected in endophyte infected hosts. It has potent deterrent activity against Argentine stem weevil, a major pest of ryegrass in New Zealand and is not known to have any adverse effects on livestock (Popay et al., 2013). Peramine is catalysed by a two module non-ribosomal peptide synthetase, known as *per*A (Tanaka et al., 2005). Non-ribosomal peptide synthetases are multimodular enzymes. It has been shown that deletion of *per*A renders the infected plants susceptible to damage caused by Argentine stem weevil. This study provides strong evidence that endophyte produced peramine has insect deterrence activity (Tanaka et al., 2005).

Indole-diterpenoids

Indole-diterpenoids such as lolitrem B are associated with tremorgenicity in mammals. Neurotoxic effects of this class of secondary metabolite are the cause of syndromes such as ryegrass staggers, which continues to have a considerable impact on New Zealand agriculture. A major effort has been directed at minimising the toxic effects of lolitrem B. It has been shown that endophyte genotype, tissue and the environmental conditions have major impact on the production of lolitrems (Siegel et al., 1997). Lolitrems are predominantly found in the leaves and seeds of the host plants. Biosynthesis of lolitrem B is driven by 10 genes within the cluster (Young et al., 2006).

All these genes are highly expressed by the endophyte when in symbiotic association with their host (Young et al., 2009). Epoxy-janthitrems (indole-diterpene), is known to confer resistance to various insects (Popay et al., 2009).

Ergot alkaloids

Ergot alkaloids such as ergovaline are secondary metabolites responsible for tall fescue toxicoses. This disorder is caused by the endophyte *Epichloë*, a symbiont of tall fescue. Tall fescue toxicoses results in vasoconstriction of blood vessels leading to loss of heat regulation, poor weight gain, reduced fertility and gangrene in livestock, causing losses of nearly $1 billion per year in the U.S alone (Panaccione et al., 2001). Toxicoses also affects sheep in New Zealand grazing on ryegrass with endophyte that produces ergovaline (Easton et al., 2001). Genes are clustered in ergot alkaloid biosynthesis pathway as well and have been identified from *Claviceps purpurea* and *Aspergillus fumigatus*. Many of the genes have been isolated for *Epichloë* as well. In the ergot biosynthesis pathway, non-ribosomal peptide synthetases catalyse the formation of ergopeptines by addition of tripeptide to lysergic acid (Fleetwood et al., 2007).

Since the end product of the ergot alkaloid pathway in *Epichloë*, ergovaline, causes toxicoses it would be advantageous to induce stop codons by nonsense mutations to block this pathway at an earlier step.

1.4 Endophyte Improvement

Early research on endophyte chemistry discovered that the predominant and naturally occurring endophyte strain in New Zealand, *Epichloë festucae* var. *lolii,* did not have a favourable combination of secondary metabolites and were toxic to grazing animals (Leuchtmann et al., 2000). *Epichloë* has a genome size of 35Mb (Schardl et al., 2012) and GC content of 45% (P. Maclean pers com). Since *Epichloë festucae* var. *lolii* are both haploid and asexual, they are not amenable to breeding for endophyte improvement. As such subsequent screening efforts were focussed on identifying naturally occurring endophytes from Europe with favourable alkaloid profiles that provided insect protection without toxic effects (Hume et al., 2007).

Number of these endophyte strains have been commercialised, such as AR1 which does not produce lolitrem B or ergovaline but only peramine (Popay, 2001). This was followed by AR37, which confers broad insect resistance due to the production of epoxy-janthitrems, an indole-diterpene alkaloid (Pennell et al., 2005).

As endophyte discovery was laborious and time consuming alternative methods such as genetic modification of alkaloid gene pathways had been explored (Fleetwood et al., 2007; Panaccione et al., 2001; Tanaka et al., 2005; Young et al., 2006) but these experiments have been largely proof of concept and cannot be transferred to the field.

1.5 Mutagenesis

With the advent of genome sequencing, the number of genes identified with unknown function have risen exponentially (Gilchrist et al., 2005). Reverse and forward genetics have emerged as a powerful tool to elucidate the function of gene products. Forward or classical genetics is based on determining the genes responsible for a particular phenotype while reverse genetics analyses phenotypic effects based on the knowledge of gene sequences. This section describes a reverse genetics approach in which TILLING technology has been used to mutagenize organisms of interest and identify mutations in specific genes of interest (McCallum et al., 2000).

Some of the reverse genetics approaches that have been widely used to induce mutations are insertional mutagenesis, physical mutagenesis, site directed mutagenesis and chemical mutagenesis (Alonso et al., 2006).

1.5.1 Insertional mutagenesis

Insertional mutagenesis involves the incorporation of a foreign DNA into the genome of interest in order to modify genes or genetic elements; this foreign DNA also acts as marker to identify the mutation (Krysan et al., 1999). Transfer–DNA mediated transformation (*Agrobacterium tumefaciens)* and transposon insertion mutagenesis have been widely used in many plant species where a DNA insertion ranging from 5 to 25kb is incorporated into the genome (Krysan et al., 1999). T-DNA also has been used as approach for insertional mutagenesis on filamentous fungi including *Aspergillus awamori*, *Aspergillus niger*, *Neurospora crassa, Trichoderma reesei* (de Groot et al., 1998) as well as *E. festucae* (Tanaka et al., 2007).

Insertions in gene of interest are random and can be screened via Polymerase Chain Reaction (PCR) using primers specific for sequences flanking the insertion, followed by cloning and sequencing. The changes brought out by the insertional mutagenesis can be used to obtain complete loss of function mutants (Settles et al., 2004).

1.5.2 Physical agents

Fast neutron mutagenesis randomly induces deletions and chromosome rearrangements throughout the genome by damaging the DNA due to production of reactive oxygen species and hydroxyl radicals (Men et al., 2002). In *Arabidopsis* and rice, a technique known as Deletagene has been used, in which seeds are mutagenized and then screened with PCR primers. Fast neutrons in *Arabidopsis* have been shown to cause G:C>A:T transitions due to reactive oxygen species mediated DNA damage and higher number of single base mutations have been observed compared to large deletions (Belfield et al., 2012). Advantages of using the fast neutron mutagenesis are that large number of seeds can be mutagenized and no transformation of plants is required (Li et al., 2001). Fast neutron mutagenesis in a *Candida* spp. strain resulted in 92 fold increase in lipase production in the mutant (Shu et al., 2010). Other physical agents such as ultra violet light and gamma radiations have also been used for mutagenesis. Ultraviolet light is known to cause base substitutions such as C to T at dipyrimidine sites by deamination of the cytosine bases (Ikehata et al., 2011). UV light was used as mutagen to increase antibiotic production of *Acremonium chrysogenum* (Ellaiah et al., 2003).

Gamma radiations causes C to G repair substitutions and cause chromosome breakage and often cause deletions due to oxidative damage to DNA due to production of reactive oxygen species (Cecchini et al., 1998). Gamma rays were used on the entomopathogenic fungi as *Isaria fumosorosea* to enhance their resistance to fungicides (Shinohara et al., 2013).

1.5.3 Site directed mutagenesis

Site directed mutagenesis has been used to elucidate gene function and genetically modify the genes of interest. It works on the principle that a sequence can be modified by using synthetic oligonucleotides, through having an internal mismatch and being complementary to the DNA template creates desirable mutations upon DNA replication (Carter, 1986).

This is a lengthy and cumbersome procedure where multiple steps are involved in the transfer of the target sequence into the plasmid vectors (Ho et al., 1989). Site directed mutagenesis was applied to *Neurospora crassa* to identify amino acid residues that play essential role for the flavin domain of nitrate reductase (González et al., 1995). In *Arabidopsis* and rice, site directed mutagenesis has been used for targeting specific genes by homologous recombination (Osakabe et al., 2010).

Plasmid mediated homologous recombination has been used in *Epichloë* to knockout secondary metabolite genes in the ergovaline pathway (Fleetwood et al., 2007), the peramine pathway (Tanaka et al., 2005), the indole-diterpenoid pathway (Young et al., 2009) and the loline pathway (Schardl et al., 2013b).

1.5.4 Chemical mutagenesis

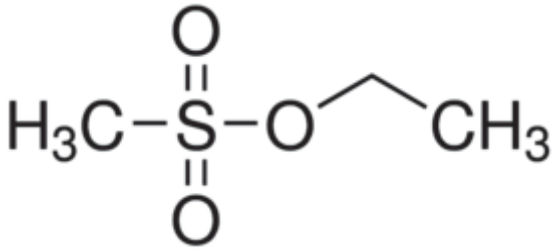
A multitude of chemical mutagenic agents are currently available for mutagenesis such as ethyl methanesulfonate (EMS), ethyl nitrosourea (ENU) and formaldehyde and these are known to cause changes ranging from point mutations to large rearrangements (Anderson, 1995). Generally, chemical mutagens form adducts with nucleotides and thus mispair with complementary bases in the templates, resulting in alteration of bases during replication (Greene et al., 2003).

Formaldehyde can also cause base substitutions and frame shift mutations. Most point mutations are transversions of G:C to C:G base pairs due to the ability of formaldehyde to react with the DNA, RNA and proteins to form adducts and cross links (Kawanishi et al., 2014).

EMS is a mono functional alkylating mutagen, that has one reactive group which may result in breaks in single strand of DNA or damage bases resulting in genetic changes such as transition mutations, base pair insertions or deletions (Figure 2A) (Sega, 1984). EMS causes both GC to AT and AT to GC transitions; however GC to AT mutations occur more commonly (Kodym et al., 2003) (Krieg, 1963). Mutagenesis using EMS generally results in high number of point mutations and less major chromosomal rearrangements such as indels (insertions and deletions) (Henikoff et al., 2003). EMS is biologically reactive due to presence of the ethyl group that can be transferred to different nucleophilic sites.

Investigating the mechanism of EMS mutagenesis it was found that O6-alkylguanine was highly responsible for the mutagenic effects (Figure 2B) (Sega, 1984).

(a)



(b)

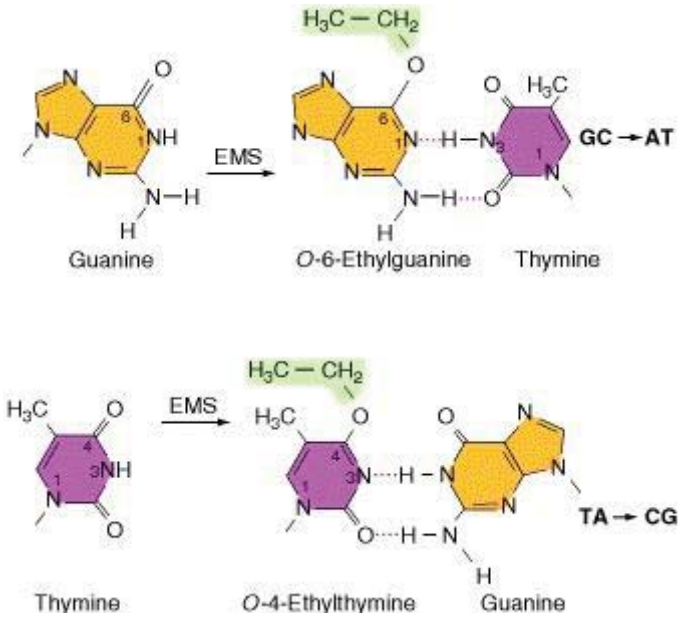


Figure 2: **a)** Structural formula of Ethyl methanesulfonate (EMS) (Sega, 1984). **b)** EMS mode of action.
EMS mutagen causes ethylation at O-6 of guanine and O-4 position of thymine that can cause mispairing leading to GC to AT transitions. Image from Griffiths et al., (2000).

ENU is another alkylating agent that causes nucleotide substitutions with bias towards A-T base pairs. Nucleotide substitutions like A-T to T-A transversions and A-T to G-C transitions alone account for 70% to 85% of the mutations induced (Noveroske et al., 2000).

EMS has been used on variety of organisms such as wheat (Uauy et al., 2009), *Arabidopsis* (McCallum et al., 2000), rice (Till et al., 2007), *C. elegans* (Cuppen et al., 2007) and *Drosophila* (Bokel, 2008).

EMS mutagenesis has also been tried on fungi with various degrees of success. EMS mutagenesis in *Aspergillus niger* was successfully carried out using spores to increase the antifungal resistance through increased production of extracellular glucose oxidase activity in mutants. An increase of 393.8% in synthesis of glucose oxidase was observed after EMS mutagenesis (Khattab et al., 2005). Spores of *Aspergillus oryzae* were also mutagenized with EMS and mutants were obtained that had antibacterial activity against *Staphylococcus aureus*. One of the mutants out of 3000 EMS treated cultures that were tested showed anti-bacterial activity (Leonard et al., 2013). Ideally a single propagule containing a single haploid nucleus of *Epichloë* would be the starting point for mutagenesis. However, because many *Epichloë* strains, including the commercial endophyte strains AR1, AR5 and AR37, do not readily produce asexual spores (conidia) in culture (W.Simpson pers com), protoplasts could be used to perform EMS mutagenesis.

EMS mutagenesis of *Claviceps purpurea* protoplasts has been successful as it yielded mutants that were higher in their alkaloid production. High alkaloid producers from EMS mutagenesis were in the range of 1 to 2% of the total mutants screened (Keller, 1983).

1.6 5-Fluoroorotic acid (5-FOA) selection

5- Fluoroorotic acid (5-FOA) selection has been used on fungi such as *Aspergillus fumigatus* (d'Enfert, 1996) , *Candida albicans* (Kelly et al., 1988), *Saccharomyces cerevisiae* (Ko et al., 2008b) and *Trichoderma reesei* (Hao et al., 2008). Gene disruptions and gene replacements resulting from insertion of different genes, have been selected by *ura4* gene encoding for orotidine-5'-monophosphate decarboxylase, that acts as a selectable marker (Grimm et al., 1988). The enzyme OMP decarboxylase catalyzes the conversion of orotidine-5'-monophosphate to uridine-5'-monophosphate, as the final step in the pyrimidine biosynthetic pathway (Hao et al., 2008). The enzyme orotidine-5'-monophosphate decarboxylase, is also known to convert 5-Fluoroorotic acid (5-FOA) into 5-Fluorouracil (5-FU). The pyrimidine analogue, 5-FU misincorporates in place of thymine or uracil in DNA and RNA and ultimately leads to cell death. This misincorporation allows positive selection of OMP decarboxylase mutants, as any loss of function mutant, will be unable to grow until exogenous supply of uridine or uracil is provided (O'Keefe et al., 2012).

Coupled with EMS mutagenesis, 5-FOA selection may be useful in screening mutants for uracil/uridine auxotrophy and for determining the frequency of mutations (Kakoi et al., 2014).

1.7 TILLING: A search engine for mutations

Reverse genetics has emerged as a powerful tool to elucidate function of a gene product and its role *in vivo* (Gilchrist et al., 2005). Targeting Induced Local Lesions In Genomes (TILLING) has revolutionised the discovery and screening of mutants (Gilchrist et al., 2006). With the availability of gene sequences, high throughput screening techniques can be applied to both forward and reverse genetics to discover gene functions and expression (Tamas et al., 2005). TILLING has been used on number of organisms such as *Arabidopsis thaliana* (Till et al., 2003), *Drosophila* (Cooper et al., 2008a), zebra fish (Moens et al., 2008) and *Phytophthora* spp. (Bhadauria et al., 2009) just to mention a few.

TILLING is a two-step procedure in which mutagenesis is followed by screening of mutants for mutations. A variety of approaches have been used for screening the mutants including the use of restriction enzymes (Cooper et al., 2008b), denaturing high-performance liquid chromatography (dHPLC) (McCallum et al., 2000), sequencing (di-deoxy and high throughput) (Colbert et al., 2001; Schmidt et al., 2013) and High Resolution Melting analysis (HRM) (Lochlainn et al., 2011). With the advent of high throughput sensitive technologies, screening for mutations has become cheaper, faster and more reliable. In the following section PCR and post PCR methods to identify mutations induced by mutagenesis are briefly described.

1.7.1 Restriction fragment length polymorphism

Restriction fragment length polymorphism analysis can be used to detect SNPs through the use of specific restriction enzymes. Digested DNA is run on agarose gel and the absence of particular fragment sizes indicates the presence of different genomic alterations. However, this method is laborious as it may require several runs and different restriction enzymes and is therefore not ideal for high throughput SNP discovery (Ota et al., 2007).

Restriction fragment length polymorphism analysis has been used to track SNPs in the *Candida albicans* genome (Forche et al., 2009) and for typing of different isolates in *Aspergillus fumigatus* (Pizeta Semighini et al., 2001).

1.7.2 Temperature gradient gel electrophoresis (TGGE) or temperature gradient capillary electrophoresis

TGGE is based on the principle that denatured DNA will travel slower during electrophoresis, as its movement is more restricted. TGGE requires two fragments, one being the target DNA containing the SNP and the other one being the native DNA. Reannealing of the denatured fragments leads to homoduplex formation if the target DNA has the identical allele as the native DNA (no SNP) or a heteroduplex formation if a SNP exists in the target DNA. These different forms can be differentiated by gel electrophoresis (Jones et al., 2009). TGGE has number of different applications such as determining the genetic diversity on soil fungi (Bidartando et al., 2005).

1.7.3 Cel-I endonuclease mutation detection

Cel-I endonuclease is a mismatch-specific nuclease (Stemple, 2004). Mismatch heteroduplexes are formed as a result of denaturing and reannealing of normal and mutated alleles. Cel-I endonuclease cuts one strand of dsDNA and the digested products are visualized on the gel. Two products of different size and colour are observed as heteroduplexes and labelled with different colours on each strand that validates the mismatch (Igarashi et al., 2000 672). This technique was very popular in early 2000 to detect induced mutations caused by chemical mutagens, for example in *Phytophthora* spp. (Lamour et al., 2006). With the development of more sensitive and high throughput techniques it has been largely superseded.

