THE EFFECT OF PRESERVATION REGIME ON THE PHYSIOLOGY AND GENETIC STABILITY OF ECONOMICALLY IMPORTANT FUNGI

A Thesis Submitted to the University of Kent

By

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For the Degree of Doctor of Philosophy
in the Faculty of Science, Technology and Medical studies

19th October 1999

Research School of Biosciences

University of Kent at Canterbury

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of learning.

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ABSTRACT

The long-term preservation of fungi has been carried out using several different methods, depending on resources and laboratory. Isolates have been deposited in culture collections for preservation and storage for periods of many years. Despite the widespread use of preservation regimes, little attention has been paid to the post-storage stability of the physiological and genetic characters of strains. The loss of viability of a biological control agent or the failure of an isolate to produce a secondary metabolite pivotal in the production of drugs or food could result in substantial economic loss for the manufacturing organisation. In this investigation the effects of five preservation protocols on fungal characters were assessed: continual sub-culture, lyophilisation, storage in water, storage at –20°C and cryopreservation in liquid nitrogen. The physiology and genetic stability of three species of economically important fungi (*Metarhizium anisopliae*, *Fusarium oxysporum* and *Serpula lacrymans*) were examined by analysis of culture characteristics, secondary metabolite profiling, extracellular enzyme tests and PCR fingerprinting over a two-year testing period. It was found that preservation regime can influence the resultant characters of the test fungi. Radial growth rate and conidial production was changed from the original isolates after preservation and storage. Secondary metabolite profiles from all of the test fungi were susceptible to change by the preservation protocols assessed. Production of some metabolites was lost, whereas others remained stable after preservation and storage. Extracellular enzyme production was also affected in a similar way. For example, some replicates of an isolate of *Metarhizium anisopliae* lost β–galactosidase activity after one year of preservation. Genetic stability was also compromised is some isolates. Polymorphisms were detected after PCR fingerprinting with a micro-satellite primer in replicates of two isolates of *Metarhizium anisopliae* that had been stored for one and two years by cryopreservation and lyophilisation and in two replicates of an isolate of *Fusarium oxysporum* maintained by continual sub-culture for sixteen weeks. The results indicate that response to preservation and storage is species- and strain-specific. Therefore, there is a need to develop new and existing preservation regimes with emphasis on strain-specific criteria. Scientists should preserve their important isolates by more than one preservation method to protect organisms from the stresses encountered during preservation and storage.

Acknowledgements

I would like to thank my supervisors, Professor Peter Jeffries (University of Kent), Professor Paul Bridge (CABI) and Dr David Smith (CABI), for all of their invaluable help, advice and encouragement. I would also like to thank my industrial supervisors Dr David Langley and Dr Brian Lane (Glaxo Wellcome) and two of the original perceivers of this project Dr D. Whitehead and Dr H.G. Wildman. I would also like to thank Dr Alan Bunch (University of Kent) for his advice and support through his role as chairman of my supervisory committee and Professor Byron Morgan (Department of Mathematics and Statistics, UKC) for his advice on statistical analysis.

The BBSRC and Glaxo Wellcome are gratefully acknowledged for the financial support. I would also like to acknowledge the co-operation of CABI Bioscience (Egham). The following are thanked for useful discussion: Dr R. Paterson (CABI), Dr N. Jenkins (CABI), Dr C.Prior (CABI), Dr M. Rutherford (CABI), Dr G. Robinson (UKC), Dr J.Dodd (IIB) and Dr J.Clapp (UKC).

I have worked with some fabulous people during my research at Egham and Canterbury and my thanks goes to them. I am particularly grateful to Jacky Kolkowski, Alan Buddie, Ethnie O'Grady, Jackson Kung`u, Minorva H. MacLawrence, Sarah Clayton, Janet Pryse, Irdika Mansur, Noah Phiri, Tim Dougall, Amanda Craven-Griffiths and Mike Rogers. A special thank-you to Denise Evans, who fetched and carried on my behalf for nearly 2 years -- I will always be grateful for your help.

Most of all, I would like to thank my family and friends who have all been so very patient and understanding over the last few years.

ABBREVIATIONS

ANOVA	Analysis of variance
Approx.	Approximately
ATCC	American Type Culture Collection
CABI	Centre for Agriculture and Biosciences International
CAP	Chloroform Methanol Propan-2-ol
CBS	Centraalbureau voor SchimmelCultures
CDA	Czapek Dox Agar
CECT	Coleccion Espanola De Cultivos Tipos
CS	Continual Sub-culture
CTAB	Cetryltrimethyl ammonium bromide
diam.	Diameter
dH_2o	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribose Nucleic Acid
DNTP	Deoxyribonucleoside-5'-triphosphate
ECCO	European Culture Collections Organisation
EDTA	Ethylenediaminetetraacetic acid
FZ	Frozen at –20°C
FD	Freeze Dried
GACA	$(GACA)_4$ oligonucleotide primer
GRC	Genetic Resource Collection
GYM	Glucose Yeast Medium
HPLC	High Performance Liquid Chromatography
IHEM	Scientific Institute of Public Health - Louis Pasteur
IMI	International Mycological Institute
ITD	Institute of Technology, Dundee
L/UV	Long wave UV
LN	Liquid Nitrogen
LUBILOSA	Lutte Biologique contre les Locusts et les Sauteriaux
min	Minutes
MA	Malt Agar
MB	Malt Broth
MEA	Malt Extract Agar
MUCL	Mycothèque de l'Université Catholique de Louvain
MINE	Microbial Information Network Europe
MP	Mycelial Plug
MR	GAGGGTGGCGGTTCT oligonucleotide primer
MS	Microsoft Corporation, Seattle, U.S.A.
mt DNA	Mitochondrial DNA
NCPF	National Collection of Pathogenic Fungi
NCYC	National Collection of Yeast Cultures
NRRL	National Regional Research Laboratories
PCA	Potato Carrot Agar
PCR	Polymerase Chain Reaction
Pers.comm.	Personal communication

PFGE	Pulsed Field Gel Electrophoresis
PSA	Potato Sucrose Agar
PVP	Polyvinylpyrrolidone
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RY	$(CAG)_5$ oligonucleotide primer
SDA	Sabouraud's Dextrose Agar
SE	Standard Error
Sec	Seconds
SSCP	Single Strand Conformation Polymorphism
SSR	Simple Sequence Repeat
S/UV	Short-wave UV
SNA	Synthetic Nutrient Agar
T.A.E.	Tris Acetic acid EDTA
T.B.E	Tris Boric acid EDTA
T.E	Tris EDTA
T.E.F	Toluene Ethylene Formic acid
TLC	Thin Layer Chromatography
TRIS	Tri (hydroxymethyl) aminomethane
TtH	Super polymerase enzyme from *Thermus theophillus*
UKC	University of Kent at Canterbury
UKNCC	United Kingdom National Culture Collection
UV	Ultra Violet
VNTR	Variable Non-Translated Repeat
WFCC	World Federation of Culture Collections
YES	Yeast Extract Sucrose
4MU	4-methyl umbelliferyl compound

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CHAPTER 1 - INTRODUCTION

1.1 Stability of characters

1.1.1 Importance

For many years, fungi have been deposited in culture collections for preservation and storage. Type strains and isolates important to academia and industry are routinely preserved and stored in national or "in-house" collections until they are required by the depositor or for use by the wider scientific community. However, despite the relative security provided by collections, there have been few attempts to determine the subsequent stability of the physiological and genetic characters of isolates after resuscitation from preservation. Long-term stability is critical if the isolate is used by the biotechnology industry. The loss of viability of a biological control agent or the failure of an isolate to produce an enzyme or secondary metabolite pivotal in the production of drugs or food could result in substantial economic loss for the manufacturing organisation. Strain stability is also important for reference strains, strain registration purposes, patent protection and tracking of fungi released into the environment, in these instances, any change of characteristics could result in legal complications. To lessen the chances of an economically important strain being lost or damaged, microbiologists may deposit their isolates in more than one culture collection or use a variety of preservation protocols or regimes. The aim of preservation is to suppress metabolic activity, so that cells have a significantly reduced rate of metabolism or become dormant (Smith and Onions 1983). To achieve successful preservation, cultures must attain good sporulation and mycelial growth and have been incubated under optimal conditions of humidity, temperature, aeration, illumination and media prior to storage (Smith and Onions 1994).

1.1.2 Influencing factors

Before any organism is preserved it should be fully characterised, only then can potential preservation protocols be considered. The choice of preservation protocol is dependent on the taxonomy of the isolate. If the fungus does not sporulate, the choice of preservation protocols is limited: for example, of 29 species of Oomycota tested for viability after preservation at IMI (now CABI Bioscience, UK Centre (Egham), Bakeham lane, Egham, Surry, U.K.) by centrifugal freeze-drying and storage for between 7 and 10 years, none were viable (Smith and Onions 1994). No method has been successfully applied to all fungi (Kolkowski and Smith 1995). Once the preservation method (section 1.2) has been selected, the isolate has to be replicated.

Accidental contamination or unfamiliarity by inexperienced workers at this stage could render the preserved material unsuitable for the use intended. Once replicated, the fungus can be preserved. However, the severity of some preservation processes can be damaging to the long-term viability and stability of the fungus (Smith and Thomas 1998). The length of storage is dependent on the preservation method used, but environmental factors such as light, temperature, background radiation and humidity could be influential. When required, cultures can be resuscitated. Protocols for resuscitation are specific to the preservation method used.

1.2 Culture collections: Preservation methods

There are many preservation methods available to scientists when considering how to preserve their fungal isolates. Continual sub-culture is the most common method of maintaining fungi in the laboratory. Longer term continuous culture methods involve the storage of cultures under mineral oil or water (or as mycelial plugs in water) and freezing at –20°C. Alternatively, fungi can be dehydrated in the presence of silica gel or stored in sterile sand or soil. More complex methods of desiccation are L-drying (dehydration from the liquid state under pressure) and centrifugal or shelf lyophilisation (freeze-drying). Finally, cryopreservation in liquid nitrogen is now considered to be the most suitable for long term preservation of fungi (Smith 1993).

1.2.1 Maintenance by sub-culture

Continual sub-culture is widely used and is perhaps the simplest and most cost effective for a small laboratory, especially if cultures are required frequently and quickly. Most laboratories will have some cultures maintained by sub-culture and they will be most commonly maintained on agar slopes rather than on Petri dishes and stored under controlled temperature depending on the genus. Refrigeration below room temperature is ideal as it reduces the number of transfers required due to the suppression of the metabolic rate of the fungus. However, Tian and Bertolini (1996) reported that spores of *Botrytis allii* and *Penicillium hirsutum* germinated earlier and their germ-tubes lengthened faster at low temperatures. Fungal transfer by sub-culture can be potentially disadvantageous, as frequent sub-culturing could result in contamination from other microorganisms such as bacteria or air-borne spores or other fungal species. The requirement to work in a class II microbiological safety

cabinet is advisable to protect the worker and strict aseptic technique must be observed to protect the organism (Smith and Onions 1994). During storage, cultures should be routinely sealed with air-permeable tape to prevent invasion from mites such as *Tyrophagus* and *Tarsonemus*. Mites not only damage cultures by utilising the fungus as a food source but can carry contaminants such as bacteria or other fungal spores from plate to plate, thus rendering cultures unusable (Smith and Onions 1994). A mite infestation can be extremely costly, as all cultures may need to be destroyed. Important cultures can be recovered using a combination of antibiotics, freezing and careful sub-culturing (Smith and Onions 1994). However, irreversible damage could be caused to the fungus due to the pressures exerted by the restorative methods (Bridge pers.com.). Frequent cleaning of laboratory work surfaces with bleach or acaricides can deter mites.

The choice of medium is an important factor, as some fungi are notoriously difficult to culture (e.g. many mycorrhizal fungi). Most fungi will survive on Malt Agar (MA) or Potato Carrot Agar (PCA) (Smith and Onions 1994), but others require more specialised media. Dermatophytes, for example, may grow better on a substrate of hair (Al-Doory 1968). Additives such as growth factors may be added to the growth medium for specific fungi (Smith 1993), for example, the yeast form of *Histoplasma* spp. requires both biotin and thiamin for growth but the mycelial form does not (Fries 1965). Similarly other nutrients may inhibit fungal growth: *Fusarium* spp. are inhibited by nutrient agars containing high glucose concentrations, but the addition of biotin alleviates the inhibition (Smith *et al.* 1994). However, intraspecific differences can occur between saprobic and pathogenic forms of some *Fusarium* spp. Saprobic isolates require biotin for spore germination, pathogenic forms do not (Smith *et al.* 1994). Variation of the nutrient source may prevent the permanent adaptation and modification of the strain to a specific medium. The effects of culture medium, light, temperature and pH on fungi in culture can be critical and has been documented for a wide range of genera (Fox *et al.* 1996; Campbell *et al.* 1996; Roger and Tivoli 1996; Tian and Bertolini 1996; Hallsworth and Magan 1996). When inoculating fresh plates, it is recommended to subculture from the periphery of the fungal colony, i.e. the region of actively growing mycelium. Media should not encourage excessive sporulation or fructification as meiotic or mitotic crossing may

promote the formation of recombinants that may differ from the parental genotypes (Smith and Onions 1994).

The precautions mentioned above should ensure that, as far as possible, fungi maintain the characteristics exhibited upon isolation from nature and do not mutate or show selection. It is a feature of the opportunistic nature of fungi, to easily adapt to the environment. However, despite the best management, this will inevitably happen if isolates are maintained in culture for long periods. Asexual processes such as conidiogenesis and sexual processes that result in genetic recombination enhance the likelihood of selection and mutation (Burdsall 1994). Characteristics may unintentionally be selected out from fungal cultures if workers sub-culture from atypical sectors on a plate (Smith and Allsopp 1993). Glockling and Shimazu (1997) report that isolates of endoparasitic hyphomycetes showed differences in morphology from those in the natural host-fungal interaction when maintained in artificial culture. Their reproductive structures altered and accessory spore forms were periodically produced. When maintaining cultures it is important to consider the culture conditions. Considerable variation can occur if appropriate procedures are not followed.