1.7.4 Denaturing high performance liquid chromatography, dHPLC

dHPLC is reversed-phase HPLC, which can detect SNPs due to differential affinity of the solid phase for single and double stranded DNA. The DNA fragments are denatured and then annealed prior to being put on HPLC column. Due to differences in the homoduplex and heteroduplex melting temperatures, the fragments are retained in the column differentially which helps to differentiate them from each other. Distinction is made easier as PCR products may be stained by an intercalating stain or unstained PCR products can be visualised by UV detector.

dHPLC has fast turnaround, higher sensitivity compared to other gel based systems described in Sections 1.7.1, 1.7.2 and 1.7.3 and can be automated but the column temperature needs to be optimised for the target (Yu et al., 2001). This technique was applied on *Candida* to identify seven different species by analysis of PCR products (Goldenberg et al., 2005).

1.7.5 High Resolution melting analysis

Mutations are detected in target genes by High Resolution Melting analysis (HRM) using PCR, followed by denaturing and reannealing of the dsDNA product which is monitored via a DNA binding fluorescent dye. On reannealing of the double stranded DNA, fluorescence increases and results in high resolution melt curve (temperature vs fluorescence) (Reed et al., 2007).

In HRM, three different scenarios, wild-type, heterozygous and homozygous are possible (Figure 3A). Temperature shifting of the normalized melting curves, helps to differentiate heterozygous samples from homozygous samples as they have lower melting temperature and different melting curve profiles (Figure 3B) (Graham et al., 2005). Data can also be represented as difference plot in which one of the samples is used as a reference with all other samples plotted relative to the reference (Figure 3C) (Wittwer et al., 2003).

HRM analysis relies upon getting different melt curves based on SNPs and melt curve differences are easier to detect in heterozygous samples.

In haploid organisms this problem can be circumvented by mixing or pooling samples of interest with other samples, mainly wild-type (Taylor, 2009). SNPs have been divided into four classes based on the shift in the melt curves (Table 1). (Venter et al., 2001).

SNP Classes	Base Change	Melt curve shift
1	C/T and G/A	Large (>0.5°C)
2	C/A and G/T	↓
3	C/G	↓
4	A/T	Small (<0.2°C)

Table 1: Different classes of the SNPs

These SNPs are classified based on their shift in melt curves (Venter et al., 2001).

The melting behaviour of the PCR products is dependent on their GC content, sequence and length. Use of smaller amplicons results in better differentiation of the genotypes, as it increases the difference in melting temperatures (Tm). Physical properties of both homozygous and heterozygous DNA strands are exploited through the HRM analysis. The fluorescence peaks for homozygous mutant and wild-type have been shown to differ by 0.3 to 1°C, which is reflected in the HRM curve shape (Erali et al., 2008).

Small amplicon size provides detection accuracy that cannot be attained using dHPLC (Yu et al., 2001). HRM is highly reproducible technique and position of the SNP need not be fixed. HRM is non-destructive process; as PCR products can be used for sequencing. Other advantage is sample pooling and high throughput (96 and 384 well format), which allows large number of samples to be processed simultaneously (Hondow et al., 2011).

For clinical applications, HRM has been applied to distinguish *Cryptococcus* species by genotyping Internal Transcribed Spacer 1 region and to identify drug resistance mutations in *Plasmodium falciparum* (Tong et al., 2012). HRM technology has been applied to screen allelic series of mutations in *Brassica rapa* for two genes (Lochlainn et al., 2011). HRM has also been applied to wheat to screen EMS-induced mutations with amplicon sizes ranged from 100 to 350 bp (Botticella et al., 2011).

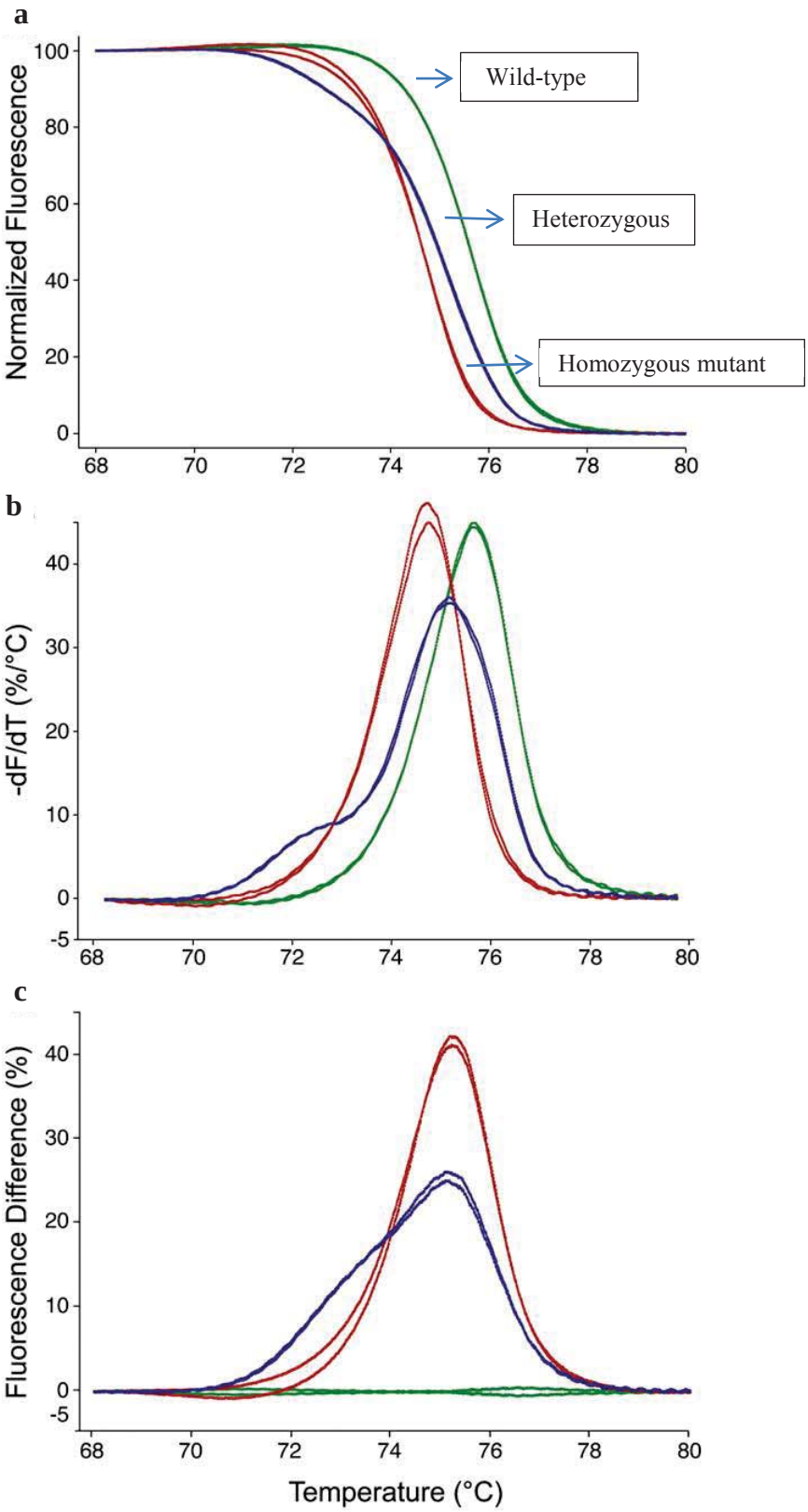


Figure 3: High resolution melting PCR analysis

Normalized melting curves showing melt curves for wild-type, homozygous and heterozygous samples **(a)**, Derivative plots showing the peaks where 50% of the samples are denatured **(b)**, and Difference plots showing one of the sample being used as reference and all other samples plotted relative to it **(c)**. Samples are green (wild-type), blue (heterozygous and red (homozygous mutant). Image from Erali et al., (2008).

Further technology improvements and chemistry development in terms of next generation sequencing is slowly introducing another approach to the high throughput, and cost-effective screening of mutational libraries.

1.8 MiSeq sequencing

The advent of next generation sequencing as an important tool for whole genome sequencing, re-sequencing and de novo sequencing has revolutionised genetic research (Quail et al., 2012). Next generation sequencing in a single reaction can be used to sequence a large number of DNA sequences. The high quality DNA is sheared into fragments of a specific size and specific adapters are ligated to the 3' and 5' ends (Buermans et al., 2014). These DNA templates are immobilised on a flow cell surface and amplified by sequential addition of nucleotides. HiSeq and MiSeq are the two most commonly used sequencing platforms. HiSeq is high throughput and has a larger turnaround time (Quail et al., 2012).

The MiSeq sequencing platform has a fast turn-around time. It is based on the principle of Sequencing by Synthesis technology (Loman et al., 2012). This process involved extraction of DNA from the sample, followed by fragmentation of the DNA into pieces no larger than 600 bases. Then oligo adapters were ligated to the DNA fragments as they were partly complementary. The libraries were pooled and barcoded prior to being run on the flow cell and a universal sequencing primer was used that binds to the adapters and was amplified by PCR amplification for sequencing (Figure 4).

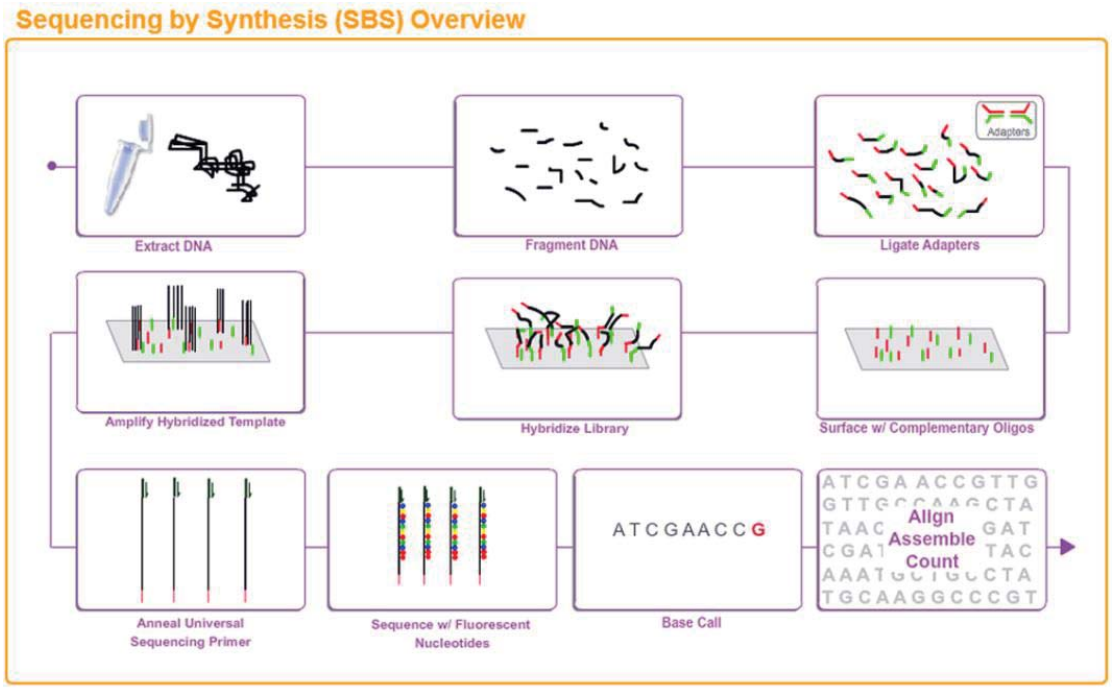


Figure 4: Next generation sequencing using MiSeq

Sequencing by synthesis technology is the basis of MiSeq sequencing. This next generation sequencing involves fragmentation of the DNA into pieces no longer than 600 bases and is followed by ligation of adapters, that are partly complementary. Libraries are barcoded and pooled prior to the run on MiSeq flow cell. Universal sequencing primer is used for sequencing (Image from Illumina, Inc.)

Libraries are barcoded and pooled then run on MiSeq flow cell with paired 300 base reads and 6-base index read (Quail et al., 2012). Quality (Q) value for each base call represents the likelihood of each call being incorrect. SNP discovery increases with increasing depth. SNP calling is recommended around 30-40x coverage, however for haploid genomes even 15x is reasonable. Paired end sequencing provides accurate read alignments thus improving accuracy and coverage of consensus sequence and SNP calling. In MiSeq read lengths of 150 bp using paired-end sequencing would give 99.83% of coverage, which would yield high quality data for analysis (Quail et al., 2012).

Faster turnaround-time and simplified sequencing on MiSeq platform makes it a cost effective approach for screening mutant libraries unlike some other approaches. Therefore MiSeq is ideal for small genome sequencing and resequencing projects (Kozich et al., 2013). MiSeq sequencing has been used on soil fungi to cover Internal Transcribed Spacer region 1 and Internal Transcribed Spacer region 2 in their genomes (Schmidt et al., 2013).

The limitation of the next generation sequencing platforms is the shorter read lengths (30-400 bp) and these shorter reads sometimes do not align properly to the reference genome leaving repetitive regions unmappable and de novo genome assembly is difficult as well (Rizzo et al., 2012).

Finding SNPs is one of the many applications for next generation sequencing. Generally, two approaches can be used to achieve this: targeted amplicon sequencing and whole genome shotgun sequencing (Daber et al., 2013). Whole genome shotgun sequencing involves shearing the genomes of cells and sequencing the fragments produced. These sequencing reads are then mapped to the genome. The main advantage of whole genome shotgun sequencing is that large parts of the genome can be analysed for mutation (van El et al., 2013). The disadvantage of whole genome shotgun sequencing is that genomic coverage by the sequencing reads can be limited in some parts of the genome, reducing confidence in the calling of mutations. In addition, there are errors inherent in the next generation sequencing process (PCR error and base call errors) (Robasky et al., 2014).

In the next generation sequencing, DNA polymerase is involved in cluster amplification and in other steps of sequencing by synthesis process; there are chances of base errors being introduced (Aird et al., 2011). However, the biggest source of error in next generation sequencing is phasing and cross-talk between sequencing clusters (Ledergerber et al., 2011) .

For these reasons, stringent filtering of sequencing data must be applied to a certain coverage and quality score to avoid false positive calls (Daber et al., 2013). Quality score (Q) reflects the probability of a base being called incorrectly by sequencer, ideally Q value of 30 is used, giving base call accuracy of 99.9%. Bases with quality scores above 30 were considered in the SNP analysis (Wan et al., 2012). In small genomes with few mutations, it is feasible to examine candidate mutants using mapping viewers such as the integrative genomics viewer (Thorvaldsdóttir et al., 2012).

1.9 Aims

This project is based on the observation that *Epichloë* secondary metabolites are expressed only *in planta* and not in culture (Fleetwood et al., 2007). As such traditional mutagenesis targeting these pathways would require all mutants to be inoculated into plants to detect the alkaloids through laborious chemical screening. The advent of TILLING has made it possible to screen for specific mutations in genes of interest using DNA and high throughput analysis.

The objectives of the project are:

- EMS mutagenesis of *Epichloë* using protoplasts
- HRM analysis to screen for mutations in specific genes
- 5-FOA positive selection to determine the mutation frequency of EMS
- MiSeq sequencing to determine the number of mutations genome-wide

Chapter 2 MATERIALS AND METHODS

2.1 Biological material

The strains used in this study were *Epichloë festucae* Fl1 wild-type and *E. festucae* Fl1 transformed with plasmid pYH2A, containing histone H2A fused to YFP.

Fungal strains	Relevant Characteristics	Reference
Epichloë festucae Fl1	Host: *Lolium perenne*	(Young et al., 2005)
EF/pYH2A Fl1	Fluorescent nuclear tag	(Gifted by Sameera Ariyawansa, PFI, AgResearch Ltd., Grasslands)

2.2 Media used

All media were sterilized by autoclaving at 121°C for 15 minutes, unless stated otherwise.

2.2.1 Potato Dextrose Agar (PDA)

A 1L Schott bottle was used to make 500 mL of Potato Dextrose Agar (Difco™ Becton, Dickinson and Co. USA) using 19.5 g of PDA powder according to manufacturer's instructions. The powder was resuspended into 500 mL of MilliQ water and agar was poured into sterile plastic petri plates in a laminar flow cabinet.

2.2.2 Potato Dextrose Broth (PDB)

Potato Dextrose Broth (Difco™) was made according to the manufacturer's instructions by suspending 24 g of PDB powder in 1 L of MilliQ water and was dissolved by heating with stirring on a hot plate. 50 mL of broth was decanted into each of the 250 mL Erlenmeyer flasks that were plugged with non-absorbent cotton and covered with aluminium foil.

2.2.3 Regeneration Agar (RG)

To make 1 L of Regeneration agar, 24 g of Potato Dextrose Broth and 273.8 g of Sucrose (0.8M) were suspended in 1 L of MilliQ water. The suspension was dissolved by heating with stirring on a hot plate and pH was adjusted to 6.5 with 1M NaOH. Bacto agar (Difco^{TM}) 1.5% (15 g) was added and media was poured into sterile petri-plates in a laminar flow cabinet.

2.2.4 0.8% RG media overlay

This Regeneration overlay (RG) was made up in 100 mL aliquots with same conditions and quantities as above, only exception was that 0.8% agar was used (0.8 g/100 mL).

2.2.5 5-Fluoroorotic Acid media (5-FOA)

Complete medium (PDA) was prepared with and without 5-FOA and uracil. To prepare this media 39 g of PDA was added to 500 mL of distilled water and autoclaved at 121°C for 15 minutes and kept at 50°C in a water bath.

5-FOA (1 g) and 1.5 mL of uracil (5 mg/mL) was added to 500 mL of distilled MilliQ water and filter sterilised and kept at 50°C for an hour. Both these solutions were mixed and poured into sterile plastic petri-plates in a laminar flow cabinet. Different concentrations of 5-FOA were used initially to find the optimal concentration (Table 2).

Conc. 5-FOA	Conc. Uracil
1mg/mL	5mg/mL
0.5 mg/mL	5mg/mL
0.2 mg/mL	5mg/mL
0.1 mg/mL	5mg/mL

Table 2: Different 5-FOA and uracil concentrations

For the 5-FOA plates, different concentrations of 5-FOA were used to make 250 mL of the solution.