1.2.2 Storage in water

Immersion in sterile water can be used to extend the life of an agar culture (Burdsall 1994). Fungi can be maintained on agar slopes in universal bottles and then submerged in water. A layer of mineral oil (10 mm deep) may be floated on top to prevent dehydration. An alternative method involves the transfer of mycelial plugs or blocks cut from cultures sustained on agar in Petri dishes to universal bottles, filled with 10ml of sterile deionised water (Boeswinkel 1976), or alternatively cryovials may be used (Burdsall 1994). The length of storage in water is variable but Figueredo and Pimental (1975) successfully stored some phytopathogenic fungi for ten years by this means. Onions and Smith (1984) stored strains of *Pythium* and *Phytophthora* in water for five years. However, only 58% of the isolates remained viable. Qiangqiang *et al.* (1998) preserved 78 isolates, belonging to seven genera, in water for 12 years. On resuscitation, 89.7 % of isolates were viable. Burdsall (1994) reported that water storage did not significantly affect growth rate, viability or genetic stability in 155 isolates of Basidiomycota stored for 7 years. As with all

methods, some fungi are better suited to individual protocols, and notably ectomycorrhizal fungi have been successfully stored by this method (Marx and Daniel 1976). However, the storage of ectomycorrhizal basidiospore slurries in water was not successful (Torres and Honrubia 1994). The advantages of storage in water are the low cost and easy application. However, the length of storage is not endless and some fungi will not survive even short periods submerged. It has been considered to be outdated and a method for short-term preservation (2-5 years) and not suitable for important organisms, where alternative methods may be available (Smith and Onions 1994).

1.2.3 Storage under mineral oil

Storage under oil involves a relatively simple and cost-effective method. Sterile mineral oil is layered over cultures of fungi maintained on agar slopes in universal bottles. The depth of oil used is an important factor: too little may not suspend metabolism sufficiently and fungal growth may penetrate the upper oil meniscus; too much and oxygen may not be able to permeate through to the living fungal cells below (Smith and Onions 1983). Compared to the water storage method, the length of time for which oil-covered cultures can be kept on the shelf is longer. Smith and Onions (1983) reported that oil-covered cultures of *Penicillium* and *Aspergillus* at IMI have remained viable for 32 years. Some cultures deteriorate much more quickly than others and some cultures have to be sub-cultured at frequent intervals to avoid deterioration. *Pythium* species for example, must be sub-cultured every two years (Smith and Onions 1984). The Basidiomycota are routinely maintained in mineral oil (Kobayashi 1984, Smith and Onions 1994).

The use of storage under oil has recently been adapted for some fungi used in biological control e.g. *Metarhizium* spp. Conidia of the entomopathogenic mitosporic fungi *Beauveria bassiana* and *Metarhizium* spp. are lipophilic and easily suspended in oil (Bateman 1992). This is advantageous as biocontrol agents can be stored and then dispersed at ultra low volumes in the field still suspended in oil (Bateman *et al.* 1993). Conidia of *M. flavoviride* have been successfully stored in oil, with no loss of pathogenic virulence when tested in bioassays against the desert locust (*Schistocerca gregaria*) for up to 30 months (Moore *et al.* 1995). The optimal method for storing conidia in oil formulations was developed under the LUBILOSA programme for

biocontrol of locusts (Prior *et al.* 1992). Conidial moisture content must be around 5 % before storage (Moore *et al.* 1996). Silica gel can be added to prevent the conidia from absorbing water from the oil and may prevent hydration of the oil (Moore *et al.* 1996). Low temperatures are preferable for extending the shelf life of cultures (Stathers *et al.* 1993, McClatchie *et al.* 1994). Botanical oils are preferable to mineral oils (Daoust *et al.* 1983) although diesel oil was found to be the premium mineral oil in the short-term (Moore *et al.* 1996). Disadvantages of oil storage were considered minimal, although superior methods may be preferable. On retrieval, viability may be impaired and growth may be slow. Genetic selection may occur, due to the adverse environmental conditions imposed on the culture.

1.2.4 Drying

Drying is a relatively easy and cost efficient method of preserving and storing fungal cultures. There are many drying techniques that can be applied to fungi. However, two basic methods that can be applied are storage on silica gel or storage in sterile sand / soil. More complex methods of desiccation involve drying cultures under vacuum, from the liquid (L-drying) or from the frozen state (lyophilisation, Section 1.25) (Tan 1998). However, these protocols are more costly, and initially more time-consuming, than storage in oil or water. Generally desiccation techniques give longer storage periods but drawbacks of desiccation are that a wide range of fungi, (i.e. non-sporulating fungi) will not survive and other storage methods may be more appropriate. Spores withstand desiccation better than mycelium as they have thicker cell walls and a lower water content than hyphae. Dehydration surpresses the metabolic activity of the conidia which reduces the production of toxins and loss of storage reserves (Moore *et al.* 1996). In nature, dehydration of the vegetative state of most fungi is a lethal process (Smith 1993).

Silica gel storage involves the drying and storing of spores on non-indicating silica gel. Spores are suspended in skimmed milk before drying. The method is better for spores that have thick walls without mycelial appendages (Smith and Onions 1983). The length of storage is variable and species-specific. At IMI some Ascomycota tested for viability between 8 and 11 years after storage gave 100 % survival. Members of the Oomycota, however, had 0% survival (Smith 1993). Working with the entomopathogenic fungus *Metarhizium flavoviride*, Moore et al.

(1996) found that the addition of silica gel to dried formulations of conidia, significantly improved subsequent percent germination especially at high temperatures (25-37°C). The ability of cultures to withstand high temperatures is important in biocontrol programmes where cultures are often stored and transported in extreme climates. However, high temperatures are not encountered in culture collections and are not recommended. The inoculation of spores into sterile sand or soil involves a very simple method. *Fusarium* species especially undergo this process with excellent results (Booth 1971), surviving for between 10 and 20 years before the need arises to sub-culture (Smith and Onions 1984).

Dried material appears to be stable over time. For example, investigations of PCR (Polymerase Chain Reaction) and RFLP (Restriction Fragment Length Polymorphism) profiles of the DNA of eleven-year-old dried material of *Ceratocystis adiposa* revealed that the DNA fragments taken from the dried samples were the same as those taken from a fresh isolate of the same species (Wingfield and Wingfield 1993). This shows that the genome of this fungus was stable whilst dried, even though it was not preserved as a living specimen.

1.2.5 Lyophilisation

Lyophilisation (freeze-drying) is a preferred method of preserving and storing filamentous fungi in many international culture collections. However, as with all preservation and storage processes, it is more suitable for some fungi than others. Although initial outlay for equipment may be expensive and the methods time-consuming, the advantages that lyophilisation provides outweigh the disadvantages. In developing countries, where a constant supply of electricity or liquid nitrogen may not be guaranteed, lyophilisation may often be the best method of preservation available. Once preserved, cultures are easy to handle and occupy little storage space with shelf longevity of 20 to 40 years (Smith and Onions 1994). Ampoules can also be dispatched to clients without having to be revitalised beforehand, cutting the cost of postal charges and avoiding potential damage to the organism during transport.

The standard method involves freezing the specimens and drying them from the frozen state by the sublimation of ice under reduced pressure (Smith 1993). Cooling of the specimen must be controlled, and a rate of 1°C min^{-1} is widely

considered to be optimal for most fungi (Smith 1993, Grout and Morris 1987, Tan *et al.* 1991b). Drying must avoid the liquid phase and be executed at temperatures below -15°C until the water content of the culture is reduced to less than 5% (Smith 1993). The rate of heat input during the drying stage and the final residual moisture content are critical factors that may affect the viability and stability of the lyophilised culture (Kolkowski and Smith 1995). Lyoinjury (Tan 1997) can occur during the cooling and/or drying stages. The phase changes encountered during the drying process can cause the liquid crystalline structure of the cell membranes to degenerate to the gel phase, which disrupts the fluid-mosaic structure of the membrane (Tan 1997). This causes leakage of the membrane, which may culminate in cell damage. Saccharides such as trehalose (Tan *et al.* 1995, Tan 1997) protect membranes by attaching to the phospholipids, replacing water and lowering the transition temperature. Tan *et al.* (1995) suggest that a mix of dextran and trehalose improves the viability of cultures after resuscitation. This lyoprotectant formed a glass during lyophilisation, which prevented rehydration of the dried formulation and subsequently improves the physical stability of the lyophilised product. Other lyoprotectants that can be added to the suspending medium to add protection to the specimen include serum, skimmed milk with inositol, peptone or saccharides

The drying stage of the procedure can be carried out by centrifugal (spin) freeze-drying (a two-stage procedure) or shelf freeze-drying. The latter method may be preferable as the protocol can be automated for individual species of fungi. Once lyophilised, cultures have a very low moisture content and will be suspended in a state of prolonged dormancy, preventing the onset of metabolic activity. Freeze-drying can also be carried out without specialist equipment but viability and stability may be uncertain. Recovery of lyophilised cultures is relatively easy. Two drops of sterile deionised water added to ampoules before transfer to a nutrient source is usually sufficient to re-hydrate cultures. Specialist resuscitation media containing peptides can be used to enhance recovery (Tan 1997).

Freeze drying of sporulating fungi such as the Ascomycota and mitosporic fungi is routinely undertaken, but is not so suitable for the Oomycota and other non-sporulating cultures. Although it is only spores and conidia that are routinely freeze dried, research has been carried out to establish whether lyophilised hyphae can be

revitalised successfully after preservation. In most cases this has met with little success, but hyphae from *Claviceps* spp. (Pertot *et al.* 1977), a limited range of basidiomycetes (Bazzigher 1962) and some arbuscular mycorrhizal fungi (Tommerup 1988) have been revitalised successfully. Investigations by Tan *et al.* (1991a and 1991 b) gave mixed results. Some cultures did not survive at all, others showed only limited viability. They found that hyphal suspensions of ascomycetes withstood lyophilisation better than those of basidiomycetes. Some cultures only revitalised if the pre-incubation medium contained the disaccharide trehalose, other species recovered better with combinations of lyoprotectants containing malt extract or skimmed milk. They also showed that melanised strains of *Alternaria* survived better than hyaline mutants of the same genus (*A.bataticola* and *A. dianthicola* respectively). The melanin may have acted as a natural protectant, due to the ability of the compound to absorb free radicals produced by dehydrated tissue. Carotene may act in the same way (Breierova 1990). A theory as to the cause of lyoinjury was proposed by Tan *et al.* (1991b). Hyphal fragments (which are fragmented into hyphal suspensions before preservation) become wholly frozen during the cooling process. Ice spreads down individual filaments through the damaged open ends of the hyphae causing ice nucleation that may irreversibly damage the hyphae.

The response of conidia to freeze-drying was observed microscopically at various stages of the lyophilisation process by Tan *et al.* (1994). They showed that conidia act differently during the lyophilisation protocol. Cooling rates that resulted in osmotic dehydration prior to dehydration were found to be optimal. However, it was also found that the size of the conidia and the thickness of the conidial wall were important parameters. Species that produce large thick-walled conidia, such as *Curvularia lunata*, had improved viability when cooled slowly at 1°C min^{-1}. Fast cooling rates (75°C min^{-1}) resulted in poor viability for species with large thin-walled conidia such as *Arthrobotrys superba*. Species with smaller thin-walled conidia (*Aspergillus candidus, Trichoderma harzianum*) were not affected by cooling rate in the investigation. Tan *et al.* (1994) concluded that species with small, thin-walled conidia dehydrated rapidly and so could tolerate faster cooling. The importance of cooling rate during preservation has been described in a previous section (1.2.6).

Higher than normal moisture content in ampoules during storage may promote suppressed growth. Mutation may occur due to extreme environmental conditions. However, if preservation protocols are strictly followed there should be few problems with lyophilisation. If an ampoule cracks, cultures may become deteriorate as they will gradually re-hydrate due to atmospheric moisture (Kolkowski pers.comm.). Temperature fluctuation and sunlight may also be inhibitory to successful storage. Working with the bacterium *Lactobacillus bulgaris*, Castro *et al.* (1995) found that cell membranes can be damaged during storage. Using gas chromatography, they discovered that, with time, the unsaturated fatty acid index changed, possibly as a result of oxidation of the lipid composition of the cell membrane. No evidence is available to show if this is also the case with fungi or whether redox processes damage other organelles. To help avoid lyoinjury, a new computer assisted freeze-drying instrument with a liquid nitrogen supply has been developed. In this device the drying stage is undertaken at extremely low temperatures (<-80°C) (Rindler *et al.* 1998).

1.2.6 Cryopreservation

The ability of living organisms to survive freezing and thawing was first realised in 1663 when Henry Power successfully froze and revived nematodes (Morris 1981). Polge *et al.* (1949) became the first "modern day" scientists to report the freezing of living organisms when they successfully froze and thawed avian spermatozoa. Cryopreservation was not applied to fungi until Hwang (1960) applied and adapted the methods proposed by Polge et al. (1949) for filamentous fungi using liquid nitrogen. Liquid nitrogen is the preferred agent for cryopreservation, although liquid air or carbon dioxide can be used. Cryopreservation is effective at temperatures of -70°C degrees and below. Mechanical freezers can run at this temperature and are often used when liquid gas storage is inappropriate. Cultures maintained on slants or petri dishes can be stored in a freezer at –20°C. This method of preservation is commonly used and is very cheap. However, it is not a method of cryopreservation, because at such temperatures water will freeze but the cell cytoplasm may not (Smith pers.comm).

Cryopreservation in liquid nitrogen at -196°C is widely considered to be the premium method for the preservation and storage of fungi (Smith 1998). For many

years it was (and still is) believed that cryopreservation is the "ultimate" method of preservation, primarily because isolates can be continually stored for an indefinite period. Like other preservation methods, cryopreservation has its disadvantages. Controlled rate coolers, cryo-refrigerators and safety equipment can be expensive. The high and variable cost of liquid nitrogen could also be a problem in some laboratories and the process may become uneconomical, especially because a reliable and continuous supply of nitrogen is required. If the nitrogen supply is interrupted, valuable cultures could be lost or irreversibly damaged. This is potentially more of a problem in developing countries where environmental conditions cause the nitrogen to evaporate more quickly. However, even in industrialised countries, the cost of this method means that only cultures important to science and industry are stored in liquid nitrogen. If finance and facilities are present for liquid nitrogen storage then the process has many advantages over other methods used by culture collections. The ultra-low temperature of the liquid gas freezes the internal water content of the cell and under these conditions no internal metabolism can occur as biochemical reactions cease. Morris (1981) reports that at temperatures above -130°C, re-crystallisation of ice can occur, which can cause damage to isolates in storage. Smith (1993) found that Oomycota stored at -20°C and -40°C showed a substantial loss of viability after two years of storage. The freezing process in eukaryotic cells was analysed by Steponkus (1984), who studied the influence of freezing on plant cell protoplasts. During the initial stages, supercooling occurs in the cell and suspending medium. Ice nucleation in the suspending medium follows until the eutectic point (equilibrium of the chemical potential of unfrozen medium with ice) is reached. The chemical potential of the intracellular fluid also reaches equilibrium with the ice and this is reached as a result of cell dehydration or intracellular ice nucleation, the plasma membrane acting as a selective membrane.

The freezing process can be potentially damaging to cells and can result in changes in the position of chemical and biochemical equilibrium. A reduced rate of molecular motion, aggregation or dissociation of macromolecules and concentration of matrix components can all combine to influence the metabolism and structure of cells (Smith 1993). The mechanisms that result in or are caused by dehydration, are collectively known as "solution effects" (Merryman *et al.* 1977) and can cause more specific damage which is referred to as cryoinjury. Solution effects include pH

changes caused by precipitation of buffers, dissolved gases, electrolyte concentration, intracellular crystallisation resulting from loss of the water of hydration from macromolecules and cell shrinkage. Membrane damage may be a result of solution effects. Smith (1993), suggests that cryoinjury in fungi is a result of the interaction of several stresses. Osmotic imbalance may occur as a combined result of solution effects. The "dynamic nature" of membranes may be compromised causing changes in the ability of the membrane to transport solutes and water (Pringle and Chapman 1981). After warming, cells may not return to their original volume, and Steponokus (1984) considers this to be sublethal injury. Dissolved gases are excluded from the crystal ice lattice and may concentrate by up to 20 times both intra- and extracellularly. This causes problems during thawing and may cause injury to fungal cells before returning to solution (Coulson *et al.* 1985). Intracellular ice formation may also be a significant cause of cryoinjury (Mazur 1970, Steponokus 1984). Roquebert and Bury (1993), suggest that ice crystals may exhort a "physical destructive pressure on membranes" causing death. Working on *Lentinus edodes* (the Shiitake mushroom) they established that loss of viability after cryopreservation was correlated with rupture of the plasmalemma, residual membrane material and organelle reorganisation.

The effects of cryoinjury can be reduced by the application of varying cooling rates and cryoprotectants. The purpose of a cryoprotectant is to reduce the time that cultures are subjected to the critical zone of freezing (Singleton and Sainsbury 1993). The use of a cryoprotectant was deemed essential by Morris *et al.* (1988). Some cryoprotectants act by being absorbed into the cytoplasm and cell membranes prior to cooling while others protect from the outside preventing mechanical ice-damage and shielding cells from concentration effects. Smith (1993) summarised the stages in cryoprotection as: non-critical volume loss by the reduction of ice formation, an increase in viscosity to reduce ice crystallisation and solution effects, and the reduction of the rate of diffusion of water caused by the increase of solute concentrations.

Fungi that grow at low temperatures can withstand freezing in the environment. Addy *et al.* (1998) reports that arbuscular mycorrhizal fungi withstand freezing better after a low temperature acclimation period. Some fungi produce

compounds under stress that may act as natural cryoprotectants (osmoregulators). For example, *Mucor* spp. synthesise glycerol when stored at low temperatures (Jennings 1990). Other natural cryoprotectants include trehalose and arabitol. Smith (1993) suggests that cultures should be stored at reduced temperatures prior to cryopreservation, to allow isolates to acclimatise to the cold environment. This may induce natural cryoprotectant production. Common cryoprotectants include glycerol, skimmed milk/inositol, dimethylsulphoxide (DMSO), polyvinylpyrrolidone (PVP) and desferrisoxamine. Cryoprotectants generally act by reducing the size of ice crystals, substituting intracellular water to prevent shrinkage and avoiding excessive osmotic stress (Smith 1993). The mode of action of cryoprotectants varies. PVP acts extracellularly by affecting the structure of water at low temperatures (Smith 1993); whereas glycerol acts both intra- and extracellularly to lower the concentration of salts in equilibrium with ice (Nash 1966). Some researchers have advocated the addition of chemicals to thawing cell suspensions. Tan and Stalpers (1996) reported that cells should be revived in 1.2M sucrose to help limit osmotic expansion and eradicate cryoprotectant from the thawed cells. Tan (1997) suggested that amino acids could be added to the resuscitation medium to repair denatured proteins and restore energy charge.

The rates of cooling and thawing was considered irrelevant for many years, due mainly to the relative success of fungal cryopreservation. Most laboratories used slow cooling rates after the original work on the cryopreservation of fungi by Hwang (1960), with a cooling rate of -1°C min^{-1} being recommended (Smith and Onions 1983). However, with poor viability following preservation for some fungi, i.e. the Basidiomycota and Oomycota, new protocols had to be determined. Many cells have an optimum rate of cooling (Mazur 1970) and react differently to the stresses of freezing and thawing. Smith (1993) concluded that variation in the physiological condition, chemical component, structure, means of osmoregulation and degree of osmoregulation associated with the extensive taxonomic diversity are the reasons for cryogenic survival disparities within the Eumycota.

The use of cryomicroscopy allows the study of the response of hyphae to freezing and thawing at a microscopical level (McGrath 1985). This has helped to define more precisely the possible mechanisms of cooling and thawing damage to

fungal isolates and the establishment of optimal cooling and thawing regimes for specific fungi. The cooling rate used for fungal hyphae influences viability after storage. Coulson *et al.* (1985) discovered that at slow rates of cooling, dehydration of hyphae will occur, but if the cooling rate is increased, intracellular ice will form because there is less time for osmotic equilibrium to be maintained, ice nucleates and the cell will not shrink. The research, carried out on *Penicillium expansum*, showed that at slow cooling rates, ($<15°C\ min^{-1}$) extensive cellular dehydration occurs, with hyphal membranes protecting the cell from damage by extracellular ice. Cells react osmotically to the increase in osmolarity of extracellular solutions, and thus upon thawing will re-expand. This is because the cell wall and protoplast shrink and expand synchronously. With relatively faster rates of cooling there is no time for these processes to arise and at a cooling rate of $>50°C\ min^{-1}$ intracellular ice nucleation will occur. This formation of intracellular ice could be due to three factors- intracellular under-cooling, seeding of ice due to structural changes in the plasmalemma and contact of ice with the cell (Smith 1993).

The variation in the responses of fungi to freezing and thawing was illustrated after a comparative study on *Penicillium expansum* and *Phytophthora nicotianae*. *Penicillium expansum* survived intracellular ice formation at fast cooling rates. Previously, this was considered to be lethal to cells (Mazur 1977). Morris *et al.* (1988) established the optimal cooling rate for 20 fungi from all the fungal sub-divisions in the presence of glycerol. Although most fungi were cooled at rates of and in excess of $1°C\ min^{-1}$ with good survival rates, an exception was the basidiomycete, *Serpula lacrymans* which had an optimal cooling rate of $0.5°C\ min^{-1}$. However this fungus showed a different response with 7 and 28 day-old cultures. *Serpula lacrymans* failed to survive cryopreservation in an investigation by Chvostov'a *et al.* 1995, but survived in an experiment by Yang and Rossignol (1998). Morris *et al.* (1988) suggested that fungi fall into two groups depending on their responses to freezing and thawing. The first group, which responded to freezing by shrinkage at slow cooling rates and intracellular ice formation at fast cooling rates contained examples of the Ascomycota, Mitosporic fungi and Oomycota. The second group, which shrunk at all rates of cooling contained examples of other fungal groups. Nevertheless, no link has been found between taxonomic group and response to freezing and thawing (Smith and Thomas 1998), especially as fungi of

the same species and cultures of different ages can respond differently. *Lentinus edodes* lost all viability when cooled at fast rates (50° C min^{-1} and 160°C min^{-1}), whereas at slow cooling rates (1°C min^{-1}) 80% of replicates recovered viability. Some cells cooled at slow rates showed similar changes to cells cooled rapidly upon ultrastructural examination, suggesting that individual cells of the mycelium do not respond to cooling in the same way (Roquebert and Bury 1993). In a study of 252 strains from 121 species of wood-inhabiting basidiomycetes, viability was assessed after cryopreservation in liquid nitrogen with 10% glycerol (w/v) as a cryoprotectant. One hundred and sixty four of the strains survived cryopreservation, and of these 103 out of 138 were Aphyllophorales and 60 out of 113 were Agaricales (Chvostov`a *et al.* 1995). Meanwhile, Yang and Rossignol (1998) report that out of 516 isolates of Basidiomycota tested for viability after cryopreservation, only 13 did not survive. The results illustrated the variation in response to cryopreservation within the Basidiomycota. Corbery and Le Tacon (1997) found that *Laccaria proxima* was more sensitive to freezing than *L. laccata*. Chvostov`a *et al.* (1995) concede the necessity to characterise the freezing conditions for every fungus, even strains of the same species in order to achieve optimal cryopreservation. Instability in pathogenicity of 3 isolates of *Entomophaga maimaga* (Zygomycota) resulted after long term cryopreservation in liquid nitrogen (Hajek *et al.* 1995). Stored as naturally occurring protoplasts, all isolates survived, but their ability to produce conidia and azygospores was diminished. All three isolates of *E. maimaga* declined in ability to infect the larval stage of the gypsy moth (*Lymantria dispar*). Monosporial cultures of the wheat bunt fungus (*Tilletia* spp.) were stored in glycerol at -70°C. 80% of cultures maintained viability after one year, with 5 out of 9 cultures retaining sexual fertility (Loomis and Leung 1995). The benefit of this method is significant as *Tilletia* spp. cultures are usually stored as sori within which the teliospores are enclosed. Although storage can be for up to twenty years, resultant mycelial cultures are usually mixed. Many workers suggest that more work is required to achieve optimal freezing and thawing protocols for a wide range of fungi, especially for strains that are scientifically and economically important. Some workers have attempted to devise new methods for cryopreservation. For example, Palagyi *et al.* (1997) maintained fungal strains on cryopreservative-immersed porous ceramic beads with some success.

1.3 Culture collections: History and organisation

1.3.1 Role of culture collections

Microbial culture collections (biological resource collections) are centres where living collections of fully-characterised microorganisms (e.g. fungi, viruses, bacteria, protozoa and algae) are preserved and stored under controlled conditions by academic, research or industrial institutions. Type specimens, scientifically significant and economically important strains are routinely stored in culture collections and are generally available to the wider scientific community. Supply of economically important strains may be restricted (Smith 1993). The need for culture collections has become more important as microorganisms increase in economic significance (Smith and Allsopp 1993). The object of storage in a collection is to ensure that the microorganism that is submitted to a culture collection can be revived from its state of preservation in the same condition as it was when deposited. This means that the processes involved in the preservation and storage of an organism must not result in changes in its morphological, genetic or biochemical profile. This is most important if the microorganism is economically important, for example, if it is used in drug production or its future use is uncertain.

1.3.2 Organisation, procedure and protocol

It is essential for scientists to utilise reputable culture collections. Major international fungal collections include the CABI Bioscience Genetic Resources Collection (GRC) (formerly the International Mycological Institute or IMI), Egham, Surrey, UK; the American Type Culture Collection (ATCC), Maryland, USA and the Centraalbureau voor Schimmelcultures (CBS), AG Baarn, Holland. Specialist collections include the National Collection of Pathogenic Fungi (NCPF), York, UK and the National Collection of Yeast Cultures (NCYC), Norwich U.K. The World Federation of Culture Collections (WFCC) issue guidelines for the establishment of culture collections and lists registered collections (Hawksworth 1990). The European Culture Collection Organisations (ECCO) provides similar information on a more regional basis. In the United Kingdom, public service culture collections may belong to the United Kingdom National Culture Collection (UKNCC (http:\www.ukncc.co.uk)), an umbrella organisation that distributes information about the collections and the services they provide. The European commission has sponsored the compilation of a database known as CABRI (Common Access to

Biotechnological Resource Information) which contains data on strains from culture collections throughout Europe (http:\\www.cabri.org). The development of the internet has allowed scientists to access more in-depth information about individual culture collections and the strains in their archives throughout the world.

One disadvantage of culture collections is that they can be expensive to maintain. Materials and equipment are costly. Full-time staff are required to receive deposits, check identifications, prepare organisms for preservation, and undertake complex procedures and subsequent viability checks. Staff should be well-trained and competent. For fungal collections, a basic knowledge of mycology is essential. Nevertheless, the security that collections provide far outweighs the expense, especially for economically important organisms. This differs from herbarium collections of fungi, which although extensive, do not keep living fungi, as the cost would be prohibitive.

1.3.3 Economically important fungi and their preservation

Examples of economically important fungi and their uses are given in Table 1. Most important organisms will be deposited in major international public service culture collections for example that at CABI Bioscience UK Centre (Egham). Alternatively, more specialist collections could be used, for example the National Collection of Wood Rotting Fungi (NCWRF).

Table 1: Economic uses of fungi (adapted from Smith 1998)

Fungus	Use	Reference
Fusarium spp.	Food ([1]Quorn™ mycoprotein)	Trinci (1992)
Aspergillus oryzae	Food (soy sauce)	Onions (1981)
Pleurotus ostreatus	Food (oyster mushroom)	Hamlyn and Temple (1997)
Saccharomyces cerevisae	Brewing	Hawksworth *et al.* (1996)

[1] Quorn™ is a trademark of Marlow foods, U.K

Thermomyces lanuginosus	Detergent (lipase)	Nielson and Oxenboll (1998)
Penicillium spp.	Cheese making Antibiotics	Isaac (1997) Langley (1997)
Aspergillus niger	Industrial enzymes Organic acids Bioremediation of uranium	Lowe (1992) Lowe (1992) Greenshields (1989)
Metarhizium anisopliae	Biocontrol of locusts	Prior (1991)
Fusarium oxysporum	Biocontrol of phytopathogens	Gullino (1995)
Thielavia terrrestris	Silage fermentations	Lowe (1992)

Depositors may request security deposit status for their culture, which enables cultures to be preserved and stored in culture collections but with the guarantee that they are not made available to the wider scientific community. Other collections are International Depository Authorities (IDA's) under the terms of the Budapest treaty (1977) for the deposit of organisms for which patents have been sought (Hawksworth *et al.*1996).