2.2.6 Czapek Dox Media (Defined minimal medium)

To make 1 L of Czapek Dox Media Agar (Difco^{TM}), 45.4 g of Czapek Dox media powder was suspended in 1 L of MilliQ water and was dissolved by heating and stirring on a hot plate. Before pouring 1 mg/L of Thiamine hydrochloride (Difco^{TM}) filter sterilised solution was added to the solution and poured into plastic sterile petri-plates in a laminar flow cabinet.

2.3 Buffers used

2.3.1 Osmotic Medium (OM) buffer

To prepare the OM buffer, 100 mL of sterile water MilliQ was used to dissolve 88.8 g of $MgSO_4.7H_2O$, with brief heating if required. The pH of the solution was adjusted to 5.8, with few drops of 100mM Na_2HPO_4. Total volume was topped up to 300 mL (pH rises to 6.3) and solution was filter sterilised (Young et al., 1998).

2.3.2 ST buffer

To prepare ST buffer, 100 mL of sterile water MilliQ was used to dissolve 10.93 g of sorbitol and pH of solution was adjusted to 8.0, with 100mM Tris-HCl (Young et al., 1998).

2.3.3 STC buffer

To prepare STC buffer, 100 mL of sterile water MilliQ was used to dissolve 72.8 g of sorbitol and 4.38 g of $CaCl_2.6H_2O$. The pH of solution was adjusted to 8.0, with 50mM Tris-HCl (Young et al., 1998).

2.4 Fungal culture methods

2.4.1 *Epichloë festucae* protoplasts preparation

Protoplasts of *E. festucae* strain Fl1 were prepared for EMS mutagenesis. An *E. festucae* colony was grown at 21ºC on 39 g/L PDA (Potato dextrose agar) medium to about 2 cm diameter. Mycelium from half the colony was cut out and placed in 750 µL of PDB (Potato Dextrose broth) and macerated using Fastprep®-24 instrument at 4 m/s for 30 seconds. 8 x 50 mL of PDB in flasks was inoculated with 300 µL of the macerate. These cultures were incubated at 22°C for 4 days on 100 rpm shaker. Mycelium was filtered out through two layers of 3M Whatman filter paper in Buchner funnel and washed with 1 L of water and 5 mL of OM buffer (Section 2.2.5). Mycelium was scraped off the filter paper and then added to a flask with 50 mL of OM buffer and 15 mg/mL of lysis enzyme (Sigma, lysing enzymes from *Trichoderma harzianum*, L1412) for digestion. The flask was incubated overnight at 30°C on a 100 rpm shaker. This protoplast solution was filtered through two layers of nappy liner in a flask into 15 mL tubes (5 mL in each). The filtrate was carefully overlaid with 2 mL of ST buffer and centrifuged for 20 minutes at 1578 x g. Protoplasts were harvested from the interface between lysis enzyme solution and ST buffer and transferred to fresh tubes.

These tubes were centrifuged at 2465 x g for 5 minutes after the addition of 10 mL of STC buffer. The pellet was washed twice with 5 mL of STC buffer and centrifuged at 2465 x g for 5 minutes. The resulting pellet was resuspended in 500 µL of STC buffer and protoplast concentration was determined by haemocytometer. The protoplasts were diluted to final concentration of 1.25 x 10^9 protoplasts/mL (Young et al., 1998).

2.5 DAPI and FM4-64 staining of fungal protoplasts

Epichloë festucae, Fl1 protoplasts were prepared and 100x diluted in STC buffer. Aliquot (10 µL) was pipetted out on a slide. FM®4-64 (Invitrogen) 100 µg of lyophilized powder was dissolved in 20 µL of STC buffer to make a working solution of 8 mM and 0.5 µL of it was added to the protoplast solution on the slide.

DAPI, 4'-6'-diamidino-2 phenylindole (Sigma Chemical Co., St. Louis) stock solution of 10 µg/mL was diluted to working solution of 1 µg/mL. 2 µL of the working solution of DAPI was placed on the slide and was mixed with a pipette tip gently to make a homogenous mixture.

After a minute, a glass coverslip was placed and sealed with nail polish. Photomicrographs were taken on a Zeiss cooled CCD camera attached to an epifluorescence microscope, Nikon MicrophotSA using Metasystem's ISIS software v.5 (Altlussheim, Germany).

2.6 Chemical mutagenesis using EMS

2.6.1 Determining the LD50

Fl1 protoplasts were mutagenized with 50 mM EMS and resulting viable colonies were counted. Different time points were used 0, 15, 30, 45 and 60 minutes. The time point at which 50% of the protoplasts were killed was used for screening of the putative mutants.

2.6.2 Mutagenizing the protoplasts

The protoplasts were prepared (Section 2.3.1) for EMS mutagenesis to create mutants. Aliquot (25 mL) was filtered through double layer of nappy liner in glass funnel into 50 mL tube for negative control. For the rest 25 mL, 50mM EMS was added (500 µL of 50 mM EMS) and time intervals used were 15, 30,45 and 60 minutes. Similar to the negative control above, all these aliquots (5 mL) were filtered through two layers of nappy liner in a flask into 15 mL tubes (5 mL in each). The filtrate was carefully overlaid with 2 mL of ST buffer and centrifuged for 20 minutes at 1578 x g. Protoplasts were harvested from the interface between lysis enzyme solution and ST buffer and transferred to fresh tubes. These tubes were centrifuged at 2465 x g for 5 minutes after the addition of 10 mL of STC buffer. The resulting pellet was resuspended in 1 mL of STC buffer and protoplast concentration was determined by haemocytometer. Aliquots (15 µL in triplicate) of these protoplast solutions were added to 4 mL of 0.8% Regeneration medium (RG) agar (50°C) and spread on 1.5% RG agar plates and 5-FOA plates (200 µL in triplicate). Plates were incubated at 22°C for a week. This period was extended for 5 weeks for 5-FOA plates.

2.6.3 Protoplast count using Haemocytometer slide

The haemocytometer slide (Neubauer depth 1/10mm, Boeco, Germany) was used to count the protoplasts that had been treated with EMS mutagen at different intervals and protoplasts without EMS were counted as well. Protoplast suspension of 10 µL was loaded onto the haemocytometer slide and counted. Using the formula below, number of protoplasts per mL was inferred.

Average x 2.5 x 10^5 x dilution = protoplasts per mL

Average is derived from the count of protoplasts from 25 large squares which further have 16 smaller squares in each. The protoplasts were counted at different time-intervals for both treated as well as negative controls to find out whether there was any decline in intact protoplasts with the exposure to EMS and with respect to the time.

To determine the viability of the protoplasts 15 µL were plated onto complete regeneration medium and the numbers of colonies arising counted and adjusted to number of colonies per mL.

2.7 Subculture for putative mutants

2.7.1 Complete media (PDA)

After mutagenesis, the viable colonies that grew on the complete (RG) media plates were subcultured onto the complete media (PDA) using a scalpel blade, by cutting a block of agar with mycelia under the dissecting microscope (Carl Zeiss, Germany). 20 colonies along with wild-type Fl1 were transferred onto each of the plates, care was taken to minimise the transfer of agar with the mycelial mass.

2.7.2 Czapek Dox media

After mutagenesis, the viable colonies that grew on the complete (RG) media plates were replica plated onto the minimal medium (Czapek Dox) using a scalpel blade, by cutting a block of agar with mycelia under the dissecting microscope (Carl Zeiss, Germany).

20 colonies along with the wild-type Fl1 were transferred onto the plates; three subcultures were performed on the Czapek Dox medium.

2.8 5-FOA media and EMS mutagenesis

5-FOA and uracil (5 mg/mL) were used to make this medium (Section 2.2.4). Different concentrations of 5-FOA were used for growth of the putative mutants (Table 2).

For the initial generation of the protoplasts, complete RG media was used supplemented with 5-FOA and uracil. For the further testing of the putative mutants these were subcultured onto complete media (PDA) supplemented with 5-FOA and uracil.

2.9 HRM and its optimisation

High Resolution Melting analysis is based on the melting of double- stranded DNA in presence of DNA intercalating dyes, which specifically bind to dsDNA resulting in high levels of fluorescence. As the temperature rises, DNA is denatured and dye is separated from the DNA, resulting in low levels of fluorescence.

2.9.1 HRM protocol

LightCycler® 480 High Resolution Melting Master from Roche Diagnostics was used. Reactions of 12 µL were routinely used for the HRM assay (Table 3) and a concentration of 3mM of $MgCl_2$ was found to be optimal for the experiments. Different temperatures and time intervals were used in the LightCycler® 480 HRM program (Table 4).

Reagents	Final concentration	Volume (µL)
LightCycler® Melting mix	1x	6
$MgCl_2$	3mM	1.5
Template DNA	10-50 ng/µL	2.5
Forward primer	0.2 µM	0.42
Reverse primer	0.2 µM	0.42
PCR grade water	-	Up to 12 µL

Table 3: Table shows the reaction components of LightCycler® 480 High Resolution Melting master mix.

Steps	Temperature	Time	Cycles
Pre-incubation	95°C	10 min	1x
Amplification	95°C	10 sec	45x
	56°C	10 sec	
	72°C	10 sec	
Melting	95°C	1 min	1x
	40°C	1 min	
	65°C	1 sec	
	95°C	continuous	
Cooling	40°C	30 sec	1x

Table 4: High Resolution Melting program

Table shows different steps involved in High Resolution Melting program.

The DNA was extracted using ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research) from the selected endophytes and pooled in different ratios and were run on the LightCycler® 480 for HRM assay both individually and as pools in ratios of 1:2, 1:5 and 1:10. Primer pair MG150/151 was used, screening known SNPs in beta tubulin gene for both the pooling experiments (Appendix, Table 8).

Another HRM experiment was setup for the same endophytes, in which mycelia was pooled in different ratios of 1:1, 1:2, 1:3 and 1:4 and DNA was extracted using ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research). These pools and individual samples were run on the LightCycler® 480 for the HRM assay.

2.9.2 Isolation of fungal genomic DNA

To isolate the genomic DNA from a library of 1000 EMS mutants the colonies were grown on complete medium PDA (Potato Dextrose Agar) until they were 2 cm in diameter each. About half of the outer periphery of the colony was cut out and added to 300 µL of PDB (Potato Dextrose Broth) and was macerated using Fastprep®-24 at 4 m/s for 40 seconds. This macerate was used to inoculate 10 mL of PDB in 50 mL falcon tube along with bead and was incubated on 100 rpm shaker for 4 days at 22 °C.
The mycelium was centrifuged at 3600 x g for 3 minutes and the resulting pellet was pooled with another mutant and transferred to bashing bead tube provided with Zymo kit (Ngaio Diagnostics Ltd.). The resulting DNA was eluted in 50 µL of elution buffer and stored at –20°C for long term storage.

2.9.3 Quantification of genomic DNA

The isolated DNA was quantified using both Qubit® 2.0 Fluorometer and NanoDrop® ND 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For Qubit® 2.0 Fluorometer, the Qubit® dsDNA broad range Assay Kit was used and readings were recorded. With NanoDrop® ND 1000 the purity of DNA was assessed from the 260/280 ratios.

2.10 MiSeq sequencing

2.10.1 Pooling of DNA from putative mutants

EMS mutagenesis was carried out on Fl1, model organism using 50mM EMS on the mycelia digest (containing lysis enzyme). Colonies from time-points 0, 15, 30, 45 and 60 minutes were subcultured onto complete PDA medium.

These plates were incubated at 25 ºC. The colonies were macerated using Fastprep®-24 for 20 seconds in 1 mL PDB. 2 colonies each from the time points 0, 15, 30 and 45 minutes and one colony from 60 minutes were inoculated into 300 mL PDB in flasks. These were incubated at 25°C on a shaker at 100 rpm for a week. The resulting mycelium from different time points was spun at 2465 x g for 5 minutes in 50 mL falcon tubes. Supernatant was discarded and mycelia was washed with sterile MilliQ water and spun again at 2465 x g for 5 minutes. The mycelium was frozen with liquid nitrogen after removal of the supernatant. These cultures were put on freeze drier overnight. The freeze dried mycelium was ground with liquid nitrogen using pestle and mortar.

The DNA was extracted using Zymo fungal DNA kit and fluorometer was used to quantify the DNA. Restriction enzyme HindIII was used to check the quality of DNA. DNA from two different colonies for time points (0, 15, 30 and 45 minutes) were pooled and for 60 minutes time point a single colony was used to a 5 µg concentration and was sent off to Macrogen for Miseq sequencing. The five samples sent for sequencing were barcoded from 1 to 5 (time points 0 to 60 minutes).

2.10.2 Analysis for high quality and Unique SNPs

Analysis for the SNPs was carried out by AgResearch Bioinformatician, Paul Maclean. The reads were mapped from time point 0 against the "NCBI Genome": *Epichloë festucae* Fl1 genomics scaffolds (Accession numbers: JH158803-JH158942) using BWA version 0.7.9a-r786 as a mapping software, with the BWASW algorithm and default settings (Li et al., 2009a). The resulting SAM file was converted into a BAM file using the samtools software suite version 0.1.19-44428cd (Li et al., 2009b). The mpileup function in the samtools was used along with the vcftools to call SNPs from the BAM file. The 893 potential SNPs were filtered in Excel to 79 SNPs. Filtering used: DP4 (high-quality reads) sum >= 30, proportion of DP4 ALT (SNP call) >= 0.98 (allow for 2% error rate in reads). Quality score (Q) is the probability of base being called incorrectly by sequencer. Quality score of 30 was used, which represents probability of calling a base incorrectly 1 in 1000 (Table 5).

Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10,000	99.99%

Table 5: Quality scores in sequencing analysis

The phred quality scores and probabilities of calling the base incorrectly in sequencing analysis are listed above.

A new reference was created from the 79 SNPs and the "NCBI genome" using the vcf-consensus command. The resulting consensus sequence is called "T0 genome".

The other samples were mapped against the "T0 genome" again using BWA mapping software, with the BWASW algorithm. The resulting SAM files were converted into BAM files using the samtools software suite. SNPs were called using the mpileup function in the samtools along with the vcftools.

2.10.3 Identification of SNPs for sequencing

Simple sequence finding program "dust" with default parameters was used to mask out the repeat regions. Blast was used to get the flanking sequences and Integrative Genomics Viewer IGV was used to verify the coverage and SNPs.

2.10.4 Primers designed for sequencing of SNPs

Flanking sequences were extracted from the genome for each of the SNPs. These flanking sequences contained added bp on each side of the SNPs (giving 1001 bp in total) to ensure that primers could be designed for capillary sequencing. Vector NTI was used to design primers targeting the SNP from these flanking sequences (Appendix, Table 11). These primers were synthesised by Custom Science (IDT DNA).

2.11 Molecular Techniques

2.11.1 Polymerase chain reaction (PCR)

PCRs were performed in 50 µL reactions using 10-100 ng of endophyte genomic DNA. The reagents in Table 6 were set up on ice and mixed well before being transferred to thermocycler (Biorad iCycler, Biorad Hercule, CA, USA). PCR conditions were used as shown in Table 7.

Reagents	Final concentration	Volume (µL)
PCR mix – $MgCl_2$	10 x	5
$MgCl_2$	1.5 mM	1.5
Template DNA	10-100 ng/µL	5
Forward primer	0.2 µM	1
Reverse primer	0.2 µM	1
Distilled water	-	35.85
dNTPs	200 µM	0.40
Taq polymerase	1.25 U	0.25

Table 6: PCR reagents and their final concentrations

Table shows PCR reagents, along with their final concentrations and volumes in 50 µL reaction.

Steps	Temperature	Time	Cycles
Pre-incubation	95°C	4 min	1x
Amplification	95°C	30 sec	45x
	56°C	30 sec	
	72°C	1 min	
Elongation	72°C	5 min	1x
Cooling	4°C	∞	Hold

Table 7: PCR program showing different steps involved

Different PCR steps represented by different time intervals, temperature and cycles are shown above.

2.11.2 Agarose gel electrophoresis

Gel electrophoresis was used to separate the DNA based on its size. To make 1% (w/v) agarose gel, 1 g of UltraPure™ Agarose (Life technologies) was dissolved in 100 mL of 1x TAE buffer. To 100 mL of this gel, 5 µL of UltraPure™ 10 mg⁄mL ethidium bromide (Life technologies) was added. 5 µL of 1kb plus DNA ladder (Life technologies) was run against the samples at 85 volts and bands were visualised on a Biorad Gel documentation system using UV light.

2.11.3 Gel extraction

The PureLink® Quick Gel Extraction Kit (Life technologies) was used on the DNA extracted from agarose gel, according to the manufacturer's instructions. The DNA was eluted into 50 µL of elution buffer and 3 µL of it was run on an agarose gel to check for quality.

2.11.4 DNA purification/ concentration

For DNA bands that were single and clean, DNA Clean & concentrator *(*Zymo Research*)* was used and eluted into 8 µL of elution buffer. The DNA resulting from the gel extraction and from the clean-up kit was quantified using Qubit® 2.0 Fluorometer (Life technologies), according to the manufacturer's instructions.

2.11.5 DNA sequencing

PCR products were sequenced using the dideoxy sequencing method with gene specific primers. Dye-terminator reactions were carried out in 20 µL reactions with 15 ng of PCR products, 0.875x ABI dilution buffer (Applied Biosystems), 3.2 pmol primer and 1.5 µL Big-Dye (Applied Biosytems). Reactions were cycled in an iCycler (BioRad) thermocycler at 96°C for 3 min then 30 cycles of 96°C for 10s, 50°C for 10s and 60°C for 4 minutes. Separation and detection were carried out on ABI 3730 automated sequencer.

Chapter 3 RESULTS

3.1 Fl1 protoplasts

The aim of my research was to develop methodology that would allow mutation of *Epichloë* genes using Ethyl methanesulfonate (EMS) based mutagenesis. As discussed in section 1, spores or protoplasts are the two options available to consider for mutagenesis but since many *Epichloë* strains, including the commercial endophyte strains AR1, AR5 and AR37 do not readily produce asexual spores (conidia) in culture (W.Simpson pers com), protoplasts were chosen as the material to perform EMS mutagenesis. Fl1 protoplasts were prepared (Section 2.4.1) (Young et al., 1998) and resuspended to a concentration of 10^7 protoplasts per mL for the study below.