To guard against changes such as mutation, strict protocols for preservation and storage methods must be followed (Smith & Onions 1993). A number of criteria have to be considered before an organism is preserved including purity, taxonomic classification, availability and cost. Not all fungi can withstand every preservation protocol and some isolates may be difficult to preserve, so organisms should be preserved by more than one method. Most sporulating fungi, for example *Fusarium oxysporum* and *Metarhizium anisopliae* can be preserved by lyophilisation and cryopreservation, although species-specific protocols may need to be established. Basidiomycetes may be preserved by storage under oil or water and some species survive cryopreservation. Oomycota are difficult to preserve and may be maintained under oil (Smith 1993). Notoriously difficult to preserve organisms may need to be stored with a host organism or substrate, for example arbuscular mycorrhizal fungi cannot be maintained by *in vitro* culture and so are stored in soil as pure pot culture (Dodd pers.comm.).

1.3.4 Examples of instability after preservation and storage

Changes in culture stability as a result of preservation and storage in culture collections have been reported. Gramss (1991a) found that the physiology of stock cultures of basidiomycetes changed during long-term storage in axenic culture. Morphological deterioration or senescence can occur as a result of continual sub-culture. Cultural degeneration from wild type cultures to intermediate, mycelial and pinnotal cultures of *Fusarium compactum* and *Fusarium acuminatum* occurred over ten transfers using single germinated macroconidium and single hyphal tip transfer methods (Wing *et al.* 1995). In a culture collection, however, isolates may be left on one plate for a longer time period than those used in the experiment of Wing *et al.* (1995). Kim (1997) observed attenuation in cultures of *Fusarium oxysporum* f.sp. *niveum*. After eighteen successive sub-cultures, sectors were detected that exhibited variation in colonial morphology and pigmentation. However, it was concluded that sector characteristics remain stable, even after further sub-culture. Hawksworth *et al.* (1995) describe sectoring as "mutation or selection in plate cultures resulting in one or more sectors of the culture having a changed form of growth". Proser (1993) summarises sector formation as atypical growth that is not well understood. Cultures of *Pholiota nameko* were inoculated from stock cultures after cultivators noticed declining harvest yields. Delayed and declined fruit body formation, altered mycelial growth and changed laccase activity were detected and concluded to be the result of cultural deterioration (Kumata *et al.* 1995). Stock cultures of wood-decaying basidiomycetes are subject to progressive senescence, even under optimum conditions of strain preservation. Over six years, isolates lost the ability to utilise non-sterile wood substrates and produce fruit bodies. Isolates also showed an increased tendency to sector in culture (Gramss 1991b). Kelley *et al.* (1984) found that an isolate of *Fusarium oxysporum* f.sp. *ciceris* was non-pathogenic to cultivars of chickpea. They concluded that the isolate had lost pathogenicity as a result of mass sub-culture for 6 years on potato dextrose agar. Similarly, *Phytothphora* lost its pathogenicity after storage in water (Smith and Onions 1994). Insect pathogens may loose their ability to infect if they are not regularly passed through an insect host (Jenkins pers.comm.) and the pathogenicity of fungal entomopathogens such as *Entomophaga maimaiga* may be changeable from repeated sub-culturing alone (Hajek *et al.* 1990). Loss of pathogenicity could be indicative of changes in enzyme and metabolite production (Bridge pers.comm). In a further context, Shinohara *et al.*

(1995) found that repeated sub-culturing of wine yeast strains (*Saccharomyces cerevisiae*) caused changes in the enological properties of wine. The use of microbial preservation and storage techniques may affect the fungal genome. Using RFLP-PCR, a polymorphism was detected between two replicates of *Fusarium merismoides* stored in soil and liquid nitrogen (Gaylarde and Kelley 1995). It was suggested that the polymorphism could be due to a plasmid associated with senescence. Kim (1997) examined DNA methylation of the ribosomal RNA gene in *Fusarium oxysporum* f.sp. *niveum*, before and after successive sub-culture. It was found that changes in DNA methylation occurred as a result of continual sub-culturing. Kuhls *et al.* (1995) noticed that synonymous strains (originating from a common isolate) of filamentous fungi distributed by different culture collections, but often through third parties, showed deviating PCR fingerprinting patterns. They undertook an investigation to assess the stability of the PCR fingerprints of ex-type strains of *Trichoderma* spp. maintained for many years in different culture collections. They found that all of the ex-type strains retained their specific PCR fingerprinting, regardless of where and how they were cultured

1.4 Aims and objectives

The aim of this research is to establish the effects of five different preservation regimes on the physiological and genetic stability of three economically important fungi. The "main investigation" to study the effects of preservation and storage time over two years, was supplemented with "subsidiary experiments" designed to assess and develop protocols used in culture collections.

Three species representing different genera of economically important fungi were selected:

- *Fusarium oxysporum* (Eumycota, Mitosporic fungi: Hyphomycetes), a phytopathogen, causative agent of vascular wilts and biological control agent.
- *Metarhizium anisopliae* (the green muscardine fungus) (Eumycota, Mitosporic fungi: Hyphomycetes), an entomopathogen of a wide variety of insect orders and a biological control agent.
- *Serpula lacrymans* (the dry rot fungus) (Eumycota, Basidiomycota: Aphyllophorales), a biodeteriogen of domestic coniferous timber products.

The maintenance and preservation methods that were investigated are those in common use in research, industrial and academic culture collections:

- Continual sub-culture. Maintenance of cultures grown on nutrient agar in Petri dishes with periodic transfer of mycelial blocks.
- Freeze-drying. Two-stage centrifugal lyophilisation with skimmed milk and inositol as a protectant. Cultures were sealed under vacuum in ampoules for storage.
- Cryopreservation. Controlled-rate cooling and storage in liquid nitrogen at -196°C with 10% glycerol as a cryoprotectant
- Freezing at –20°C. Storage of slope cultures at -20°C in glass universals without cryoprotectants.
- Storage in water. Mycelial plugs were cut from Petri dish cultures and stored in sterile deionised water in universal bottles at 20°C.

To examine the stability of physiological and genetics characters, the following features of the test fungi were monitored:

- Culture characteristics using quantitative and qualitative analysis.
- Assays of extracellular enzymes using APIZYM and a fluorogenic-assaying system.
- Chromatography of secondary metabolites using Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC).
- Genetic stability using PCR fingerprinting.

These characters were examined before and immediately after preservation, and then further monitored at a range of time intervals of storage. The overall aims of the investigation were to:

- Compare the relative effectiveness of a range of preservation and storage methods across a range of fungi.
- Investigate how different characters are affected by the preservation method used and/or the method of storage

The supplementary investigations were intended to:

- Compare the stability of replicate *Metarhizium* isolates stored in different culture collections.
- Determine if immobilisation within polymers can be used to store fungi that do not survive traditional methods of preservation.
- Investigate the effects of sectorisation on the physiology and genetics of an isolate of *Metarhizium.*

CHAPTER 2 – MATERIALS AND METHODS

2.1 Selection of isolates

In order to increase the relevance of the work, three economically important species were selected:

***Serpula lacrymans* S.F Gray** "the dry rot fungus".

***Metarhizium anisopliae* (Metschnikoff) Sorokin** "the green muscardine fungus" an entomopathogen and biological control agent.

***Fusarium oxysporum* Schlecht.** a widespread and significant phytopathogen

For each species, a number of isolates were chosen to evaluate interspecific and intraspecific variation (Table 2)

Table 2: Details of isolate number, source and histories of the isolates used in this study.

Fungus	Isolate code	Project code	Isolation date	Origin	Isolated From	Source
Serpula lacrymans	UKC[•] 1	S1	1994	Whistable U.K.	Infected timber	Dr P. Jeffries
Serpula lacrymans	ITD[*] 9403	S2	1994	Scotland U.K.	Infected timber	Dr F. Runne
Serpula lacrymans	UKC[•] 2	S3	1997	Egham U.K.	Infected timber	Dr P. Bridge
Fusarium oxysporum	IMI[**] 370367	F1	1996	U.S.A.	Water body	CABI Bioscience
Fusarium oxysporum f.sp. *cubense*	UKC[•] K19	F2	1996	Kenya	*Musa* spp.	Dr J.N. Kung`u
Fusarium oxysporum f.sp. *cubense*	UKC[•] K54	F3	1997	Kenya	*Musa* spp.	Dr J.N. Kung`u
Metarhizium anisopliae	CC[∞] Ma9107	M1	1996	Tropics	Tropical fodder	Cenecafe, Colombia
Metarhizium anisopliae	CC[∞] Ma9233	M2	1996	Philippines	*Teleogryllus* spp.	Cenecafe, Colombia
Metarhizium spp.	I[Δ] 97-1123	M4	1997	Unknown	*Cochiliotus melolonthiodes*	CABI Bioscience

[*] Institute of Technology, Dundee, Scotland U.K.; [**] International Mycological Institute, Egham, Surrey, UK.; [•]University of Kent at Canterbury, Kent, U.K.; [∞]Cenecafe, Colombia; [Δ] International Institute of Biological Control, Silwood Park, Ascot, U.K

Before preservation, each isolate was routinely maintained by continual sub-culture on its respective nutrient agar under the conditions listed in Table 3. None of the isolates had been previously maintained or preserved by any other method.

Table 3: Media and temperatures used for culturing fungi (see appendix for media recipes)

	Fusarium oxysporum	*Metarhizium anisopliae*	*Serpula lacrymans*
Maintenance media	Synthetic Nutrient Agar (SNA)	Malt Agar (MA)	Malt Extract Agar (MEA)
Features media*	Potato Sucrose Agar (PSA)	Sabouraud's Dextrose Agar (SDA)	MEA
Liquid media	Glucose Yeast Media (GYM)	Glucose Yeast Media (GYM)	Malt Broth (MB)
Alternative media	Czapek Dox Agar (CDA)	Potato Carrot Agar (PCA)	Malt Agar (MA)
Maintenance temperature	20°C	25°C	20°C

* Media that promotes expression of culture characteristics

2.1.1 Preparation of liquid cultures

Glass universals containing 10ml GYM were inoculated with a $1cm^2$ fragmented block of mycelium and agar, and incubated at 30°C on an orbital shaker at 180rpm for three days. The contents were then transferred to a conical flask containing 60ml of GYM and incubated for a further 4 days. Mycelium and spent culture fluid were then separated by filtration through Whatman No 3 filter paper.

2.2 Preservation experiment: basic protocol

2.2.1 Design and number of replicates

The methods selected for investigation were continual sub-culture, centrifugal lyophilisation (spin freeze-drying), storage of mycelial plugs in water, freezing at –20°C and cryopreservation in liquid nitrogen. Three isolates of each fungus were subjected to each preservation regime and are detailed in Table 2. It was intended to use up to 5 replicates of each fungus preserved by each method at each testing time.

Enough replicates were prepared (30 for each method) so that the reaction of the fungus to preservation and storage could be monitored at five intervals post-preservation (day one, week one, week sixteen, year one and year two) and compared to results obtained immediately prior to preservation (day zero). To assess the stability of the isolates, four sets of characters were monitored: culture characteristics, secondary metabolites, extracellular enzymes and DNA "fingerprinting" profiles. Methodology is described in section 2.4. The effect of a longer recovery period after resuscitation from preservation was also assessed. Replicates were left for an additional 21 days (28 days in total) on their respective maintenance media (Table 3) before being transferred to media suitable for an assessment of characters.

Fig. 1: Summary of culturing procedure.

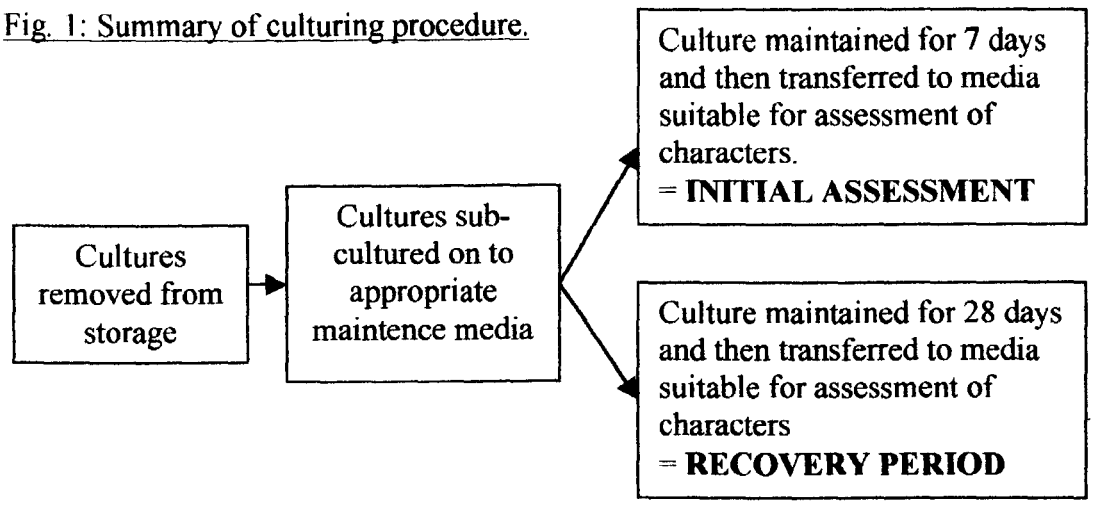


Table 4. Summary of identification codes

Preservation method	Abbreviation	Replicate(s)
Continual sub-culture	CS	A1 to A5
Lyophilisation (freeze-drying)	FD	B1 to B5
Mycelial plugs in water	MP	C1 to C5
Freezing at –20°C	FZ	D1 to D5
Cryopreservation in liquid nitrogen	LN	E1 to E5

2.3 Preservation protocols

To protect replicates from contamination, aseptic technique and a laminar flow cabinet was used for all procedures. All cultures were grown under pre-determined growth conditions (Table3) to achieve good hyphal growth and sporulation, as required for preservation. Cultures were maintained for 28-days on Petri dishes for preparation of mycelial plugs and continual sub-culture. Cultures were maintained for 35-days on slopes in glass universal bottles for preparation of replicates for cryopreservation, lyophilisation and freezing at –20°C.

2.3.1 Continual sub-culture

This method was adopted from the method described by Smith and Onions (1994). Petri dishes, half-filled with Synthetic Nutrient Agar (SNA) for *Fusarium* isolates, Malt Extract Agar (MEA) for *Serpula lacrymans* isolates and Malt Agar (MA) for *Metarhizium* spp. isolates were inoculated centrally with a $10mm^2$ agar block cut from the edge of growing colonies. Each plate was sealed (to prevent contamination from mites) and incubated in the dark at 20°C (25°C for *Metarhizium* spp.). When hyphal growth reached the periphery of each plate, cultures were transferred to a refrigerator and stored at 5°C for 2 months. After 2 months each replicate was sub-cultured onto fresh medium and the procedure repeated. Six replicates of each isolate were maintained.