3.1.1 Majority of protoplasts of *E. festucae* strain Fl1 are uninucleate

In order to discover mutations after the mutagenesis process, it was important to determine what percentage of live protoplasts contained only a single nucleus. Multi-nucleate protoplasts could regenerate into chimeric colonies if more than one nucleus survives and can cause under estimation of the mutagenesis rate. In addition during sequence analysis the reads reflect different mutations corresponding to each nuclei present. Majority of uninucleate protoplasts will reduce the number of individuals required to screen for mutations and no subculturing will be required.

The number of nuclei in protoplasts was therefore assessed using two different techniques in two experiments. In the first experiment DAPI was used to stain the nuclei in protoplasts (Section 2.5). DAPI stain has affinity for the A-T rich region and binds the minor groove of dsDNA (Chazotte, 2011). The vital dye FM®4-64 was used to determine the percentage of viable protoplasts. This dye is taken up through the cell membrane by endocytosis, but by viable cells only (Bolte et al., 2004). Three independent Fl1 protoplast preparations were prepared. Protoplasts were counted using a haemocytometer (Section 2.6.3) and the protoplast suspensions were adjusted to 10^7 protoplasts per mL. A total of 186 protoplasts from the three preparations were analysed.

Using an epifluorescence microscope it was determined that all 186 protoplasts were viable as indicated by FM®4-64 fluorescence. The majority of protoplasts were uninucleate (85%) while the remainder had two or more nuclei in them (Figure 5). Figure 7 shows examples of stained protoplasts.

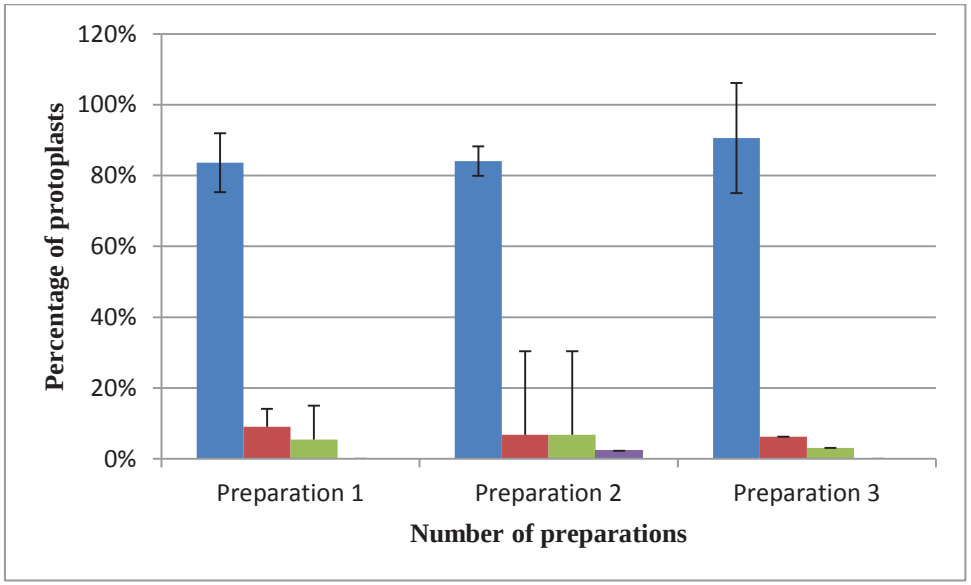


Figure 5: Percentages of nuclei in Fl1 protoplasts determined by DAPI

The graph shows percentage of uninucleate protoplasts compared to multinucleate protoplasts that were stained with DNA specific fluorescent dye, DAPI and vital dye FM®4-64. Blue bars represent percentages of uninucleate protoplasts in each preparation, red bars represent percentage of dinucleate protoplasts, green bars represent percentage of trinucleate protoplasts and violet colour bars represent percentage of quadrinucleate protoplasts. 110, 44 and 32 protoplasts were observed in the preparations 1, 2 and 3 respectively.

3.1.2 Fluorescent nuclear tag (pYH2A) analysis

Using DAPI and FM®4-64 stain the Fl1 protoplasts were found to be largely uninucleate (85%) and the remainder had two or more nuclei in them. Another approach was used to verify the above findings by using a recombinant plasmid pYH2A (gifted by Sameera Ariyawansa) expressing yellow fluorescent protein (YFP) that localizes in nucleus (Rech et al., 2007). Protoplasts were prepared from an Fl1 strain containing the recombinant plasmid, pYH2A.

Protoplasts were generated as described in Section 2.4.1 and concentrations were adjusted to 10^7 protoplasts per mL and observed using confocal microscope. A total of 582 protoplasts from three independent preparations were analysed. Using the vital stain FM®4-64 (Section 3.1.1), all the protoplasts that were analysed were found to be viable. Using the nuclear tag it was shown that more than 85% of protoplasts were uninucleate with the remainder being bi or trinucleate (Figure 6) (Figure 7f).

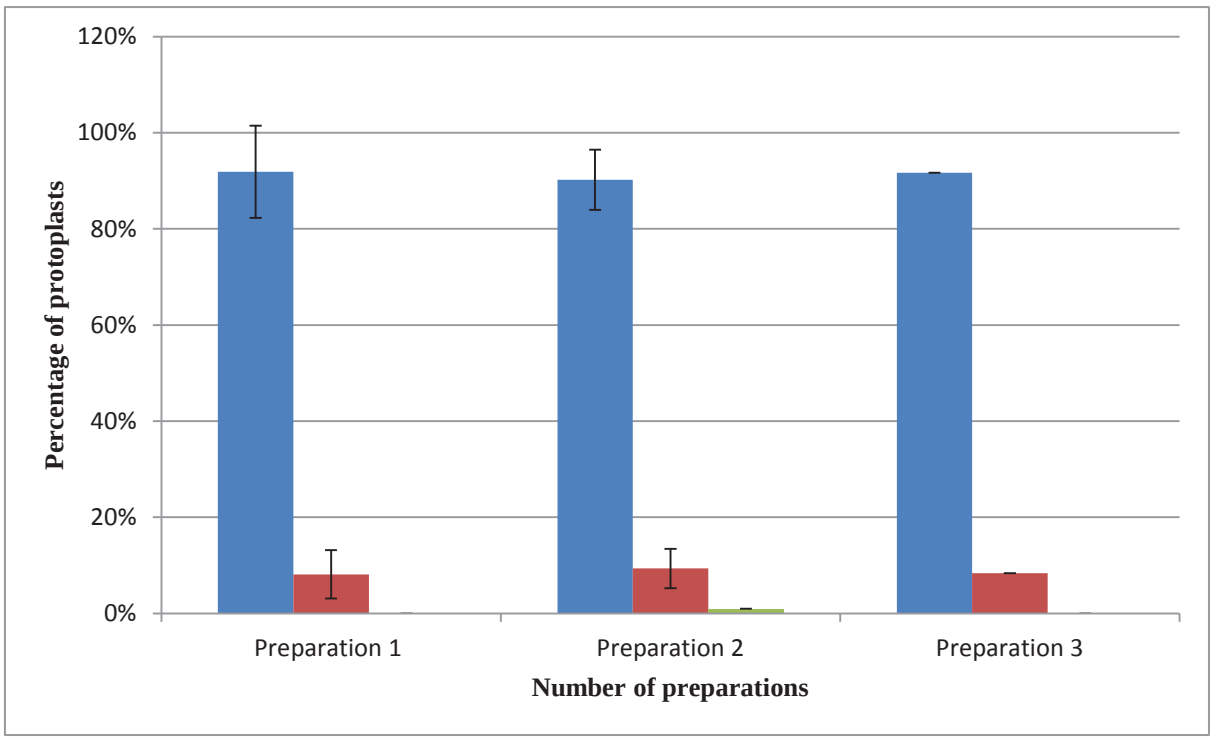


Figure 6: Percentages of nuclei in Fl1 protoplasts determined through nuclear tag

The graph shows percentage of uninucleate vs. multinucleate protoplasts created from an Fl1 strain that was expressing a fluorescent protein to act as a nuclear tag. Blue bars indicate the percentage of uninucleate protoplasts in each preparation, red bars represent percentage of dinucleate protoplasts and green bars represent percentage of trinucleate protoplasts. As shown majority of the protoplasts were uninucleate. Three different preparations were used with total number of protoplasts analysed being 308, 214 and 60 in preparations 1, 2 and 3 respectively.

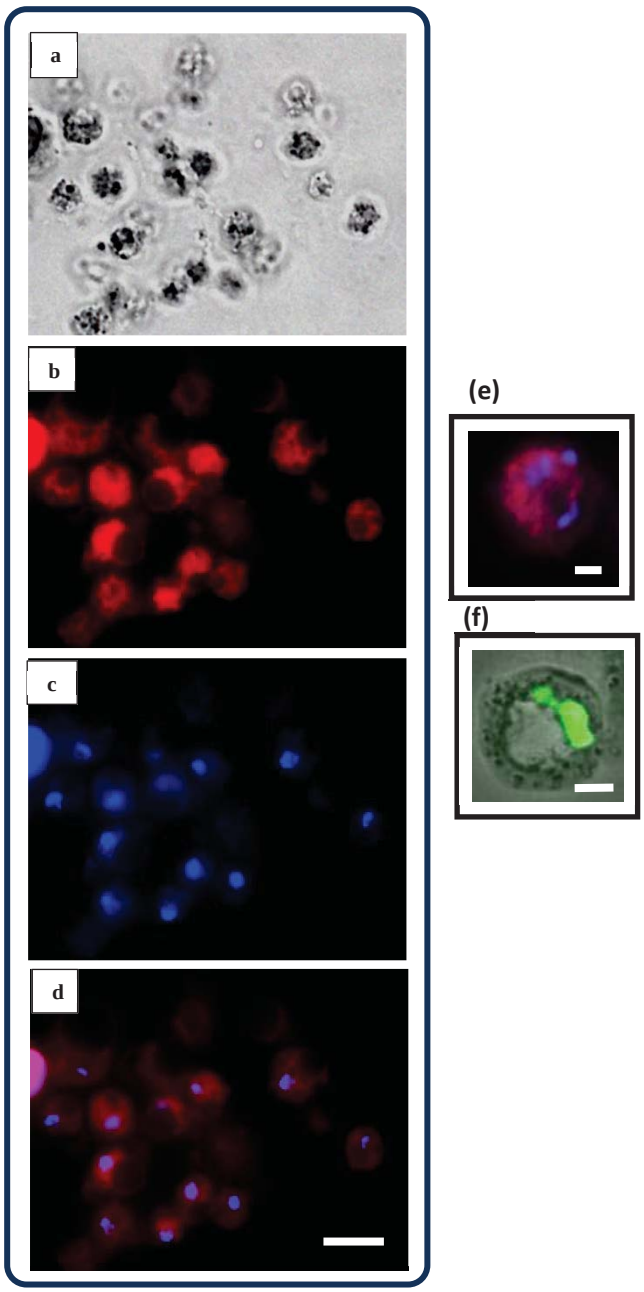


Figure 7: Nuclear staining of protoplasts

(a) Phase contrast image; **(b)** same field of view as in **(a)** showing cytoplasm stained with dye FM®4-64; **(c)** the same nuclei stained with DAPI; **(d)** merged image of the images b and c used to determine the number of nuclei in viable protoplasts. Note that all protoplasts showing nuclear staining also have taken up the vital dye. Image **(e)** shows a merged image of a quadrinucleate protoplast stained with DAPI and FM®4-64 stain; **(f)** a trinucleate protoplast of the Fl1, visualized by the nuclear tag. Bars represent 10µm for (**a, b, c** and **d**), 1µm for **(e)** and 3µm for **(f)**.

Both experiments indicated that the majority of protoplasts were uninucleate and thus suitable as a starting material for EMS mutagenesis.

3.2 Establishing a procedure to allow recovery of EMS mutants

As shown in chapter 3.1, the vast majority of protoplasts prepared for the mutagenesis were uninucleate. Based on the literature, 50mM was the most commonly used concentration of EMS and I used it to perform subsequent time course experiments. Effective EMS mutagenesis requires establishing a lethal dose at which 50% of the organisms are killed in a given timeframe and the surviving population have accumulated enough mutations that are not lethal (Kumar et al., 2013). It has been found that increasing the dose of mutagen has severe impact on survivability, while the lower dose does not yield enough mutations (Rawat et al., 2012). To successfully recover EMS mutants the mutagenesis must be balanced against having a high mutation frequency and an acceptable survivability rate (Barbour et al., 2006). Based on the literature 10 to 50% survival rate produces viable mutants containing a number of mutational events; most of them single nucleotide changes (Lawrence, 2002). Even outside this range (>50%), one would still expect to obtain surviving colonies but the higher mutation frequency could be deleterious to the organism.

3.2.1 Time course of the decline of viability resulting from EMS treatment

To establish the time course of EMS-induced decline in viability, a number of approaches were tried, such as addition of EMS to purified protoplast suspensions and plating these onto complete RG medium plates. Another method that was used involved pelleting, after the addition of EMS and the protoplasts were resuspended before being plated on complete RG media. Both these methodologies gave irreproducible results and were not used as the number of viable colonies that grew on complete regeneration were inconsistent between replicate experiments, most likely due to protoplast bursting. A new approach was devised that involved exposure of crude unpurified mycelium digest containing protoplasts to 50mM EMS and it was successfully repeated several times. The protoplast suspension (mycelial digest) was purified immediately prior to addition of EMS (time point 0) and a time standardised purification of other samples after 15 minutes, 30 minutes, 45 minutes and 60 minutes of exposure to EMS was carried out (Section 2.6.2). The number of protoplasts was determined from the protoplast suspensions from each time-point using the haemocytometer slide, by loading 10 µL of suspension for each treatment.

As a control and to check for any decline in viability by factors other than EMS, the experiments also included a control procedure in which no EMS was added during the time course.

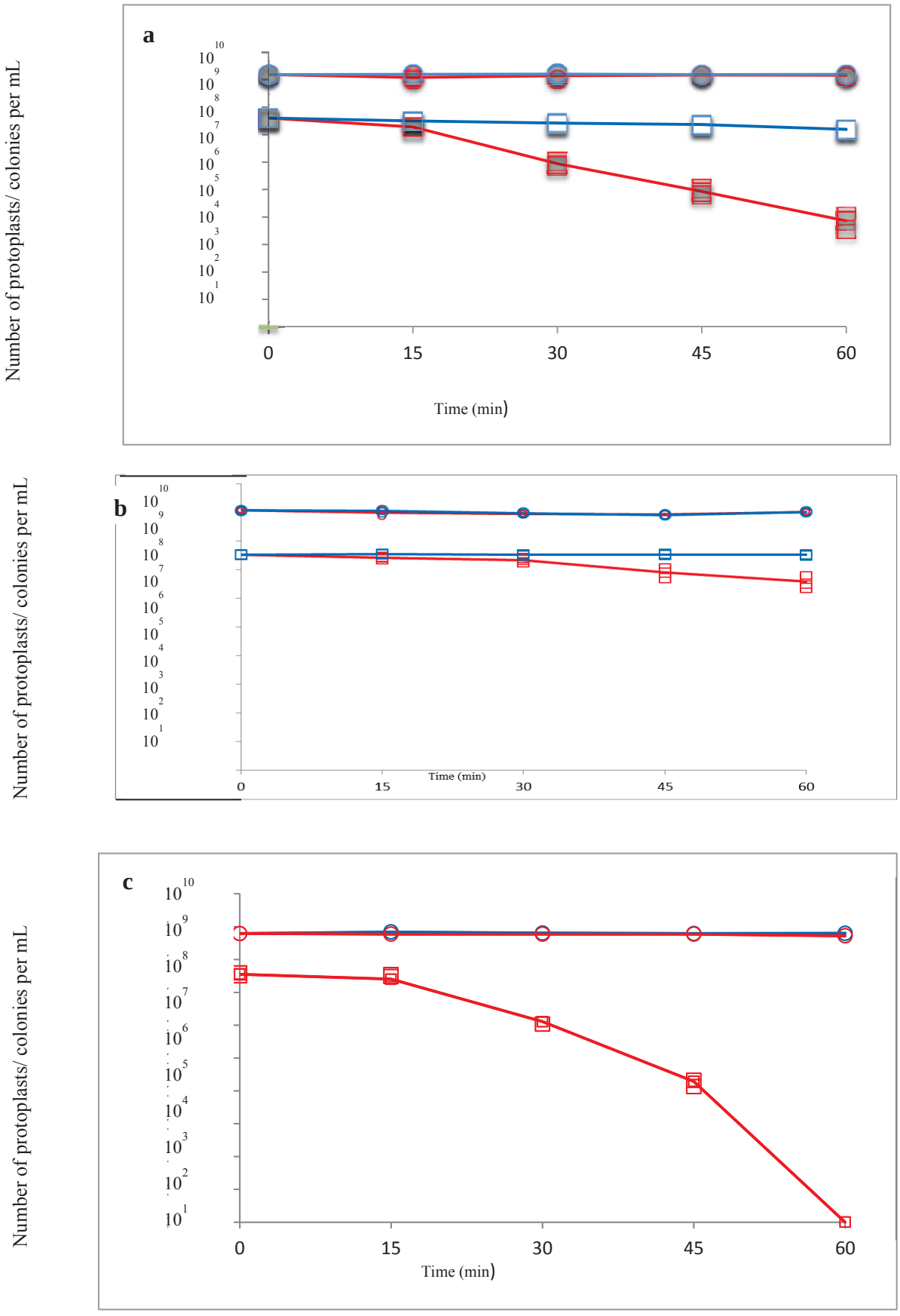
At each time point the following numbers were determined: (i) the number of protoplasts in the suspension, and (ii) the number of colonies formed 2 weeks after plating mutagenized and non-mutagenized protoplasts on regeneration medium. Four experiments were carried out in which most of these parameters were assessed (Figure 8).