2.3.2 Lyophilisation

This method was adapted from the method described by Kolkowski and Smith (1995). Glass (neutral glass) freeze-dry ampoules (0.5mm diam) (Adelphi Tubes, Sussex) were labelled and placed in an aluminium tray. Lint caps were placed over groups of ampoules and the whole tray encased in aluminium foil. The tray was then sterilised in a domestic oven for 4 hrs at 200°C and then allowed to cool to room temperature. Spore suspensions of *M.anisopliae* and *F.oxysporum* were prepared by dislodging spores with a glass rod, from a mature slope culture into a 10% (w/v) skimmed milk / 5% meso-inositol (w/v) (BDH) mixture. Mycelial suspensions of *S.lacrymans* were prepared by dislodging mycelium with a glass rod, from a mature slope culture into a 10% (w/v) skimmed milk / 5% meso-inositol (w/v) (BDH) mixture. Aliquots (approx. 0.2ml) of each suspension were dispensed directly into the sterilised glass freeze-dry ampoules and covered with lint. The ampoules were then loaded onto the ampoule rack in the spin-chamber of an Edwards™ (Super Modulyo 12K) freeze drier. The ampoules were spun, the chamber evacuated and then cooled at approximately $-10°C\ min^{-1}$. The ampoules were spun for 30 min. The frozen suspensions were then dried for 3.5 hours at a pressure vacuum of between 5×10^{-2} - 8×10^{-2} mbar. The apparatus was then returned to atmospheric pressure, the ampoules removed from the chamber and transferred to a safety cabinet where each tube was plugged with sterile cotton wool, approx. 20 mm above the culture. A gas/ air torch was used at a position of 10mm above the cotton wool plug to partially constrict each

ampoule. After cooling the ampoules were re-attached to the manifold of the freeze dryer. Three lots of 10g anhydrous phosphorous pentoxide (BDH) were placed in a tray inside the apparatus as a dehydrating agent. The apparatus was then evacuated to allow the secondary drying phase to commence. The apparatus was left for 17hr overnight. Whilst still attached to the freeze drier manifold to maintain the vacuum, the ampoules were sealed across the constriction. The ampoules were then tested to ensure that the internal pressure was maintained after the sealing using a high voltage spark tester. A blue/purple illumination appearing inside the ampoule indicating the low pressure required for successful storage. Cultures were then stored in the dark at 18-20°C in a controlled temperature room.

When required for testing, ampoules were removed from storage and opened in a laminar flow cabinet. Each ampoule was scored in one direction, in the middle of the cotton wool plug using a steel cutting blade. Heat was applied to the score line with a warmed glass rod to crack the ampoule, which was then opened by applying outwards pressure above and below the score line. The cotton wool plug was removed with forceps and 3 drops of sterile distilled water was added to the ampoule to rehydrate the freeze-dried specimen. The cotton wool plug was re-applied and the ampoule left for 30 min. The suspension was mixed thoroughly and drops inoculated centrally to plates of the appropriate medium and incubated.

2.3.3 Storage in water

Mycelial plugs were taken using a sterile 10mm cork borer, from cultures maintained on Petri dishes for 28 days. Twelve plugs of each replicate were immersed in sterile glass universal bottles containing $10cm^3$ distilled water. The tops were flamed and the caps tightly fastened. Cultures were stored in darkness in a controlled temperature room at 20°C. When required, plugs were removed with blunt-end forceps, dried on sterile filter paper and transferred to the appropriate growth medium.

2.3.4 Storage in a freezer at –20°C

Isolates were grown on slopes in glass universal bottles for 35-days. The universals were then tightly sealed, placed in a suitable container and transferred to a domestic freezer at -20°C. When required, a cube of mycelium and agar

(approximately $10mm^3$) was removed with a scalpel, thawed on sterile filter paper and transferred to the appropriate growth medium.

2.3.5 Cryopreservation

This method was adapted from the method described by Smith and Onions (1994). Spore/mycelial suspensions were prepared by dislodging the spore/hyphal matrix from a slope culture into 8ml of 10% (v/v) glycerol solution. Aliquots of suspension (0.5ml) were dispensed into labelled 2ml plastic cryotubes (LabM, Lancs.) and the tops securely tightened. The vials were left in a cool place to allow the cells to equilibrate with the glycerol for at least one hour. The vials were then placed in a controlled rate cooler (Kryo 16™ Planer Products. Ltd U.K.) equipped with a liquid nitrogen supply. The apparatus was then cooled at $-1^{0}C\ min^{-1}$. Once cooled, the vials were immediately transferred to storage racks and positioned in the nitrogen vapour phase of a liquid nitrogen refrigerator. The location of each vial was recorded and noted in the inventory control system of the International Mycological Institute (CABI Bioscience). Vials were stored until required. On retrieval, the contents of each vial were rapidly thawed for approximately 2 min by immersion in a 37°C water bath. Once thawed the vials were immediately removed from the water bath to prevent the contents from reaching damaging temperatures. After thorough mixing, aliquots were taken from each vial with a sterile Pasteur pipette and 2 drops inoculated onto an appropriate growth medium.

2.4 Analytical methods

2.4.1 Gross culture characteristics

Replicates were inoculated centrally onto Petri dishes containing the respective features medium (Table 3). A suite of culture characteristics was recorded at regular intervals (Table 5).

Table 5: Criteria recorded for analysis of gross culture characteristics

Observation	Criteria
1. Hyphal growth	Aerial, sub-medial, dense, sparse, aggregation, strand formation
2. Surface appearance of culture	Felted, floccose, flocculose
3. Sporulation	Colour, type, abundance

4. Pigmentation	Colour, density, distribution
5. Abnormalities	Formation of sectors, culture degradation
6. Culture margin	Smooth, serrated, irregular

2.4.2 Quantitative culture characteristics

2.4.2.1 Colony radius

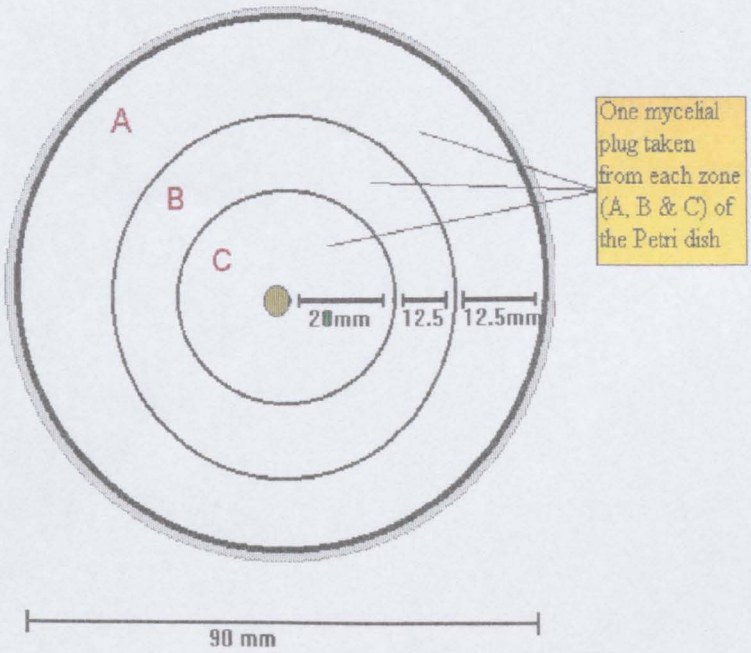
Each replicate was centrally inoculated onto Petri dishes (9cm diameter) containing the respective features media (Table 4). The inoculation area was marked on the bottom of each plate with a permanent marker. The extent of growth was measured (mm) from the edge of the central inoculation point towards the periphery of each plate at regular intervals. The average of four radial measurements was recorded at each time for each plate. Measurements were taken until fungal growth reached the periphery of each plate. Radial growth rates were plotted against time and statistically compared by regression analysis and ANOVA using MS Excel Statistca[5] or Systat[7].

2.4.2.2 Conidial density

A) *Metarhizium* spp.

Three agar plugs were removed from each Petri dish (Fig. 1), with a cork borer (5mm diam).

Fig. 1: Illustration of zonation of plates used for conidial density



Each plug was suspended in 1.5ml eppendorf tube containing 1ml of 5 % (w/v) Tween 80 (Sigma) solution. Each eppendorf tube was vigorously agitated for 3 min using a benchtop homogeniser to dislodge conidia from the agar plug. A sample was removed with a Pasteur pipette, and a drop extruded onto the central grid of a haemocytometer slide (Improved Neubaur). Conidia were counted in five of the large squares and an average determined. Conidial density was calculated for each replicate using the formula below (Fig. 2).

Fig 2: Formula used to determine conidial density for *Metarhizium.*

$$\text{Step 1.} \quad \frac{\sum x+y+z}{3} = A$$

$$\text{Step 2.} \quad \frac{A}{V} = cpm$$

$$\text{Step 3.} \quad \frac{cpm}{ba} = C$$

where:- $x\ y\ z$ = number of conidia counted per zonal division,
A = average conidial count.
V = volume of liquid occupied ($1.0 \times 10^{-4} cm^3$ under central grid).
cpm = conidia ml^{-1} tween solution
ba = borer area
C = conidia per mm^2 culture area

B) *Fusarium*

Petri dishes (49mm diam) containing SNA were inoculated centrally with a $1mm^2$ block of mycelium and agar from 7 day-old cultures of SNA and incubated in the dark for 14 days at 20°C. The plates were then flooded with 1 ml of sterile distilled water. Spores were dislodged from the hyphal matrix by vigorous agitation for one min with a glass rod. The conidial suspension was transferred to a 1.5ml eppendorf and stored in a freezer until required. An aliquot was then extracted with a Pasteur pipette and a drop placed on the central grid of a haemocytometer. Conidial counts of microspores, macrospores and chlamydospores were taken and counted by the method described

above for *Metarhizium*. Conidial density was calculated for each replicate using the formula below (Fig. 3).

Fig 3: Formula used to determine conidial density for *Fusarium.*

$$\text{Step 1. } \frac{A}{V} = cpm$$

$$\text{Step 2. } \frac{cpm}{ap} = C$$

where:- A= average conidial count.

V = volume of liquid occupied ($1.0 \times 10^{-4} cm^3$) under central grid.

cpm = conidia ml $^{-1}$ of distilled water

ap = area of plate

C = conidia per mm^2 culture area

Conidial density results were plotted on bar charts and compared statistically by ANOVA using MS Excel.

2.4.3 Extracellular enzyme tests

2.4.3.1. APIZYM

Extracellular enzyme profiles (Table 6) were determined using APIZYM™ strips (Biomerieux). Strips were placed in plastic trays containing 5ml of distilled water. Each well in the strip was inoculated with two drops of culture fluid (approx. 50 µl, dispensed with a Pasteur pipette) taken from liquid cultures incubated at 30°C for 7 days (see section 2.2.1). Strips were incubated in the dark for 4 hrs at 37°C. The strips were then developed. One drop of ZYM A™ (Biomerieux) and one drop of ZYM B™ (Biomerieux) were added to each well. Each strip was then placed under bright light (100w) for ten minutes to allow full development of positive photochemical reactions. Enzyme activity was graded on a scale of 1 (weakest) to 5 (strongest) using colour charts provided by the manufacturer. Enzyme profiles were analysed for similarity. Enzymes were scored as "1" for the positive utilisation of a substrate (graded 1-5 on the APIZYM reference scale) and "0" when no utilisation of a substrate. A binary matrix was compiled. Dendrograms were constructed by cluster analysis (hierarchical clustering, unweighted average linkage on a percentage scale) using the Statistica[5] statistical package.

Table 6. Panel of substrates and respective enzymes assayed in the APIZYM system

Well	SUBSTRATE	ENZYME ASSAYED
1	CONTROL	-
2	2-NAPTHYL PHOSPHATE	ALKALINE PHOSPHATASE
3	2-NAPTHYL BUTYRATE	ESTERASE
4	2-NAPTHYL CAPRYLATE	ESTERASE LIPASE
5	2-NAPTHYL MYRISTATE	LIPASE
6	L-LEUCYL-2-NAPTYHLAMIDE	LEUCINE ARYLAMIDASE
7	L-VALYL-2-NAPTHYLAMIDE	VALINE ARYLAMIDASE
8	L-CYSTYL-2-NAPTHYLAMIDE	CYSTINE ARYLAMIDASE
9	N-BENZOYL-DL-ARGININE-2-NAPTHYLAMIDE	TRYPSIN
10	N-GLUTARYL-PHENYLALANINE-2-NAPTHYLAMIDE	CHYMOTRYPSIN
11	2-NAPTHYL PHOSPHATE	ACID PHOSPHATASE
12	NAPTHOL BI-PHOSPHATE	PHOSPHO-AMIDASE
13	6-BR-2-NAPTHYL-α-D-GALACTOPYRANOSIDE	α-GALACTOSIDASE
14	2-NAPTHYL-β-D-GALACTOPYRANOSIDE	β-GALACTOSIDASE
15	NAPTHOL-AS-BI-βD-GLUCORONIDE	β-GLUCURONIDASE
16	2 NAPTHYL α-D GLUCOPYRANOSIDE	α-GLUCOSIDASE
17	6 BR-2 NAPTHYL β-D GLUCOPYRANOSIDE	β-GLUCOSIDASE
18	1-NAPTHYL N-ACETYL-β-D GLUCOSAMINIDE	N-ACETYL-β-GLUCOSAMINIDASE
19	6 BR-2 NAPTHYL α-D MANNOPYRANOSIDE	α-MANNOSIDASE
20	2 NAPTHYL-α-L FUCOPYRANOSIDE	α-FUCOSIDASE

2.4.3.2 Fluorogenic tests

Eleven 4-methylumbelliferyl (4MU) derivatives (Sigma) (Table 7) were prepared according to the method of Barth and Bridge (1989). Stock solutions were formulated by dissolving 1mg of each substrate in 1.6ml of dimethylformamide (BDH). Working solutions were prepared by diluting 0.15ml of each stock solution in 9.85ml of 0.05M sodium acetate (Sigma: analytical grade). Fifty µl of culture fluid, taken from cultures grown in GYM for 7 days at 30°C was mixed with 50µl of substrate working solution in a well of a 96 well microtitre plate. This was repeated for all substrates. Controls of 50µl substrate working solution / 50µl water and 50µl culture fluid / 50µl water were included on each plate. Plates were incubated for 4 hrs at 37°C. Fifty µl of saturated sodium bicarbonate (BDH) solution was then added to each reaction and the plates examined on a UV transilluminator (UVP). Enzyme activity produced a blue fluorescence, whereas the absence of enzyme activity resulted in no fluorescence. Profiles were statistically analysed using the same method described for APIZYM testing.