3.2.2 EMS treatment does not significantly alter the protoplast count over time

In the mutagenesis experiments protoplasts were counted using a haemocytometer slide (Section 2.5.3) to ensure that protoplasts stayed intact over the time course (0, 15, 30, 45 and 60 minutes). It was shown that there was no significant drop in the number of protoplasts at 0, 15, 30, 45 and 60 minutes during EMS exposure (Figure 8). Negative controls were included in this experiment in which protoplasts with no EMS also showed no decline over time (Figure 8).

3.2.3 Decline in number of colonies from EMS treatment

The number of colonies from EMS treatments and controls that regenerated on complete RG media for 0, 15, 30, 45 and 60 minutes after 2 weeks were counted. The LD50 was found to be between 15 and 45 minutes exposure to EMS (Figure 8). Longer exposure to EMS caused a further drop in the number of surviving protoplasts to the point where only 1-11% of the population was able to survive after 60 minutes (Figure 8). The decline in the protoplast viability should be attributed to the accumulation of EMS-induced mutations over time since the controls with no EMS showed no drop in viability. However, it was important to establish that the decline in viability was not due to protoplasts bursting in the EMS solution over time, so the protoplast counts were also performed across the time course.
The number of colonies resulting from the control material with no EMS was also counted at different time intervals. It was found that there was no significant difference in the number of colonies regenerating at 0, 15, 30, 45 and 60 minutes (Figure 8).


a
Number of protoplasts/ colonies per mL
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
0
15
30
45
60
Time (min)
b
Number of protoplasts/ colonies per mL
Time (min)
c
Number of protoplasts/ colonies per mL
Time (min)

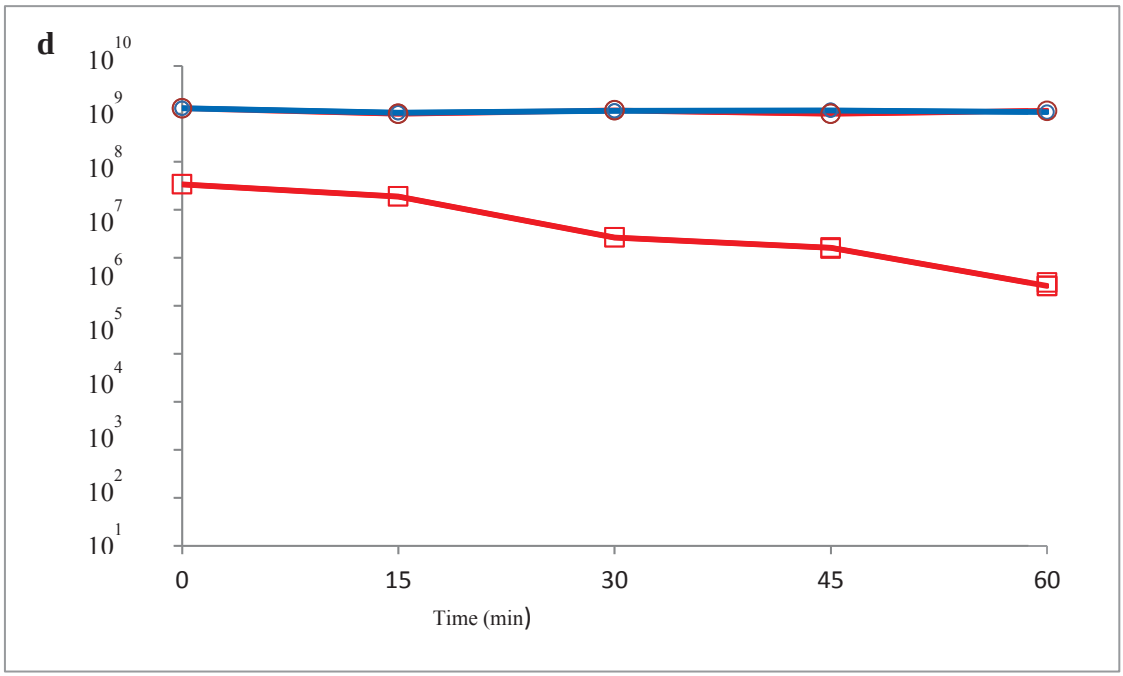
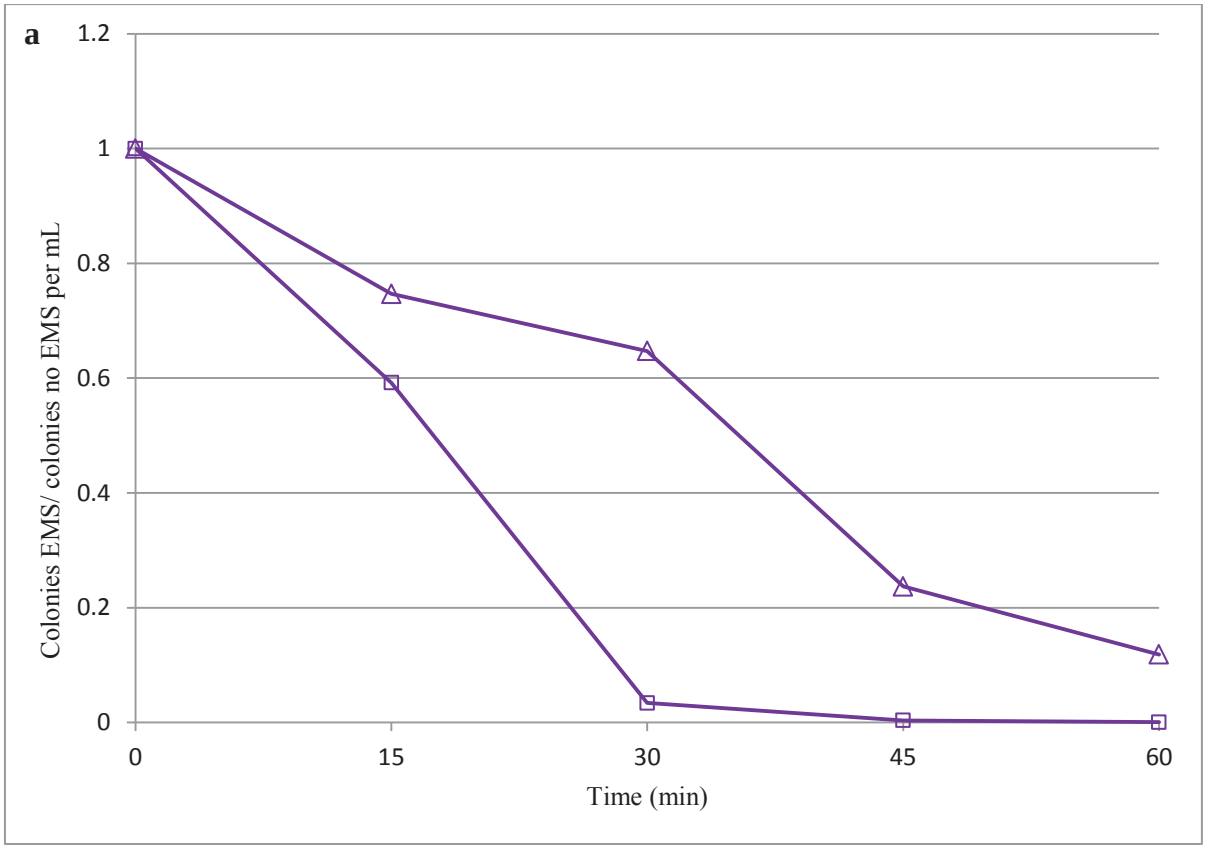


Figure 8: EMS mutagenesis experiments showing EMS treated and untreated protoplasts and colonies

Red circles represent the number of protoplasts per mL after addition of EMS and blue circles are the number of protoplasts per mL without EMS, at different time intervals. Red squares represent the number of colonies per mL treated with EMS and blue squares are the colonies per mL without addition of EMS, at different time intervals. Four independent mutagenesis experiments **a, b, c** and **d** were carried out, with 50mM EMS concentration and at time points of 0, 15, 30, 45 and 60 minutes. Panel **(a)** shows the number of protoplasts with and without EMS and number of colonies with and without EMS treatment per mL. Similar to panel **(a)**; panel **(b)** represents number of protoplasts and colonies with and without EMS, respectively. Panel **(c)** and Panel **(d)** show number of EMS treated colonies and number of protoplasts per mL with and without EMS at different time points. Controls were used where no EMS was added and number of protoplasts and colonies were counted. Lower number of red circles (number of protoplasts without EMS treatment) was the result of overlapping due to close data points.


a
1.2
1
0.8
0.6
0.4
0.2
0
Colonies EMS/ colonies no EMS per mL
0
15
30
45
60
Time (min)

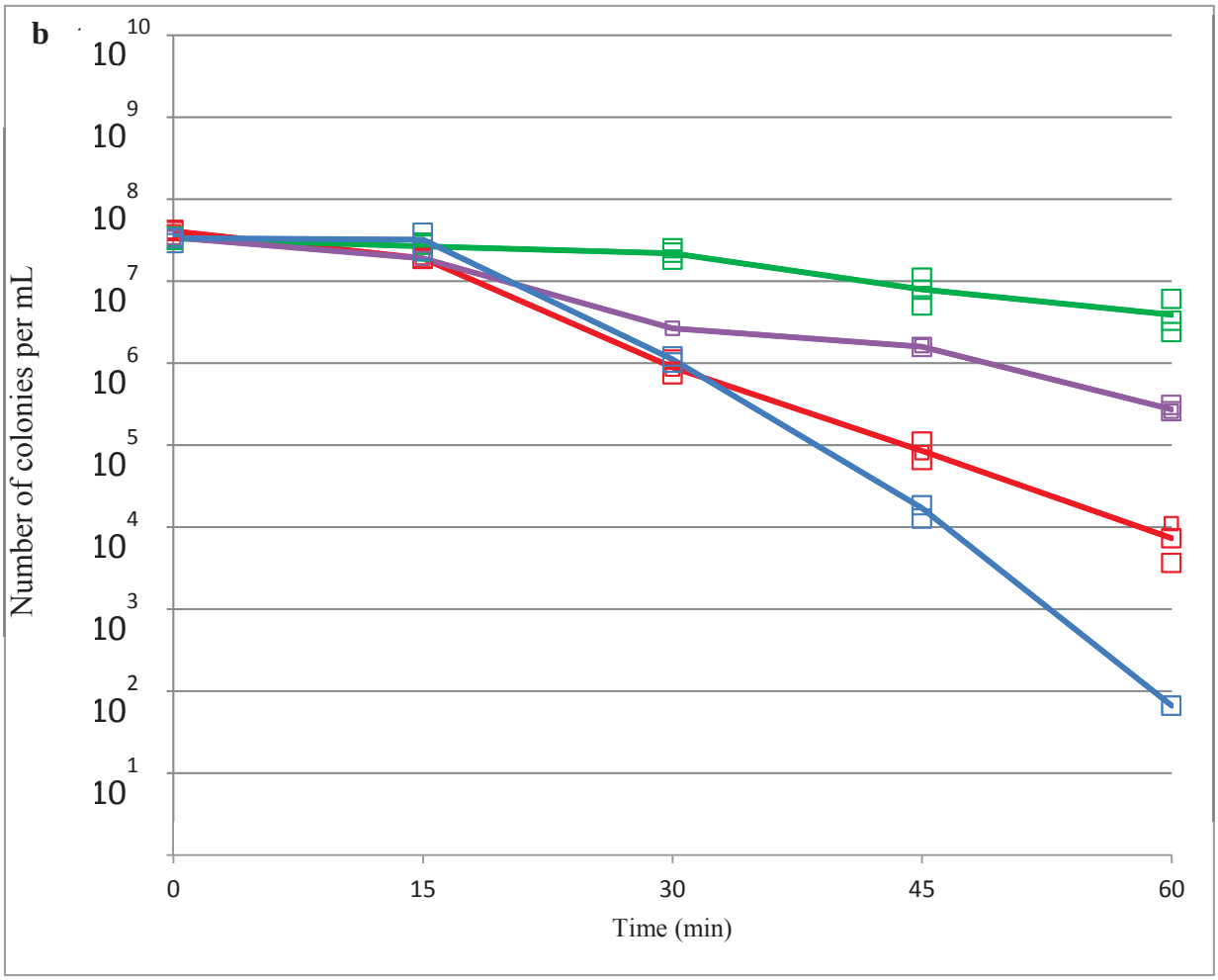


Figure 9: Ratios of EMS treated and untreated colonies at different time points

a) Purple squares represent the colony count proportions of EMS treated protoplasts vs. non-EMS ones in two experiments a (triangle) and b (square).

b) Starting point for the four experiments at 0 time point and kill curve values at different time points (15, 30, 45 and 60 minutes) are represented. The 50% kill rate is achieved between 15 and 45 minutes for all the experiments. The green line represents experiment a, violet line is experiment b and red and blue lines represent experiments d and c respectively.

The results presented here (Figure 8) indicates a timeframe of 15-45 minutes in which 50% of mutagenized protoplasts are killed. This is supported by the ratio of no EMS and EMS treated protoplasts that show the viability over different time points as shown in Figure 9a whereas Figure 9b shows the viability decline for different experiments at different time points (0, 15, 30, 45 and 60 minutes). After establishing the LD50 in the experiments above, 5-FOA selection was used to verify the effectiveness of EMS mutagenesis.

3.3 EMS mutagenesis and selection of 5-FOA colonies

5-FOA selection offers a feasible option to find loss-of-function mutations as well as gain of function resistance mutations (Section 1.7). I used this system to deduce the mutation frequency for the EMS mutagenesis in this study. 5-FOA selection was applied on the EMS mutagenized and non-mutagenized viable protoplasts to verify the effect of EMS as a mutagen.

As described in Section 1.7, inactivation of the gene encoding orotidine-5'-monophosphate decarboxylase confers resistance to 5-Fluoroorotic acid (5-FOA). Thus on growth medium containing uracil mutant colonies with a non-functional orotidine-5'-monophosphate decarboxylase will be able to grow, whereas wild-type colonies with a functional enzyme will not grow (O'Keefe et al., 2012). In addition, 5-FOA selection may also identify mutants that confer resistance to 5-FOA through unrelated mechanisms (Boeke et al., 1984).

5-FOA resistant mutants were screened on regeneration medium containing 1 mg/mL FOA and 5 mg/mL uracil. This was based on a dose response experiment in which .1, .2, .5 and 1 mg/mL of 5-FOA were tested. Doses of up to .5 mg/mL concentration lead to significant colony growth of Fl1 wild-type, for which too many colonies to count (lawn) were observed. However at 1 mg/mL of concentration, growth of the wild-type was entirely inhibited, for this reason the concentration was chosen for plating EMS-treated colonies.

This is in agreement with the literature whereby 1 mg/mL 5-FOA has been used for selection of 5-FOA resistance in *Saccharomyces cerevisiae* (Ko et al., 2008a). Protoplasts were plated from time points 0 and 45 minutes from EMS treated suspensions (Section 3.2.1) for experiment e that was based on the guidelines of experiments a, b, c and d. I plated 15 µL of protoplast suspensions from both the time points 0 minutes and 45 minutes onto complete regeneration medium and counted the resulting colonies. 490450 colonies were obtained at 0 minutes and 11000 colonies were obtained at 45 minutes corresponding to the kill rate of 97.75%. I also plated 200 µL of the same suspensions in triplicate onto regeneration medium containing 5-FOA. An average of one 5-FOA resistant colony arose on 0 minutes (i.e. 3 per 600 µL) and 66 colonies arose on 45 minutes (i.e. 200 per 600 µL).

This equates to 6 resistant colonies per 1000 viable cells for 45 minutes and 0.002 of resistant colonies per 1000 viable cells at 0 minutes. This suggests a 3000-fold EMS-induced increase in the frequency of mutations. 200 5-FOA resistant colonies along with wild-type Fl1 were replated from three plates onto minimal medium and PDA (Figure 10). The three colonies which failed to grow on minimal medium but had normal growth on PDA were good candidates to contain mutations in either the *pyr4* or *pyr2* genes of the pyrimidine pathway.

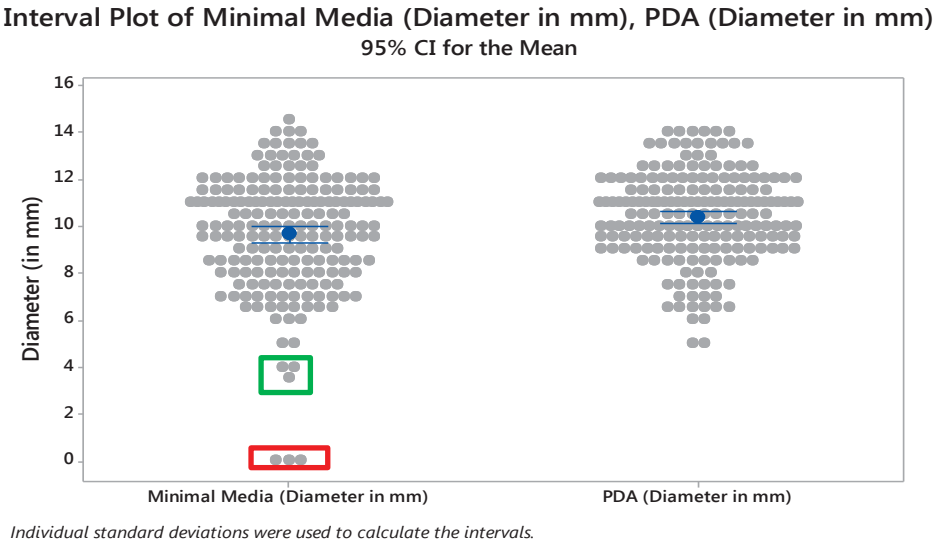


Figure 10: 5-FOA resistant colonies on minimal and complete media

Interval plots here represent the distribution of 5-FOA resistant colonies on minimal and complete media, PDA. Majority of the colonies on both media grew in range of 6-14 mm in diameter. On minimal medium three colonies showed no growth (red box), while three colonies showed slow growth (green box). Using the paired t-test it was found that the % difference in the colony growth between the two media was 7.47% and was statistically significant (p value, $p<0.001$).

These three colonies along with two that showed slow growth on minimal medium were transferred onto 5-FOA plus uridine plates. DNA was extracted from these colonies and the entire open reading frames from both genes were PCR-amplified and sequenced (see Figure 11 for primers) (Appendix, Table 9). Alignment of the sequences with the wild-type sequence, which was obtained at the same time, showed that none of the mutant colonies contained any mutations in the open reading frame of either the *pyr4* and *pyr2* genes (Figure 12).

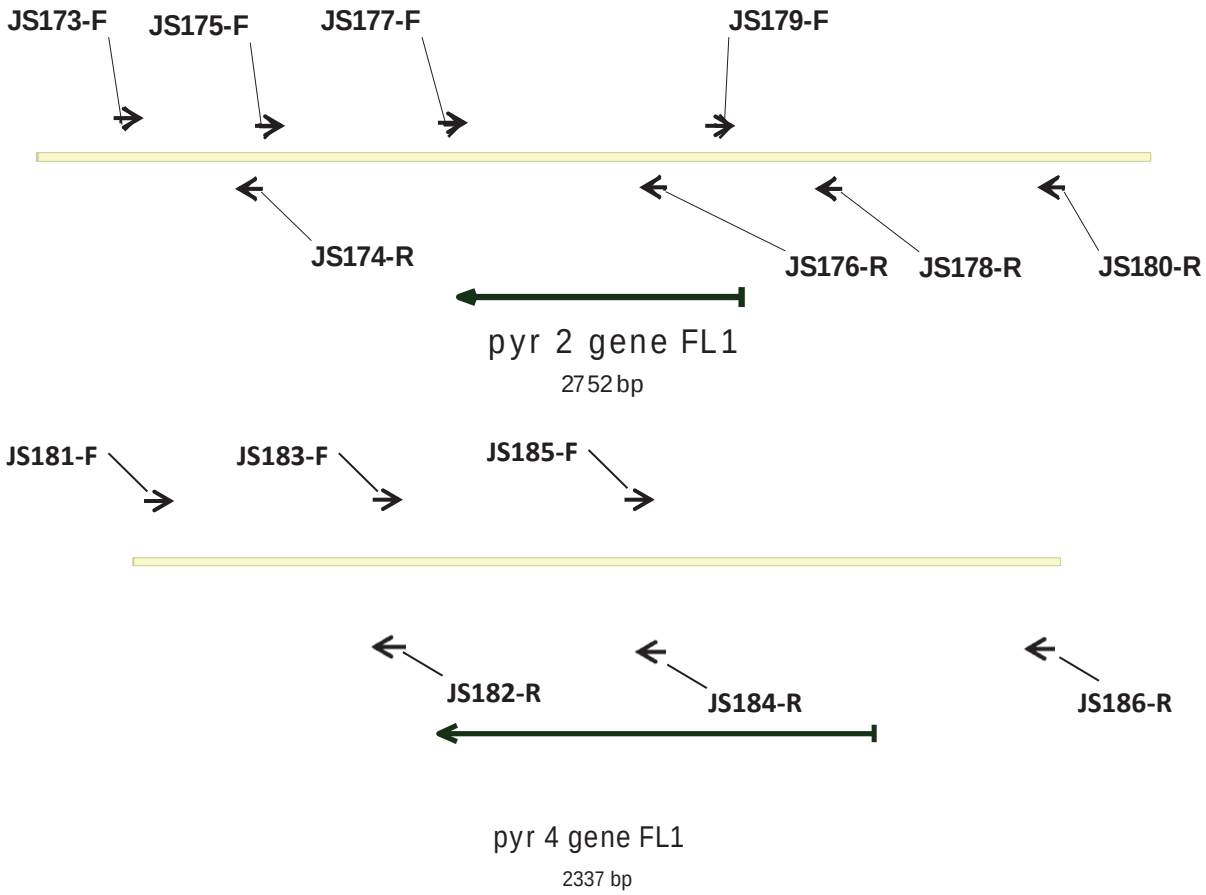


Figure 11: Pyrimidine pathway genes *pyr2* and *pyr4* in *Epichloë*

Based on 5-FOA selection, primers were designed to amplify the sequence of *pyr4* and *pyr2* genes. These primers were used to sequence the entire genes along with promoter region 800 bp upstream of the gene to locate and validate mutations.

Mutant

Fl1

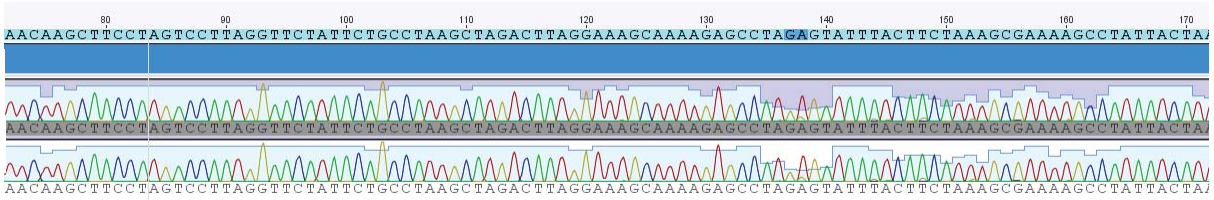


Figure 12: Alignment of Fl1 and mutant sequences showing chromatograms

Figure shows the sequence alignment of Fl1 wild-type and a representative 5-FOA resistant mutant of *pyr2* gene.

Inability to find any mutations in *pyr2* and *pyr4* genes of pyrimidine biosynthetic pathway was not expected. This 5-FOA selection confirmed the effectiveness of EMS as a mutagen. The Sangar sequencing may be limited in detecting mutations if they are at low frequency among the population of nuclei. To address this issue a highly sensitive method, HRM was used to identify SNPs.

3.4 HRM analysis of known SNPs is able to differentiate samples to the point mutation

3.4.1 Proof of concept by pooling DNA

To optimise the HRM assay, DNA from *Epichloë* strains with known SNPs in the beta-tubulin gene were used (Section 2.9). To determine how sensitive the assay was, DNA of the different strains was pooled at different ratios. The advantage of pooling is to reduce the numbers of samples, that would otherwise need to be screened individually but still enable identification of the subsets that contain a SNP (Gady et al., 2009). To determine the extent of pooling, initial HRM experiments were setup (Section 2.9). Sequencing of the beta-tubulin gene from various endophyte strains by the Plant Fungal Interactions team at AgResearch, Grasslands, identified a number of SNPs within that region. When aligned using Vector NTI, it was shown that some of the samples had a T instead of a C residue in a particular position (Figure 13).

Figure 13: Alignment of sequences showing a base pair difference

Vector NTi was used to align these six endophyte strains and it was found that three of the strains had C instead of T at position 281 in their sequence.

DNA from different endophyte samples (AR3006 and AR3007) were pooled in different ratios such as 1:2, 1:5 and 1:10 and were run individually as well. Using HRM analysis different melting curves (genotypes) were obtained (Figure 14) (Section 2.9).

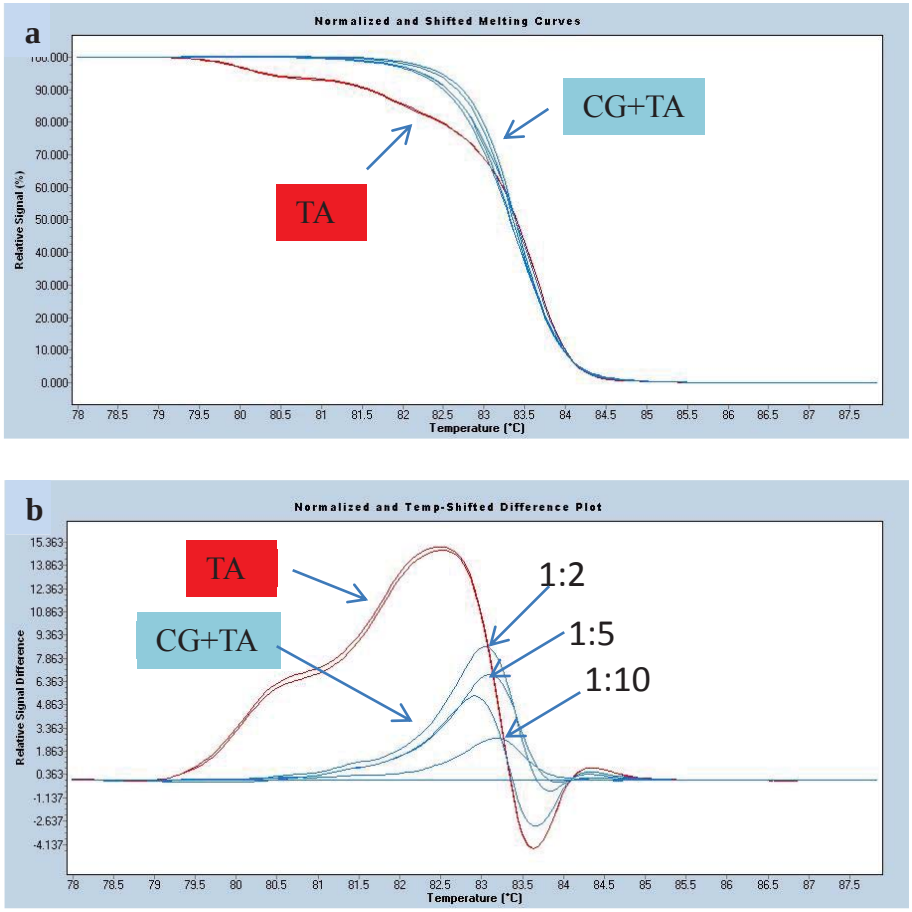


Figure 14: HRM proof of concept by DNA pooling

DNA from the endophytes (AR3006 and AR3007) with a known difference of a single base can be pooled and resolved by HRM at ratio of 1:2, 1:5 and even 1:10. The figure shows the normalized melting curve **(a)** and difference plot **(b)**. The different curves are result of the different melting profiles based on the difference in samples and different proportions of DNA in the combinations.

3.4.2 Experimental design by pooling mycelia

It was shown that when DNA was pooled from the endophytes above with a known SNP, HRM could resolve up to 1:10 ratio in a mixture as it had different melting curves (Figure 14). In order to subject these endophyte samples to experimental conditions where cultures were pooled before DNA extraction, these endophytes (Section 3.4.1) were grown in 1.5 mL eppendorfs in Potato Dextrose Broth (PDB) for 5 days at 25 °C. The mycelia was pooled in different ratios of 1:1, 1:2, 1:3, 1:4 and then DNA was extracted (Section 2.9). It was shown that HRM could easily resolve even 1:5 pool mixtures including other pools of 1:1, 1:2, 1:3 and 1:4 (Figure 15). This demonstrates that HRM is a very suitable technology to screen putative mutants from the *Epichloë* mutagenesis experiments.

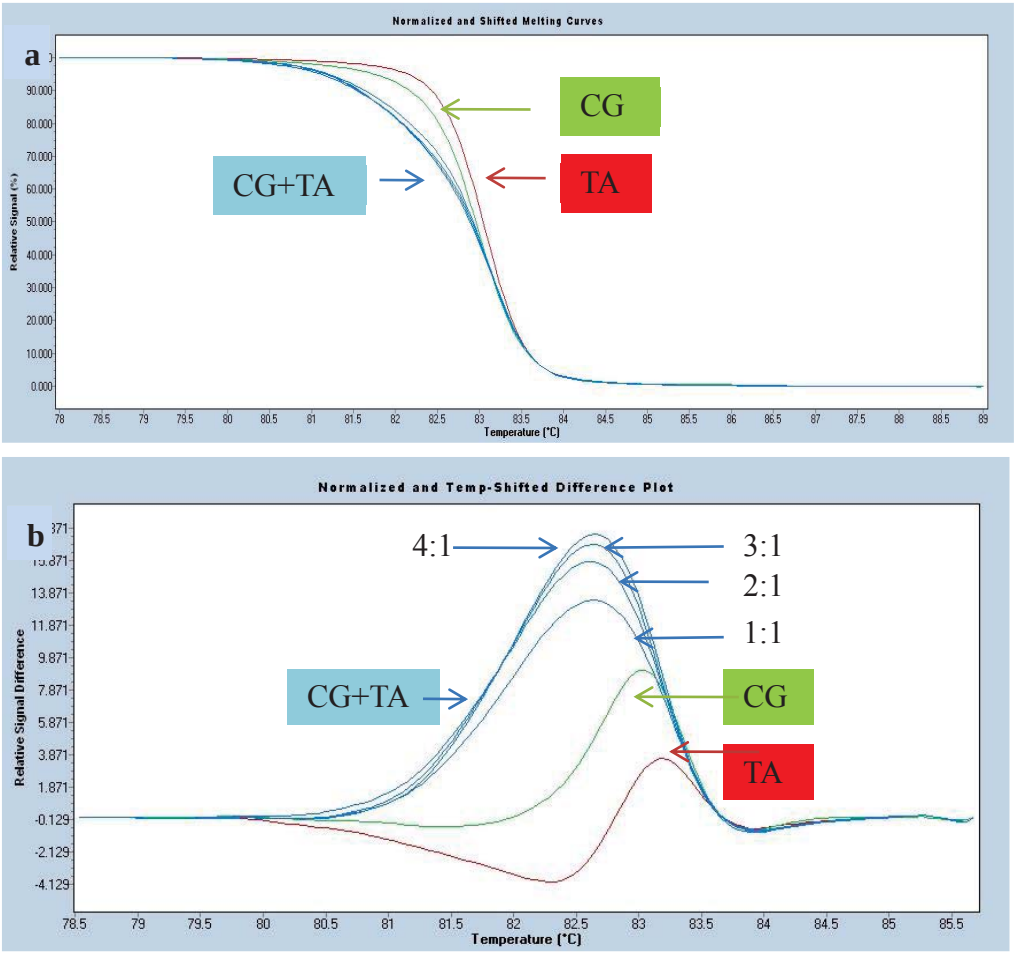


Figure 15: HRM proof of concept by pooled mycelia

Mycelia from the endophytes (AR3006 and AR3007) which have a known base pair difference were pooled in ratios of 1:1, 1:2, 1:3, 1:4 and HRM was able to resolve them individually and even to ratio of 1:4. The figure shows the normalized melting curve **(a)** and difference plot **(b)**. Both genotypes CG and TA were run individually as well.

3.4.3 HRM to screen pooled EMS mutants for genes in ergovaline pathway

From section 3.4.2, it was determined that HRM was sensitive enough to screen mutants pooled to at least 1:5. On these basis primers (Appendix, Table 10) were designed to two genes, *cloA* (Accession: FJ594408.1) and *easG* (Accession: KC989590.1), in the ergovaline secondary metabolite pathway. 1000 mutants derived from 45 minutes exposure to EMS were pooled in a ratio of 1:1 instead of 1:5 to reduce the number of false negative results in the assay and DNA was extracted from each of the 500 pools. These pools were used to perform HRM analysis with the wild-type Fl1 included as a control in all the experiments. Three primer pairs were used to screen for mutations in the *cloA* gene and one primer pair for the *easG* gene from the ergovaline pathway (Appendix, Table 10). HRM analysis failed to identify any candidate mutations in these genes (Figure 16).

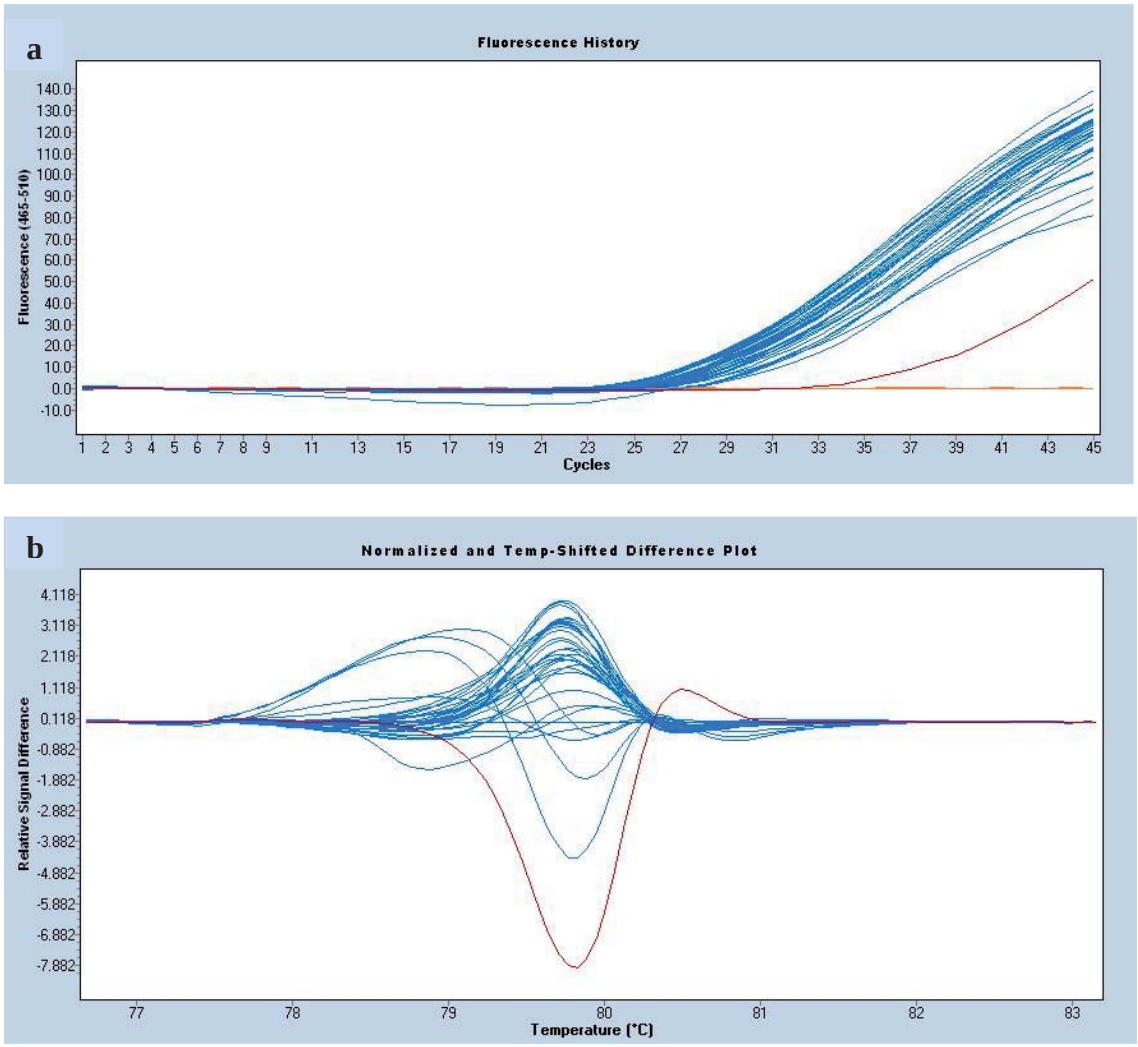


Figure 16: HRM screening for mutations in *cloA* gene from the ergovaline pathway

This figure represents the results from a primer pair that was used to screen mutations in the gene *cloA*, of ergovaline pathway. **(a)** majority of the samples had amplified after 25 cycles; **(b)** in the difference plot, four pooled samples appeared to be different. They were reanalysed and no SNPs were found.