Table 7: Methyl umbelliferyl (4MU) substrates used to detect a panel of eleven enzyme activities

No	SUBSTRATE	ENZYME ASSAYED FOR
1	4MU N-ACETYL-β-D-GLUCOSAMIDE	β GLUCOSAMIDASE
2	4MU α-ι-ARABINOFURANOSIDE	α ARABINOFURANOSIDASE
3	4MU β-D-N-N'DIACETYLCHITOBIOSIDE	β CHITOBIOSIDASE
4	4MU α-ι-FUCOSIDE	α FUCOSIDASE
5	4MU β-D-GALACTOSIDE	β GALACTOSIDASE
6	4MU β-D-GLUCOSIDE	β GLUCOSIDASE
7	4MU β-D-GLUCURONIDE	β GLUCURONIDASE
8	4MU α-D-MANNOPYRANOSIDE	α MANNOSIDASE
9	4MU P-TRIMETHYLAMMONIUM CINNAMATE CHLORIDE	CHYMOTRYPSIN
10	4MU β-D-XYLOSIDE	β XYLOSIDASE
11	4MU BUTYRATE	ESTERASE

2.4.4 Secondary metabolite profiling

Two techniques were employed for the analysis of secondary metabolites, Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC).

2.4.4.1 Thin Layer Chromatography of extracellular secondary metabolites

The methods used were adapted from those described by Paterson and Bridge (1994). Isolates were grown in the dark on YES agar for 21 days at 21°C for *Fusarium* isolates and 28 days at 25°C for *Metarhizium* isolates. A TLC plate (Silica gel 60, Merck) was prepared by drawing a horizontal line 10mm from the bottom of the plate. A horizontal line was marked 10mm from the top of the plate. Agar plugs were cut with a cork borer (5mm diam.) in reduced light conditions (to prevent photo-oxidation). The agar end of each plug was applied to its relevant origin using a dissecting needle with slight pressure for 10 sec. Spots were allowed to dry before further applications were administered. This was repeated five times. A reference standard (griseofulvin in chloroform/methanol (2:1 (v/v), Sigma) was also applied to the plate. Once dry, plates were placed in an equilibrated TLC tank containing 100ml of TEF (Toluene/Ethylene/Formic acid 80:10:10 v/v/v) solvent for approximately 45 min. Maximum saturation was achieved with saturation pads. The plates were removed and the solvent front marked with a pencil. Plates were allowed to dry in a darkened fume cupboard. Plates were examined under white light, long-wave UV (365nm), short-wave UV (254nm) and a combination of short-wave followed by long-wave UV again and spot characters recorded. To allow the detection of a wider range of secondary metabolites the plates were then sprayed with 0.5% (v/v) p-anisaldehyde (Sigma) in ethanol-acetic acid-concentrated sulphuric acid (17:2:1 v/v/v) and heated for 8 min at 105^{0}C. The visualisation procedure was repeated.

2.4.4.2 Thin Layer Chromatography of intracellular secondary metabolites

A similar methodology was used as for extracellular metabolites except that one drop of chloroform:methanol (2:1) was added to the colony end of the plug and this was applied directly onto the plate. The tank solvent used was CAP (chloroform: acetone: propan-2-ol (85:15:20 v/v/v)).

iii) Statistical analysis of TLC results

To ensure correct classification of metabolites, profiles were compared after the mean and standard error Rf values were calculated for individual spots showing similar properties (colour and Rf value). A binary matrix, which scored "1" for the presence of a secondary metabolite and "0" for its absence, was constructed from each profile. Dendrograms were then constructed using heirachichal cluster analysis and the unweighted average linkage method on a percentage scale, with the statistical package Statistica[5].

2.4.4.3 HPLC of Secondary Metabolites

Isolates were grown in the dark on YES agar in 90mm Petri dishes for 21 days at 20°C for *Fusarium* replicates and 28 days at 25°C for *Metarhizium* replicates. The contents of each plate were extracted into 50ml of HPLC grade methanol (BDH) using a vertical macerator at high speed. Extracts were then rotary evaporated and re-suspended in 5 ml of HPLC grade methanol and stored at 4°C until required. Extracts were run on an HPLC (Varian 5000) with a wavelength detector at 210nm through a Spherisorb C18 column. A volume of 50 ml was injected at a flow of 1.5ml.min^{-1}. The gradient solvent system was solution A (0.1M Ammonium dihydrophosphate: 3ml/L Phosphoric acid) and solution B (75% Acetonitrile: 25% 0.1M Ammonium dihydrophosphate:3ml/L phosphoric acid). The solvent programme for the A/B mixture was: A at 100% initially, lowered to 0% in 20 min, held for 10 min, raised to 100% in 2 min and held for 5 min. Mobile phases were prepared with MilliQ 18 ohm water, filtered though a Noorganic cartridge (Whatman). HPLC profiles were visually compared and a binary matrix constructed by scoring peaks (1 when peak present, 0 when peak absent). Dendrograms were then constructed using heirachichal cluster analysis and the unweighted average linkage method on a percentage scale, with the statistical package Statistica[5].

2.4.5 PCR "fingerprinting"

2.4.5.1 Organism growth and harvest

Frozen mycelium was transferred to a small Petri dish (50mm diam) and lyophilised in an Edwards™ (Super Modulyo 12 K) freeze drier. The mycelium was ground to a fine powder using a pestle and mortar that had been kept on ice. Ground

mycelium was collected into sterile eppendorf tubes and stored in a freezer at –20°C until required.

2.4.5.2 DNA extraction

This method was adapted from the method described by Zolan and Pukkila (1986). Ground lyophilised mycelium was transferred to an alcohol-sterilised solvent-resistant centrifuge tube and agitated with 2.5ml of 2% (w/v) (cetryl) hexadecyltrimethyl ammonium bromide (CTAB) solution containing 1% (w/v) of β-mercaptoethanol (Sigma). This was incubated at 60°C in a water bath for 30 min. 2.5ml of chloroform:isoamyl alcohol (24:1) was added and the contents gently agitated until homogenous. The constituents were then separated at 1100G in a Beckman centrifuge at room temperature for 10 min. The upper aqueous layer was collected with a wide bore pipette and transferred to an Eppendorf tube to which 0.54 volume iso-propanol (BDH) was added. The precipitate was collected by centrifugation at 1100G for 5 min at room temperature. The pellet was dried and resuspended in 700 µl of TE buffer and held at 37°C in a dry block heater for 20 min. Twenty units of ribonuclease A (Calbiochem) were added as 25 µl from a stock solution and incubated for a further 30 min at 37°C. An equal volume of chloroform:isoamyl alcohol was then added, the mixture gently agitated and then separated at 1100G at room temperature for 10 min. The upper aqueous layer was collected with a wide bore pipette and transferred to a fresh eppendorf tube. Ammonium acetate (7.5M) was added to give a final concentration of 1.5M. A double volume of 95% ethanol was then added and the DNA collected at 1100G for 5 min. The supernatant was discarded and the pellet re-dissolved in 500µl of 200mM ammonium acetate. The DNA was precipitated by the addition of 95% ethanol, and collected by centrifugation at 1100G for 5 min. The pellet was dried in a desiccator, re-suspended in 50-100µl of TE buffer and frozen at -20°C until required.

2.4.5.3 Assessment of the quality and concentration of the DNA

A 1% (w/v) LE agarose (Seakem™ FMC Bioproducts) mini-gel (10 x 10cm) was prepared using TBE buffer. The gel was set in a minigel tank (Biorad) with a 10µl capacity comb. The tank was flooded with 350ml of TBE buffer. 1µl of DNA preparation from each replicate was mixed with 9µl of loading buffer and loaded into

each well on the gel. The gel tank was run at 75 volts for approx. 1 hr. The gel was then stained with ethidium bromide (Sigma) solution (0.5 μg ml^{-1} in water) for 30 min, then viewed on a UV transilluminator (UVP) and captured electronically using Biorad Geldoc™ equipment.

2.4.5.4 The PCR (Polymerase Chain Reaction)

DNA samples were diluted by 1 in 50 or 100 in TE buffer according to the quality and concentration (determined from the results of the mini-gel). (Typically samples were diluted to 1 in 100 but samples producing bands on the mini-gel of lesser intensity were diluted to 1 in 50ul TE Buffer). The PCR was formulated by the addition of 5μl primer (MR (M13 5`GAGGGTGGCGGTTCT 3`)(50 nmol) or RY (5`$(CAG)_5$ 3`)(10 nmole) or GACA (5`$(GACA)_4$ 3`) (50 nmol) (Pharmacia Biotech)), 4μl (2.5mM each) dNTPs (Pharmacia Biotech), 31.75μl HPLC water (BDH), 3μl (1.875μmole) magnesium chloride (Sigma), 5μl Tth reaction buffer (HT Biotechnology), 0.25 μl (1.25units) Super Tth Enzyme (HT Biotechnology) and 1μl of diluted template DNA. A control was set up with 1μl sterile water in place of template DNA.

2.4.5.5 PCR conditions

The PCR was carried out in a Perkin Elmer Geneamp 2400® thermo cycler. One of two programmes was employed depending on the primer used. Programme A (for RY and MR primers) consisted of 5 min at 94°C, 45 cycles of 1 min at 94°C, 1 min at 35°C and 1 min at 72°C and a completion stage of 5 min at 72°C. Programme B (for the GACA primer) consisted of 5 min at 95°C, 30 cycles of 30 sec at 94°C, 30 seconds at 48°C and 90 sec at 72°C and a completion stage of 5 min at 72°C and then cooled to 4°C. Samples were then transferred to a freezer at –20°C for storage.

2.4.5.6 PCR product separation

PCR products were separated on a 1.5% LE agarose (Seakem, FMC Bioproducts) gel prepared with TAE buffer. A 15x10cm midi-gel tank (Flowgen) was used with a 20 well capacity comb set into the gel. The tank was flooded with 850 ml of TAE buffer. 20μl of PCR product from each replicate was mixed with 5μl of loading buffer and loaded into the gel. Size markers were prepared using 8μl 100bp

ladder (GIBCO) solution (1 part ladder in 19 parts water) and 4µl stop solution. The gel was run at 75 volts for approx. 2 hrs. The gel was then stained with ethidium bromide (Sigma) solution (0.5 µg ml^{-1} in water) for 30 min. Gels were viewed on a UV transilluminator (UVP) and photographed with a Polaroid MP4 land-camera or captured electronically using Biorad Geldoc™ equipment

2.4.5.7 Gel quantification

The banding patterns for each replicate were analysed for similarity. Bands were scored as "1" for the presence of a band and "0" for the absence of a band. A binary matrix was compiled. Dendrograms were constructed by cluster analysis (hierarchical clustering, unweighted average linkage on a percentage scale) using the Statistica[7] statistical package.

2.5 Assessment of similarity using dendrograms

Cluster analysis allows an overall assessment of similarity to be made between a population of replicates. Characters are scored into a binary matrix ("1" for the presence of a characteristic and "0" for the absence of a characteristic). Each replicate is then assessed for its similarity to other replicates in the population using a correlation, which assess the linkage distances of all replicates within the population (matrix). If no changes occur during preservation and storage all replicates will exhibit 100% similarity. However, if characters are altered they will group separately on the dendrogram from the replicates whose characters are unchanged. The more the difference, the more the replicate will group away from the other replicates. This is expressed as percentage similarity, which decreases with increasing heterogeneity. Individuals are linked using graphical interpretation. For example, if there are 20 replicates in a population, 10 replicates exhibit a secondary metabolite profile typical of the original isolate, 5 loose the ability to produce a single metabolite, whereas the remaining 5 loose the ability to produce 2 metabolites, three distinct groups will be visible on the dendrogram, the later being the least similar from the original.

CHAPTER 3 – THE EFFECT OF PRESERVATION AND STORAGE ON CULTURE CHARACTERISTICS

3.1 Introduction

Descriptions of fungi maintained in culture on nutrient agars are used for taxonomic classification and physiological studies. A number of criteria are used to analyse fungal growth and behaviour in culture, these include growth rate and pattern, pigmentation, mycelial morphology and sporulation. However, these criteria can be affected by a number of independent parameters including temperature, light, humidity, pH, nutrient source, storage container, inoculum type and culture age. The state of an isolate in culture can give an indication of its morphological stability only if its behaviour under a set of defined parameters is known. Okuda (1994) found that culture age, choice of medium, the type of Petri dish, the density of conidial suspensions used for inoculation and temperature could all affect the behaviour of *Penicillium* spp. in culture. The use of a conidial suspension of lower density for inoculation, reduced the diameter of colonies after 7 days for most strains but induced better sporulation. A 2°C difference in temperature affected the colony diameter. Hall *et al.* (1994) demonstrated that culture age had an effect on the rate of conidial germination in *Metarhizium anisopliae*, *Paecilomyces fumoso-roseus* and *Verticillium lecanii*. Conidia harvested from younger cultures (2-3 day-old) germinated more rapidly when inoculated onto fresh media than older cultures (14 days old). Working with *Metarhizium flavoviride*, Moore *et al.* (1995) found that conidial viability decreased over 37 months of storage in soya and groundnut oils. Stability of culture characteristics is essential if the fungus is to be used as a type strain for taxonomic reference, for teaching purposes, for registration as a patent or as reference of a strain that is to be released into the environment.

3.1.1 Culture anatomy and morphology of *Serpula lacrymans*

In plate culture, *Serpula lacrymans* produces floccose mycelium, which is white in colour and may become fanned. Maturing mycelium may have burgundy patches or produce a yellow pigment, possibly as a result of stress (Nuss *et al.* 1991). Clamp connections, the same diameter as the hyphae, are characteristic in young undifferentiated mycelium (Nuss *et al.* 1991). In older cultures, hyphae may aggregate into strands. Strands are formed from the aggregation of three types of hyphae: vessel, fibre and tendril, which differentiate from the secondary mycelium.