HRM was also used to screen mutations in *pyr4* and *pyr2* genes of pyrimidine pathway, to screen 5-FOA mutants and no mutations were found (Section 3.3).

3.5 NGS approach to the SNP analysis

3.5.1 Searching for point mutations

The 5-FOA selection and HRM had conflicting results. The use of next generation sequencing was proposed to see accumulation of mutations if there were any. Next generation sequencing in all its variations is rapidly becoming a method of choice when it comes to genomic research and sequence comparison.

To address the inability of HRM to confirm the presence of the SNPs in the mutants obtained from EMS mutagenesis, we decided to use MiSeq sequencing to establish the presence of mutations on genome wide basis unlike HRM that was used for a few genes and to determine the frequency in several different surviving colonies over a time course of EMS mutagenesis. Mutagenesis experiment (a) was used (Figure 8) to select for the mutants form time points 0 (0% kill rate), 15 (5% kill rate), 30 (18% kill rate), 45 (76% kill rate) and 60 (88% kill rate) minutes. DNA from these mutants was pooled as described in (Section 2.10.1, and 3.2.1) and sent to Macrogen (Macrogen; http://www.macrogen.com), Korea, for MiSeq sequencing. Sequence data was analysed for the presence of SNPs by Paul Maclean (AgResearch Bioinformatician). Microsoft excel spreadsheet was used to filter the SNPs and Integrative Genomics Viewer (IGV) was used to verify the coverage and SNPs location (Section 2.10.3).

The number of reads for each time-point was highly similar (8.4 million reads for each) and using Q30 value, reads with SNPs below 30% threshold were filtered out. Three high quality independent SNPs were found using IGV, (Figure 17) when reads from 0 minutes were aligned with 60 minutes. For these high quality SNPs, almost all of the reads showed the mutation event in the form of a transition and all three SNPs were in exons. A number of other SNPs were detected by IGV as well but the frequency of the reads exhibiting the mutations was substantially below the set threshold. This low frequency of reads with SNPs was a more alarming result and indicated the influence of some other factors.

In MiSeq next generation sequencing, first SNP (gi|347366901|gb|JH158842.1|_281804) had G in 60 minutes sample instead of A when compared to 0 minutes sample. This SNP was in a hypothetical gene that resides in contig_923 (http://csbio-l.csr.uky.edu/endophyte/) and resulted in an amino acid change from serine to proline.

The second SNP (gi|347366862|gb|JH158881.1|_66967) had an A in 60 minutes sample instead of G when compared to 0 minutes sample. This SNP resides on gene that encodes for cefP transporter protein in *Acremonium chrysogenum* (http://csbio-l.csr.uky.edu/endophyte/) and resulted in an amino acid change from valine to isoleucine. The third SNP (gi|347366848|gb|JH158895.1|_41816) had T in 60 minutes sample instead of C in 0 minutes sample. This SNP was positioned within the oxidase gene bearing high similarity with the *Glomerella graminicola* oxidase (http://csbio-l.csr.uky.edu/endophyte/) and was a silent mutation which did not result an amino acid (lysine) change. These SNPs are all transitions, indicating they are likely to have been induced by EMS mutagenesis.

In order to validate these SNPs, PCR primers spanning these regions were designed and the PCR products were sequenced using Massey Genome Service Centre.

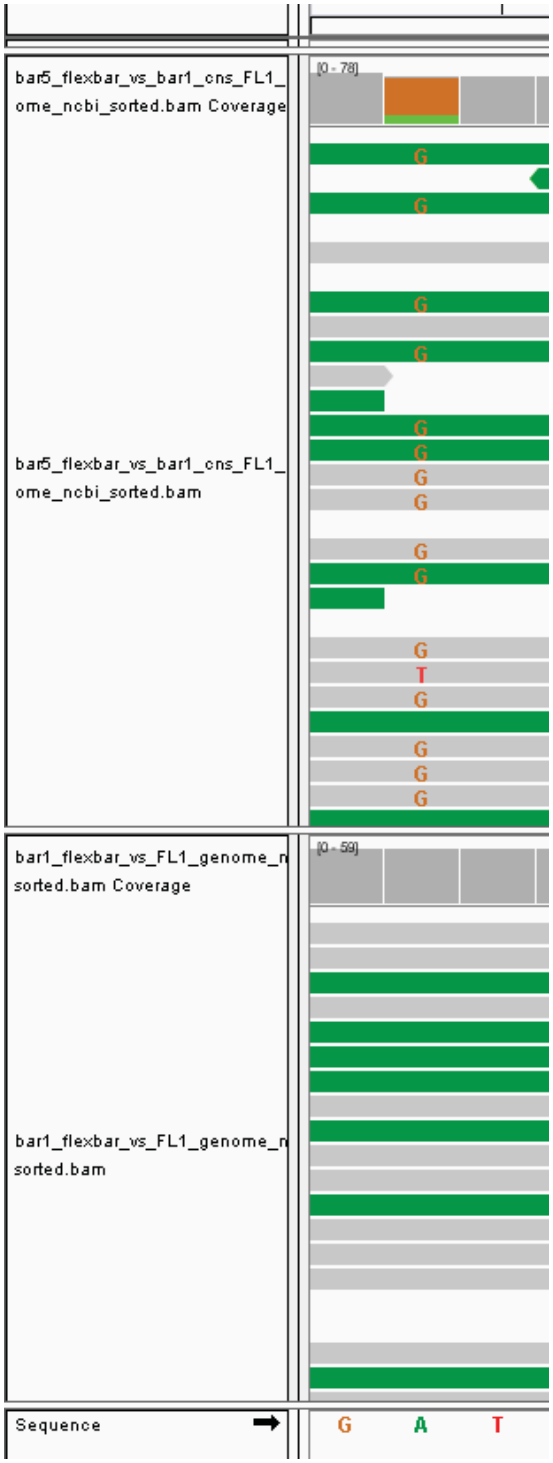


Figure 17: Integrative Genomics Viewer (IGV) alignment

Alignment of reads from barcode 1(0 minutes) and barcode 5 (60 minutes) using IGV software, to locate the SNPs from the MiSeq data of EMS mutagenesis experiment.

3.5.2 Validation of SNPs

Three primer pairs were designed using Vector NTI (Life technologies) (Section 2.9.4), flanking the regions where SNPs were located for 60 minutes time point (Section 3.6.1). The PCR products of the 3 primer pairs in both 0 and 60 minute time points were sent to Massey Genome Service Centre for sequencing and sequences were aligned using Vector NTI (Life technologies) (Appendix, Table 11). All 3 predicted SNPs were found to be present in almost all of reads in the 60 minute time point and absent in all of the reads in the 0 minute time point (Figure 18), indicating EMS induced mutations. Identification of only three SNPs in 60 minutes time point suggests that EMS mutagenesis gave rise to very low frequency of mutations. However, 5-FOA positive selection showed 3000 fold mutation frequency induced by EMS. There is a discrepancy in the number of SNPs identified and the mutation frequency of 5-FOA selection. Some other SNPS were also identified by MiSeq but they were present in low number of reads. For this reason it is possible that the three high quality SNPs were not EMS induced but were present in the 60 minutes sample prior to mutagenesis.

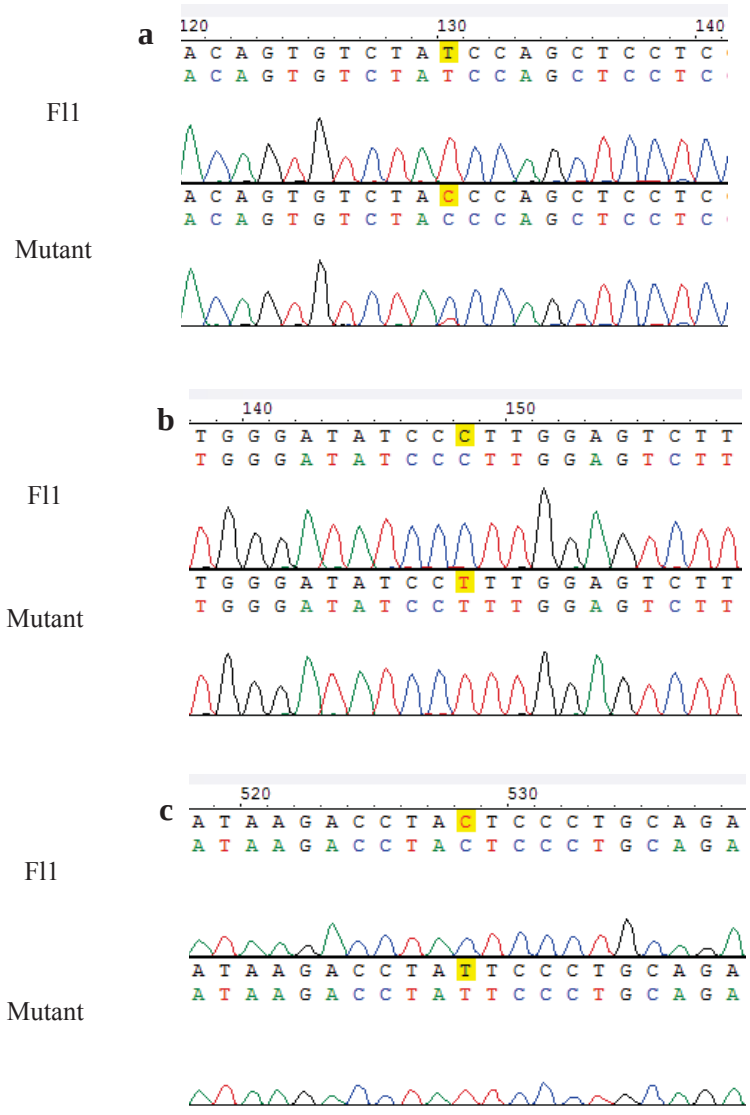


Figure 18: Validation of the SNPs in barcode 5 (60 minutes) sample

Alignment of a part of the sequence from the three mutants **(a, b, c)** showing transitions of nucleotides C to T believed to be induced by EMS mutagenesis in time point 60 minutes (exposure to EMS) compared to 0 minutes (Fl1 wild-type).

HRM was also used to validate the presence of these SNPs in the mutants.

3.5.3 HRM analysis of the mutagenized fungal protoplasts

To confirm HRM could detect the SNPs, pools of two, consisting of DNA from each time point (0, 15, 30, 45 and 60 minutes) mixed with the equal concentrations of DNA from 0 minute time point (Section 2.9.1).

Pools were restricted to two samples in order to increase the sensitivity of the HRM screening technology. For the HRM assay, primers were designed to amplify the product under 400 base pairs (Appendix, Table 12). In the HRM analysis it was shown that by pooling the mutants from different time points with DNA from 0 minutes (untreated) sample, SNP can be validated in the 60 minutes time point (Figure 19).

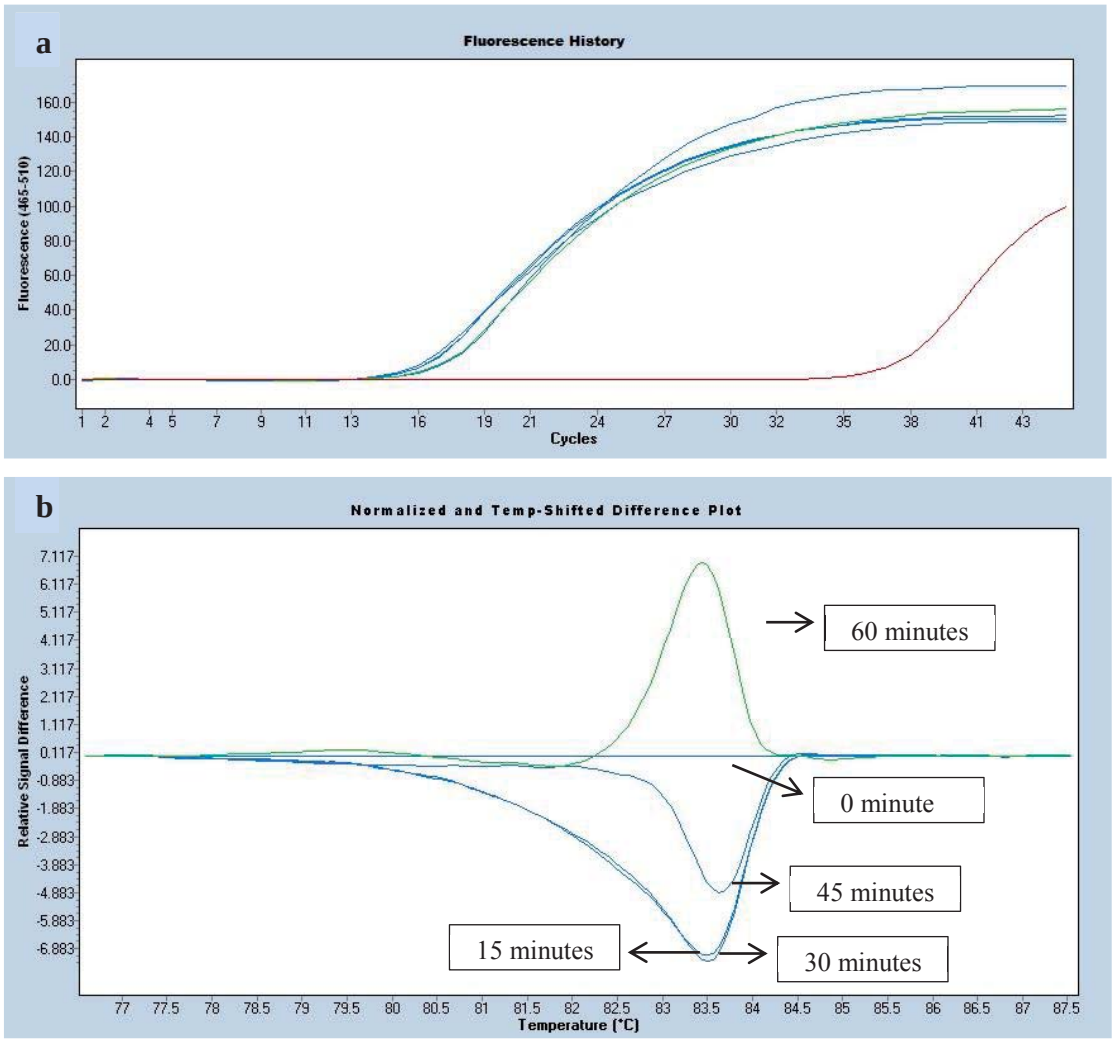


Figure 19: HRM was used to validate the transition mutations

HRM analysis on one of the mutants (347366862) that had a SNP (transition G to A) is validated by separately pooling DNA of all the time points of 0, 15, 30, 45 and 60 minutes with DNA from 0 minutes. **(a)** all the samples had amplified after 14 cycles; **(b)** in the difference plot, due to presence of the SNP, 60 minutes curve has different melting profile and is labelled as a different genotype.

EMS mutagenesis in *Epichloë* resulted in kill curve values that should give high frequency of mutations in the viable mutants (Lawrence, 2002). However on analysis of MiSeq data from the mutagenized Fl1 in this study, only 3 independent high quality SNPs were validated using sequencing and HRM. This was unexpected and suggested that multiple nuclei may have been present which masked the presence of SNPs in the samples.

Given that, I analysed protoplasts that had been mutagenized for up to 60 minutes, resulting in a greater than 90% kill and 3000-fold EMS-induced mutation frequency by 5-FOA selection, I believe that the presence of multinucleate protoplasts and clumping was the most likely explanation (as described in Section 3.5) giving rise to highly chimeric colonies.

Chapter 4 DISCUSSION

The present study is based on technology known as TILLING, which involves mutagenesis and screening of mutations using high throughput techniques. Prior to the advent of these screening techniques, analysis of *Epichloë* secondary metabolite mutants from chemically induced mutagenesis was too laborious to be practicable. This was because *Epichloë* secondary metabolites are produced only *in planta* (Schardl et al., 2013b) and there is a significant bottle neck in the inoculation of *Epichloë* endophytes into plants to evaluate their alkaloid profiles. Random mutagenesis on *Epichloë* protoplasts was performed and the mutants were screened using high throughput screening techniques.

Based on this study three hypotheses were proposed that either there were no mutations and EMS was ineffective or majority of the protoplasts that survived mutagenesis were multinucleate and the third possibility was that there was clumping of protoplasts. *Epichloë* protoplasts were exposed to 50mM EMS for different time points (0, 15, 30, 45 and 60 minutes). Out of these, 50% of the protoplasts were killed in between 15 and 45 minutes and at 60 minutes over 90% of the protoplast were killed (Figure 8). The values from the kill curves suggested that EMS should have induced mutations in Fl1. These mutations were screened using high throughput screening techniques.

HRM was used to screen mutations as a part of the TILLING procedure. The ultimate aim of my research was to screen for mutations in the ergovaline secondary metabolite pathway in order to identify mutants that could no longer make this toxic compound. For example knockout of *easG, cloA* or *lpsA/lpsB* genes would result in chanoclavine production but would inhibit the formation of ergovaline that causes mammalian toxicity (Panaccione et al., 2001) (Fleetwood et al., 2007). For this reason HRM was performed on 1000 EMS mutagenized *Epichloë* mutants using specific primers from *cloA* and *easG* genes (Schardl et al., 2013b) of ergovaline pathway from the *Epichloë* genome (Section 3.4.3). Unfortunately no mutations were detected in either of the genes. This was a surprising result and led to a no mutation hypothesis suggesting that there might be no mutations present and EMS was ineffective.

This hypothesis of no mutation was refuted by 5-FOA selection that yielded 200 mutants at 45 minutes time-point. This positive 5-FOA selection suggested EMS-induced frequency of mutations to be 3000-fold of the non-mutagenized protoplasts. The colonies from 5-FOA medium (supplemented with uracil) were subcultured onto minimal medium and complete medium, PDA. Vast majority of these colonies grew on minimal medium as well as PDA, suggesting that they were not auxotrophs for uracil or could have mixed nuclei that allow them to grow without exogenous supply of uracil. Three colonies lacked growth on minimal medium compared to PDA. These three colonies should be auxotrophs for uracil and were sequenced for *pyr4* and *pyr2* genes in the pyrimidine biosynthetic pathway, along with two intermediate growing colonies and wild-type, Fl1. No mutations were found in the *pyr4* and *pyr2* genes, which was a surprising outcome. However it has been shown earlier that in yeast (*Saccharomyces cerevisiae)* only 5-10% of 5-FOA resistant mutants had mutations in the *ura3* gene (an orthologue of *pyr4*) (Boeke et al., 1984). Though effectiveness of EMS mutagenesis was shown via 5-FOA selection, the lack of mutations in *pyr2* and *pyr4* genes was surprising. However, 5-FOA selection lead to several classes of mutants. Positive selection may reflect that wild-type and mutated nuclei might complement each other, as there were some colonies that had some growth and a few that did not grow at all (wild-type nuclei). This led me to the hypothesis that the majority of multinucleate protoplasts should have survived the mutagenesis.

Although it was shown using DAPI and FM®4-64 stains and a nuclear tag, YFP that the majority of *Epichloë* protoplasts were both viable and uninucleate (85%), with only a small fraction being multinucleate (Section 3.1.1 and Section 3.1.2). Lack of mutations was attributed to either the presence of multinucleate protoplasts or clumping of protoplasts or both. Considering the inability to find any SNPs using HRM in ergovaline genes and 5-FOA selection, there is a distinct possibility that the protoplasts which survived the mutagenesis were actually multinucleate, subsequently giving rise to chimeric colonies. These protoplasts had higher chances of survival as compared to uninucleate protoplasts, due to the fact that a lethal mutation in one nucleus would be complemented by the other viable nuclei thus giving rise to a colony.

Statistical model was made by AgResearch Statistician, Catherine Lloyd-West using the number of protoplasts form Figure 5 and number of viable colonies and protoplasts from experiment b in Figure 8. From Figure 5 the proportion of uninucleate (85%), binucleate (8%), trinucleate (5%) and quadrinucleate (2%) was used in this statistical model. This model showed that approximately 50% of the viable protoplasts at the time point 60 minutes would contain 2 or more nuclei (Figure 20). Though at this time point only three high quality SNPs were detected, my assumption was that the number of SNPs should be higher but the multinucleate nature of protoplasts was making it difficult to detect. There could be a possibility that these three high quality SNPs were not EMS-induced and were spontaneous instead. Based on my observations; protoplasts with more than 4 nuclei were not seen. In a worst case scenario it would mean that 25% of the reads map to each nuclei.

Although the selection of multinucleate protoplasts is a possibility, earlier results showed that 85% of the protoplasts were uninucleate, 8% were binucleate, 5% were trinucleate and 2% were quadrinucleate. This suggested that other factors may also be contributing to our inability to detect SNPs.

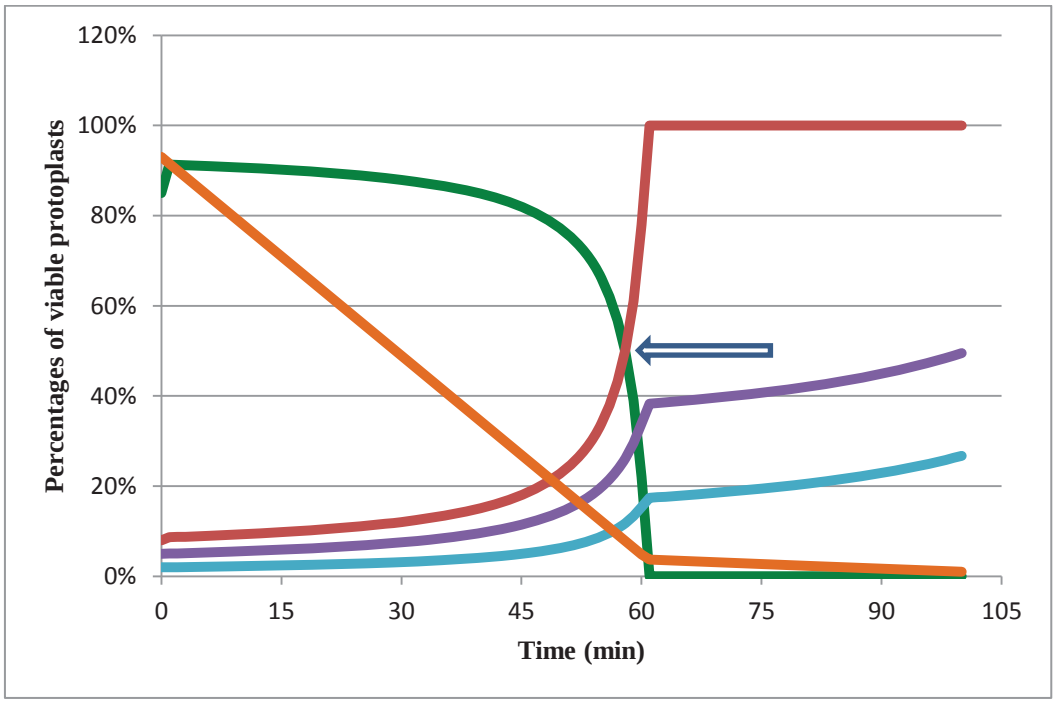


Figure 20: Statistical modelling of the survival ratios between uninucleate and multinucleate protoplasts over the course of EMS mutagenesis

This statistical model shows that multinucleate protoplasts have higher chances of survival as compared to uninucleate protoplasts at 45 and 60 minutes time points. The probability of binucleate protoplasts to be killed was 50% of that for uninucleate protoplasts and it was 33% and 25% for trinucleate and quadrinucleate protoplasts respectively, compared to uninucleate protoplasts. For the purposes of the model kill rate was assumed to be 1.65%, 0.88%, 0.58% and 0.44% for uninucleate, binucleate, trinucleate and quadrinucleate protoplasts respectively. The overall kill rate was 1.5%. Green, red, violet and blue lines indicates the percentages of uninucleate, binucleate, trinucleate and quadrinucleate protoplasts at different time points, respectively. Orange line represents kill rate of the protoplasts that were treated with EMS. The arrow represents 60 minutes time point, where three high quality SNPs were detected.

Clumping of protoplasts was also observed in the experiments performed, with the clumps sometimes consisting of 15-30 protoplasts each (Figure 21). These clumped protoplasts, could potentially give rise to chimeric colonies as well. If this is the case, the mutations within the nuclei/ protoplasts would be generated independent of each other. Based on the number of nuclei in protoplasts, it was assumed that in quadrinucleate protoplast, 25% of the reads would map to each nuclei. Considering the possibility of clumping, for example if 15-30 quadrinucleate protoplasts were to clump together, 1.6%-0.84% of the overall reads would show a SNP if there was one present. The resulting SNPs would therefore be filtered out as they would be below the level of background noise.

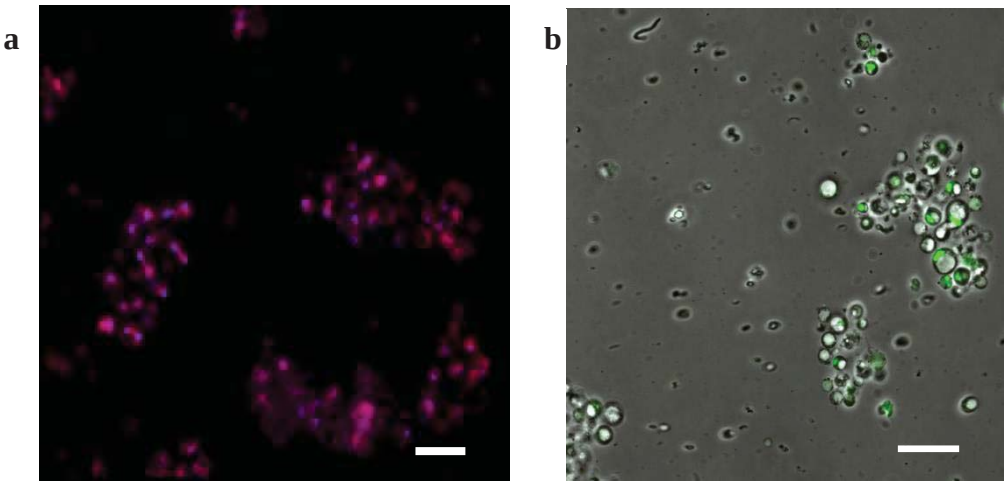


Figure 21: Protoplast clumps of *Epichloë*, Fl1

Protoplasts resuspended in STC buffer were stained with DAPI and FM®4-64 **(a)** and protoplasts of Fl1 with a nuclear tag **(b)**, form clumps with the numbers varying from 15 to 30 protoplasts. Bar represents 10µm in Figure 21 **a** and 30µm in Figure 21 **b**.

To give credibility to the presence of mutations as shown by death curves from EMS mutagenesis (Figure 8) and mutation frequency of 5-FOA selection, next generation sequencing was used. To screen *Epichloë* mutants for SNPs, mutagenized colonies were 2x pooled for time points (0, 15, 30 and 45 minutes) and MiSeq, whole genome sequencing (Matsuda et al., 2013) was used to sequence the genome. Quality score of 30 and coverage of 140x was used for this analysis to filter out the repetitive regions and over amplified regions (Section 3.5.1). Using IGV, only three high quality transition mutations were located and validated by sequencing.

By applying stringency on the MiSeq data with quality score of 30 and coverage of 140x, we were able to reliably predict only three SNPs in the entire genome. A number of factors could be responsible for such a low frequency of mutations. Most obvious one was the existence of multinucleate protoplasts and clumping of protoplasts in the colonies chosen for the Miseq sequencing (as discussed above).

Ultimately, the existence of chimeric colonies as the result of multinucleate and/or clumped protoplasts could have made the SNP analysis difficult and unreliable. This would explain the absence of unique SNPs from the MiSeq data analysis and would also explain why mutants were only identified using positive 5-FOA selection.
To address this issue in the future, consequent subculturing should be done on all the colonies destined for the whole genome sequencing, in order to purify the culture so that the progeny of only one protoplast is sequenced.

The mutation density induced by EMS mutagenesis is found to be different in different organisms depending on the size of the genome, complexity of the organism and mutagen concentration. In *Drosophila* the number tends to be around 1 mutation in every 156 kb (Winkler et al., 2005), whereas in *C. elegans* it is 1 mutation per 100 kb (Cuppen et al., 2007). Amongst plants, the mutation rate tends to stay around 1 per 300 kb as it has been shown in *Arabidopsis*, tomato and rice (Greene et al., 2003; Minoia et al., 2010; Till et al., 2007). In tetraploid wheat, mutational rate of 1 mutation per 51 kb has been detected (Uauy et al., 2009). This lower rate of mutagenesis could have been the consequence of the lower mutagen concentration used. According to these numbers stated above, 1000 *Epichloë* colonies would produce on average 4 to 20 mutations per kilobase. That was not the case for the reasons discussed earlier.

This project has a number of implications for New Zealand agriculture. As discussed in the introduction (Section 1.5), secondary metabolites also cause mammalian toxicity, along with conferring resistance to biotic and abiotic stresses. To overcome toxicity issues associated with secondary metabolites produced *in planta* by the endophytes, gene knock outs were performed using homologous recombination. The endophytes after undergoing these procedures are considered to be genetically modified and due to strict New Zealand legislations genetically modified organisms cannot be freely commercialised.

EMS mutagenesis of seeds is considered to be non-genetically modified and can be commercialised without any restrictions (Dumbleton et al., 2012). By using the EMS mutagenesis, mutations can be induced and screened by high throughput screening techniques that are less time consuming and less laborious. Whilst this study did not identify mutations in the genes of interest we have identified, the likely cause and in the future this technology, may be useful to mutagenize a range of *Epichloë* endophyte strains.

APPENDICES

Primers used	Sequences of primers	Product size (bp)	Gene name
MG150	CATCGTTCTCTTTCCGTCTCA	145	Beta-tubulin
MG151	CATCGTTCTCTTTCCGTCTCA	145	Beta-tubulin

Table 8: Beta-tubulin primers

A primer pair was used to screen for known SNP in endophytes using HRM.

Primers used	Sequences of primers (5' to 3')	Product size(bp)	Gene name
JS173-F	GCA TCG GTT GAC CCT GTT TT	365	*pyr2*
JS174-R	CCG AGT TTG TGT CAC GTT GT	365	*pyr2*
JS175-F	ACA ACG TGA CAC AAA CTC GG	1020	*pyr2*
JS176-R	AAG ACC ATC CTT GAT GCC CA	1020	*pyr2*
JS177-F	CCC GCC ATT GAT AGC TGA AC	997	*pyr2*
JS178-R	CGA TTT AGC GCC CCA TTG AA	997	*pyr2*
JS179-F	TTC AAG ACT CCG CCC TCA AT	880	*pyr2*
JS180-R	CTG AAG ATT CCG AGC TTG GC	880	*pyr2*
JS181-F	GCA ATT CGG AAA GAG GGC TA	669	*pyr4*
JS182-R	GGT ACG CAT CAT TCT CCG TT	669	*pyr4*
JS183-F	GGA GAA TGA TGC GTA CCT CC	806	*pyr4*
JS184-R	CTG TCA CTT CCC TGG CAA AT	806	*pyr4*
JS185-F	ATT TGC CAG GGA AGT GAC AG	899	*pyr4*
JS186-R	AGC AAG AAA ACA CAG CTC CA	899	*pyr4*

Table 9: Primers to screen mutations based on 5-FOA selection

Table shows names and sequences of the primer pairs that were used to screen 5-FOA resistant colonies for *pyr4* and *pyr2* genes from the 5-FOA pathway. For *pyr2* gene four pairs of primers and three pairs of primers for *pyr4* gene were used.

Primers used	Sequences of primers	Product size (bp)	Gene name	NCBI Accession
JS103 F	CACTTCTAAACTGTGGAGGGCTCA	303	*cloA*	FJ594408.1
JS113 R	GTGTTAAGCCATCAATGGAGCG	303	*cloA*	FJ594408.1
JS122 F	CGCTCCATTGATGGCTTAACAC	232	*cloA*	FJ594408.1
JS107 R	GAGAGAGCGGGATGTCCTGC	232	*cloA*	FJ594408.1
JS126 F	GCTTCGTATCTCTTGTCCGG	281	*cloA*	FJ594408.1
JS119 R	GCGATATTCCACTTCTCAATCG	281	*cloA*	FJ594408.1
JS90 F	GCGATTAAGAACGAGAACAAGAT	166	*easG*	KC989590.1
JS91 R	CCTGGTCGTATGTTATTAGCTCA	166	*easG*	KC989590.1
MG156	CGCACATTAACGGATGAGAAGTCA	157	*easG*	KC989590.1
MG157	GCCGAGAACCGTGGACAATG	157	*easG*	KC989590.1

Table 10: Mutation screening in ergovaline pathway

Three pairs of primers were used to screen for mutations in *cloA* and *easG* gene of ergovaline pathway.

Primers used	Sequences of primers (5' to 3')	Product size (bp)
JS158 F 347366901	GCTGATTGCGTTGGTCGTGTG	351 bp
JS159 R 347366901	CAGACACCACCGAAACCGAACTC	351 bp
JS160 F 347366848	CTCTGGCTTATGGCTATGGCTTATT	594 bp
JS161 R 347366848	GTTCTCCTCGCTGTTGCCTCAC	594 bp
JS162 F 347366862	GGCTCTCGAACCATCAAGGCA	437 bp
JS163 R 347366862	CACCCATCCCTCCACCAGAAC	437 bp

Table 11: Three primer pairs to validate SNPs in barcode 5 (60 minutes) sample

Three primer pairs were used to amplify and sequence the high quality SNPs in 60 minutes time point from EMS mutagenesis experiment.

Primers used	Sequences of primers (5’ to 3’)	Product size (bp)
JS158 F 347366901	GCTGATTGCGTTGGTCGTGTG	351 bp
JS159 R 347366901	CAGACACCACCGAAACCGAACTC	351 bp
JS166 F 347366848	CGACCTATCGGCGAGAGAGAACT	212 bp
JS167 R 347366848	CCTCGTGGACAAGAAGCTGGTG	212 bp
JS165 F 347366862	CCAAAGCTCATCGCTCAAGACG	194 bp
JS168 R 347366862	CCGTACTGGAATGTTGGTGGCT	194 bp

Table 12: Primers to screen for mutations using HRM

Three primer pairs were used for HRM assay, to confirm the high quality SNPs in 60 minutes time point from EMS mutagenesis experiment.

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