Point growth (a sector of the culture margin that yields faster growing mycelium) may also occur (Jennings 1991). Basidiomes can be formed under specific conditions (Schmidt & Moreth-Kerbernik 1991) producing yellowish-brown basidiospores (Pegler 1991). Basidiospores germinate to produce monokaryotic mycelium (Nuss *et al.* 1991). Arthrospores can be produced from monokaryotic mycelium

3.1.2 Culture anatomy and morphology of *Fusarium oxysporum*

Fusarium species have septate mycelium; in culture the mycelium appears striate, felted to floccose (Booth 1971a). In plate culture, hyphal growth may be initially slow and sparse before becoming more vigorous, abundant and dense. The hyphae may aggregate to form a sporodochiumentous stroma, which bears a surface layer of conidiophores (Singleton and Sainsbury 1993). Phialides are produced that are characterised by a "foot" cell with a "heel" (Booth 1984). Three types of spore are produced by *Fusarium* species: microconidia, macroconidia and chlamydospores. Microconidia are small unicellular or bicellular spores, borne on simple conidiophores. They are 5-12 μm x 2.2-3.5 μm in size and appear oval to ellipsoidal or cylindrical in shape, with straight to curved borders (Booth 1971a). Macroconidia of the "elegans" type (Booth 1971b) are borne on "elaborately branched conidiophores" (Booth 1971a). Macroconidia are commonly divided with 3 to 5 septa. Larger macroconidia tend to have more septa and some spores have been recorded with 7 septa. Macroconidia vary in size from 27-66 μm x 3-5 μm and appear falcate, occasionally fusoid-falcate, and are "pointed" at both ends. Chlamydospores (thick-walled resting spores) are "terminal", formed within the macroconidia or "intercalary", formed within hyphal cells (Booth 1971). Terminal chlamydospores are borne on short lateral branches and may be solitary or occasionally in chains. The chlamydospore wall may appear either smooth or rough. All spore types are hyaline. (Booth 1971). In culture, *Fusarium* isolates produce the red to purple vinaceous pigment, bikaverin (Brayford pers.comm.)

3.1.3 Culture anatomy and morphology of *Metarhizium anisopliae*

Metarhizium forms septate hyphae. The conidiophores are formed in compact to stromatic patches, appearing mostly monomatous but also synematous, conidiogenous cell phialides in whorls often aggregating in a candle-like fashion

appearing clavate to cylindrical. The conidia are one-celled and are smooth-walled and may be slightly coloured but are mostly hyaline, they may form long strands or aggregate into prismatic columns (Samson *et al.* 1988 after Rombach *et al.* 1970) and Tulloch (1970). A key to distinguish the species of *Metarhizium* using the size, shape and colours of conidia as taxonomic criteria was published by Tulloch (1976). Rombach *et al.* (1987) considered that the colour of the fungus in culture was a variable factor and not a suitable taxonomic criterion. The formation of appressoria in culture has been reported in *Metarhizium* species, but are probably the result of viral infection (St Leger *et al.* 1989). Blastospores can be formed when *Metarhizium* is maintained in liquid culture (Kleespies and Zimmerman 1992). In culture, the mycelium appears dense, with submerged and/or aerial hyphal growth. Sporulation is usually abundant and of herb-green colour, although mutants are reported to be yellow and pale vinaceous (Bogo *et al.* 1996). There is evidence to suggest that *Metarhizium* spp. can cease to sporulate in culture, producing a hyphal mass (Prior pers.comm.)

This chapter aims to describe the effects of preservation and storage time on the qualitative (hyphal growth, surface appearance, culture margin, abnormalities, pigmentation) and quantitative (radial growth rate, sporulation) culture characteristics of the test fungi.

3.2 Materials and methods

Materials and methods are described in chapter 2, section 2.4.1 to 2.4.2

3.3 Results

3.3.1 *Serpula lacrymans*

Viability

No isolates survived lyophilisation. Replicates maintained by continual sub-culture retained viability at all testing times and their culture characteristics remained stable throughout the investigation. Over the period of the investigation, contamination by *Penicillium* spp. was a problem, and several replicates were lost due to their relatively slow growth rate. Initial viability of replicates stored as mycelial plugs in water was good, with 100% viability for all isolates after 1 day and 1 week of preservation. However, after 16 weeks of storage, just 7.5% (3 out of 21) of S3 replicates were viable. About 55% (22 out of 40) of isolate S1 replicates were viable. After 1 year of preservation, 1 out of 5 replicates were viable from isolates S1 and S2. No S3 replicates were viable. After 2 years of storage no S2 replicates were viable and only 1 S1 replicate was viable. Initial viability of replicates of isolates S1 and S2 stored at –20°C for 1 day and 1 week was very good (100% viability), although there was a 10 day delay in the onset of growth. After 1 and 16 weeks of storage just 2 out of 10 replicates of isolate S3 were viable. After 1 year of storage, no isolates stored at –20°C were viable. Viability of replicates resuscitated from liquid nitrogen was variable. After 1 year of storage, 66% of replicates of isolate S1 were viable and 100% of S3 isolates were viable. After 2 years no replicates were viable.

Culture morphology

Before preservation, *Serpula lacrymans* isolates (Fig.1) produced dense floccose mycelium, white to white/yellow in colour, with a regular-smooth, occasionally fanned culture margin with a lemon-yellow coloured exudate. Hyphal stranding was visible from the inoculum site after 8-12 days of growth. After preservation, most viable isolates maintained a similar appearance in culture. However, after 16 weeks of storage replicates stored as mycelial plugs in water had sparse and often highly pigmented growth. Viable replicates stored at –20°C and by cryopreservation initially exhibited highly pigmented sparse mycelial growth.

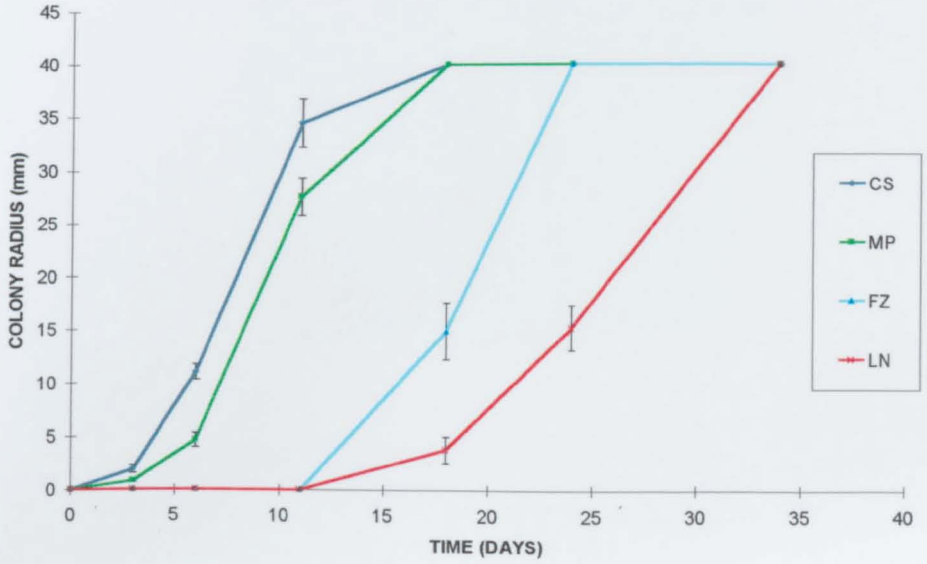
Radial growth

After 1-day of preservation there was no significant difference between mean radial growth rates from viable treatments of isolate S1 ($P>0.05$). Mean growth rates ranged from 3.7 mm day^{-1} for replicates stored as mycelial plugs in water to 4.0 mm day^{-1} for replicates maintained by continual sub-culture, 4.0 mm day^{-1} for replicates

stored at –20°C to 4.4 mm day^{-1} for replicates that had been cryopreserved. The difference between mean radial growth rates of isolate S2 was significant ($P<0.05$). For isolate S2 (Fig. 2) growth rates for ranged from 1.2 mm day^{-1} for replicates that had been cryopreserved and stored at –20°C, 1.3 mm day^{-1} for replicates maintained by continual sub-culture and 1.6 mm day^{-1} for replicates stored as mycelial plugs in water.

Fig.1: *Serpula lacrymans* isolate S1 after 28 days of maintenance on MEA

Fig 2: Radial growth of *Seprula lacrymans* isolate S2 after 1-day of preservation. Maintained on MEA at 20°C. (CS, continual sub-culture; MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation in liquid nitrogen.)



3.3.2 *Metarhizium anisopliae*

Viability

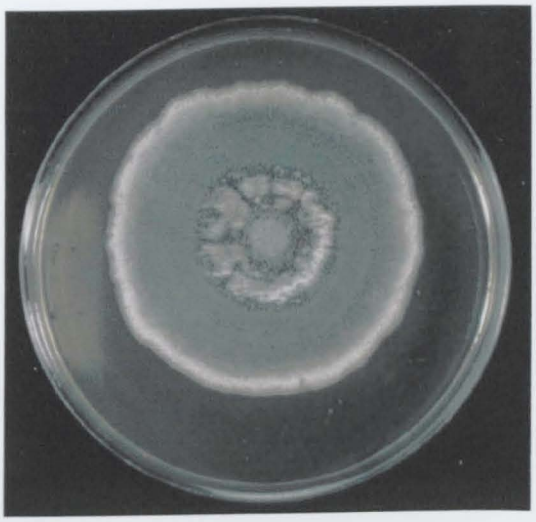
After 2 years of preservation all replicates of *Metarhizium* isolates M1 and M2 stored by lyophilisation, cryopreservation, as mycelial plugs in water and at –20°C were viable. Replicates of both isolates, stored by continual sub-culture were lost between the 1 and 2 year testing times because of contamination by *Penicillium* spp. Replicates of *Metarhizium* isolate M4 stored for one year by continual sub-culture, lyophilisation, cryopreservation and at –20°C were viable. However, only 2 out of 5 replicates stored as mycelial plugs in water were viable. A viability study of 80 mycelial plugs of isolate M4 stored for 1 year showed that 48 plugs (60%) were viable and 32 plugs (40%) were non-viable. After 16 weeks all replicates had been viable.

Culture morphology

Before preservation.

Mycelial growth was characterised as surface hyphal growth with a smooth-regular culture margin, preceding a maturing mycelium, white with occasional yellow pigmentation and developing aerial conidiophores. Sporulation, abundant and herb-green in colour (Fig. 3).

Fig. 3: Typical culture morphology of *Metarhizium anisopliae* isolate M4. After 18 days of growth on SDA at 25°C.



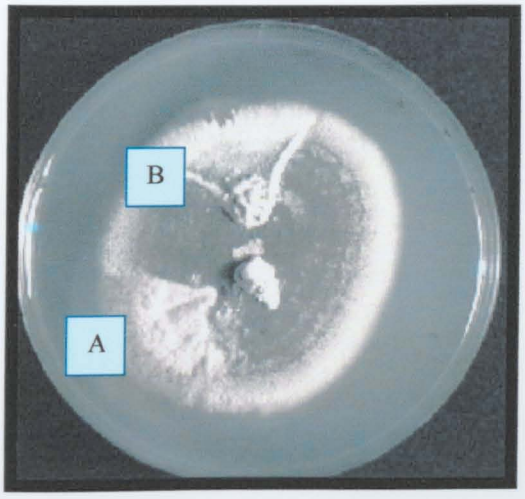
After 1 day of storage.

Replicates stored by most methods retained the characteristics exhibited prior to preservation. However, replicates stored by cryopreservation showed an increased tendency to produce "patchy areas" of floccose hyphae.

After 1 week of storage.

Replicates of isolate M4 maintained by continual sub-culture and lyophilisation exhibited typical culture characteristics. Replicates stored by cryopreservation and at –20°C produced areas of floccose hyphae. Three of the replicates that had been cryopreserved gave rise to sectors (Fig 4), 2 were abnormal and did not sporulate. Sectorisation also occurred in all of the replicates that had been stored as mycelial plugs in water. After a further sub-culture, replicates of all the treatments recovered to the pre-preservation cultural state of *Metarhizium*.

Fig. 4: Sectorisation in an isolate of *Metarhizium*, showing non-sporulating sector (A) and sporulating sector (B). Maintained on SDA for 18 days at 25°C.



After 16 weeks of storage.

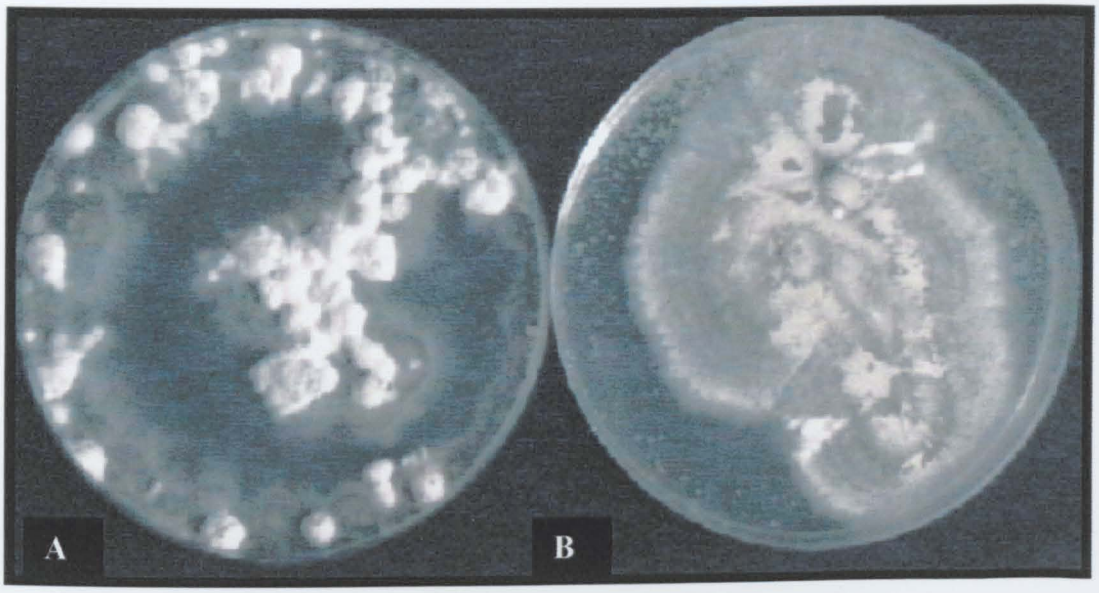
The culture morphology of replicates of isolate M4 stored as mycelial plugs in water deteriorated further. Two replicates produced masses of floccose hyphae, and another sectored, 1 replicate exhibited yellowing mycelium with an irregular culture margin and poor sporulation. Only 1 replicate exhibited typical culture morphology. Replicates that had been stored by lyophilisation and continual sub-culture exhibited typical culture morphology. Replicates that had been stored at-20°C and cryopreserved produced areas of floccose hyphae. Three of the replicates that had been cryopreserved produced sectors, 1 of the sectors produced non-sporulating mycelium. A further sub-culture did not result in any recovery of the replicates stored as mycelial plugs in water or by cryopreservation.

After 1 year of storage.

Replicates of isolates M1 and M2 stored as mycelial plugs in water showed variable sporulation with 1 replicate of isolate M2 undergoing sectorisation. Of the 2

replicates of isolate M4 which were viable, both had sectors and exhibited poor sporulation. Replicates of isolates M1 and M2 maintained by continual sub-culture and stored lyophilised displayed typical culture morphology. However, 2 replicates of isolate M4 maintained by continual sub-culture had poor sporulation and one gave rise to sectors. Replicates of isolates M1 and M2 stored by cryopreservation and at –20°C produced areas of floccose hyphae. Replicates of isolate M4 that had been stored at –20°C all sectored. Those replicates stored by cryopreservation produced masses of floccose hyphae, yellow pigments and grew poorly (Fig 5).

Fig. 5: Replicates of *Metarhizium* isolate M4 stored for one year by cryopreservation and grown on SDA for 18 days. A, plate showing patchy areas of floccose mycelium; B, plate showing sporulating culture and sectors



After 2 years of preservation.

Only replicates that had been lyophilised retained the characteristics that *Metarhizium* exhibited prior to preservation. Replicates that had been cryopreserved and stored at –20°C produced areas of floccose hyphae. Two replicates of isolate M1 stored in liquid nitrogen sectored. One replicate stored at –20°C showed very poor growth with little visible sporulation. Replicates stored as mycelial plugs in water exhibited very poor sporulation with "yellowing" of the mycelium. A further sub-culture failed to improve the condition of the cultures that had been stored as mycelial plugs in water. However, the areas of floccose hyphae that had been evident in replicates stored in liquid nitrogen and at –20°C had diminished. A further sub - culture of replicates of isolate M1 that had been lyophilised produced areas of floccose hyphae and sectors in 2 of the 5 replicates analysed.

Radial growth

After 1 week of storage

Fungal preservation method affected radial growth ($P<0.05$) in *Metarhizium* isolate M4 (Fig 6A). The mean growth rate of unpreserved replicates (maintained by continual sub-culture) was 1.7 mm day^{-1}. The mean growth rates of replicates from the other preservation treatments was lower and varied from 1.4 mm day^{-1} for replicates stored by mycelial plugs through to 1.6 mm day^{-1} for replicates stored in a freezer at –20°C. There was a 3-day delay in the onset of growth for replicates of isolate M4 that had been lyophilised. After a recovery period there was no significant difference in growth rates between preservation treatments ($P>0.05$).

After 16 weeks of storage

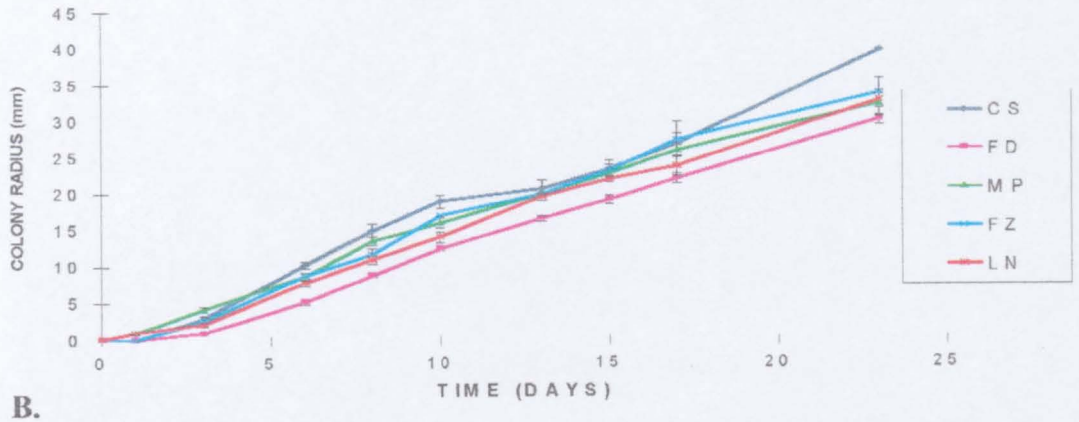
The radial growth rates of replicates of isolate M4 were changed upon recovery from storage. The mean growth rates of replicates from the various preservation treatments were significantly different ($P<0.05$) (Fig 6B) and ranged from 1.8 mm day^{-1} for replicates stored in liquid nitrogen to 1.9 mm day^{-1} for replicates that had been lyophilised, 2.0 mm day^{-1} for replicates stored at –20°C and as mycelial plugs in water and 2.3 mm day^{-1} for replicates maintained by continual sub-culture. There was a 2-day delay in the onset of growth for replicates that had been lyophilised and stored at –20°C. After a recovery period, radial growth rate became more even between preservation treatments and differences were not significant ($P>0.05$) (Fig 6C). There was no delay in the onset of growth for any treatments and mean growth rate ranged from 1.6mm day^{-1} for replicates stored lyophilised to 1.8mm day^{-1} for replicates that had been cryopreserved.

After 1 year of storage

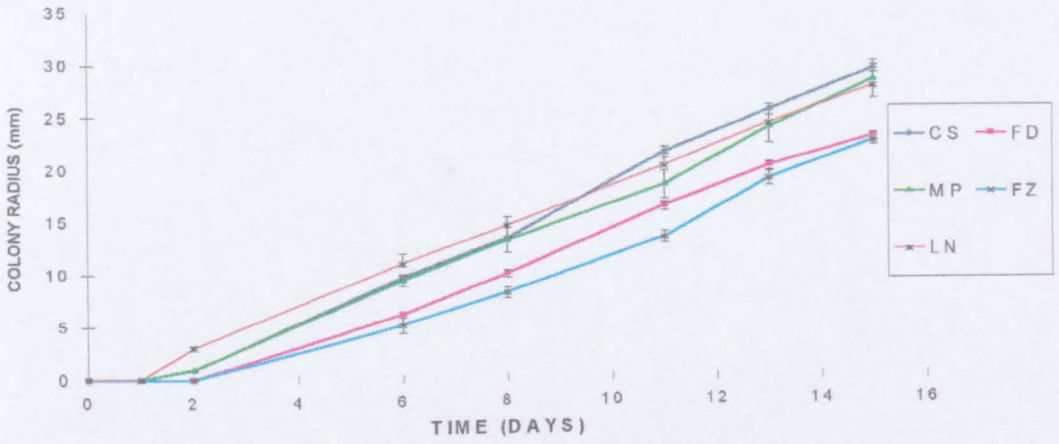
There was no significant difference in growth rates between preservation treatments ($P>0.05$) for replicates of isolate M1. The mean growth rates varied from 1.7 mm day^{-1} for replicates stored as mycelial plugs in water, 2.0 mm day^{-1} for replicates stored in liquid nitrogen, 2.2 mm day^{-1} for replicates maintained by continual sub-culture and 2.4 mm day^{-1} for replicates stored lyophilised. However, radial growth in isolate M2 was changed by preservation and storage ($P<0.05$), with mean radial growth rates varying from 1.9 mm day^{-1} for replicates maintained by continual sub-culture to 2.2 mm day^{-1} for lyophilised replicates.

Fig. 6: A. Radial growth of *Metarhizium* isolate M4 after 1 week of preservation. B. 16 weeks of preservation and C. 16 weeks of preservation and a recovery period Maintained on SDA at 25°C

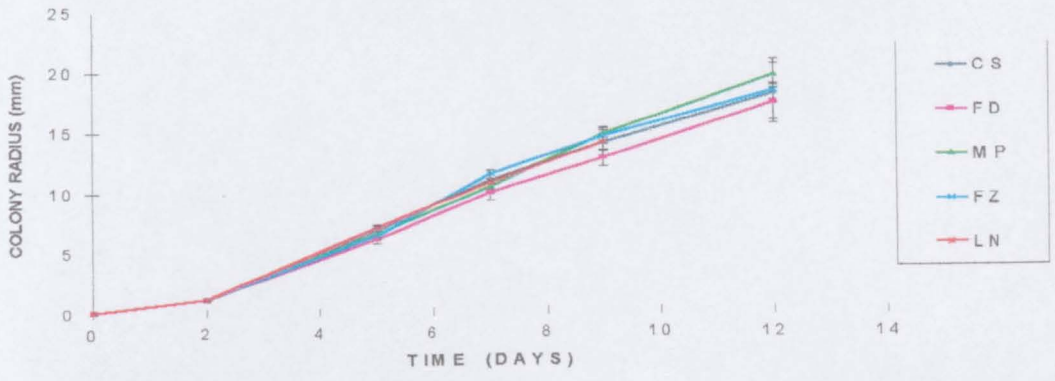
A.



B.



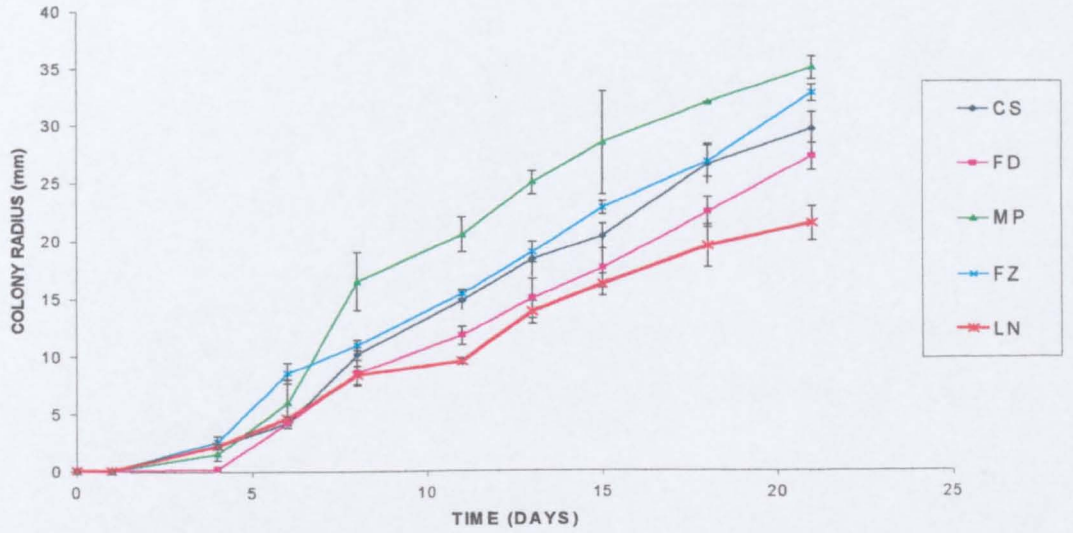
C.



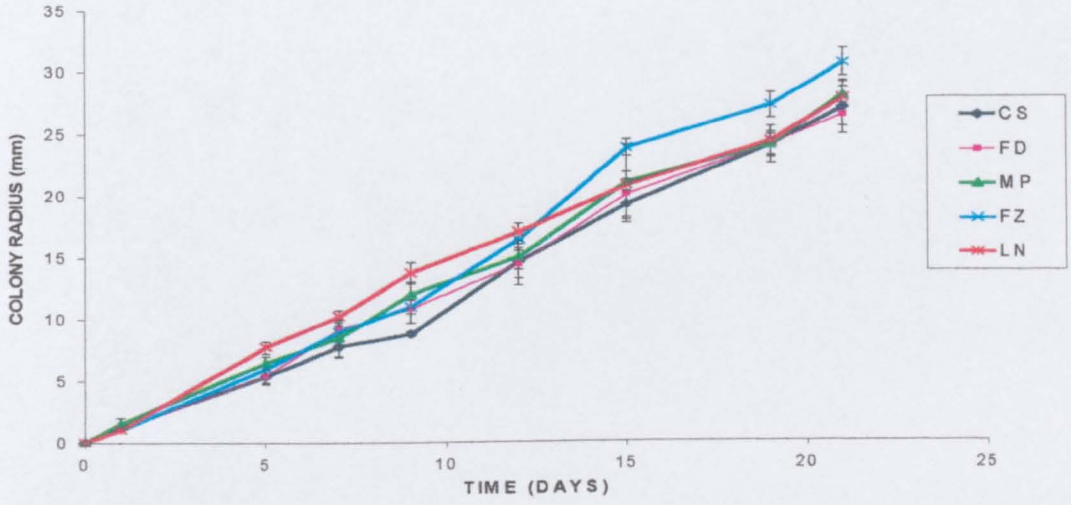
Key: CS, continual sub-culture; FD, lyophilisation; MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation in liquid nitrogen.

Fig. 7: A. Radial growth of *Metarhizium* isolate M4 after 1 year of preservation. and B. after 1 year of preservation and a recovery period. Maintained on SDA at 25°C

A.



B.



Key: CS, continual sub-culture; FD, lyophilisation; MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation in liquid nitrogen.

There was a short lag period of two days before growth was initiated in lyophilised replicates. There was a highly significant ($P<0.005$) difference in radial growth between the preservation treatments for replicates of isolate M4 after 1 year of storage (Fig. 7A). Replicates stored in liquid nitrogen had a slow mean growth rate of 1.2 mm day^{-1}. The mean growth rates of replicates of the other treatments was 1.7 mm day^{-1} for replicates maintained by continual sub-culture and 1.5, 2.0 and 1.7 mm day^{-1} respectively for replicates maintained by lyophilisation, mycelial plugs in water and at –20°C. There was less difference between the mean growth rates after a recovery period, although there was still a significant difference between preservation treatments ($P<0.05$) (Fig 7B). Growth rates ranged from 1.2 mm day^{-1} for replicates stored by cryopreservation to 1.5 mm day^{-1} for replicates stored at –20°C. There was no delay in the onset of growth amongst replicates from any of the preservation treatments.

After 2 years of preservation

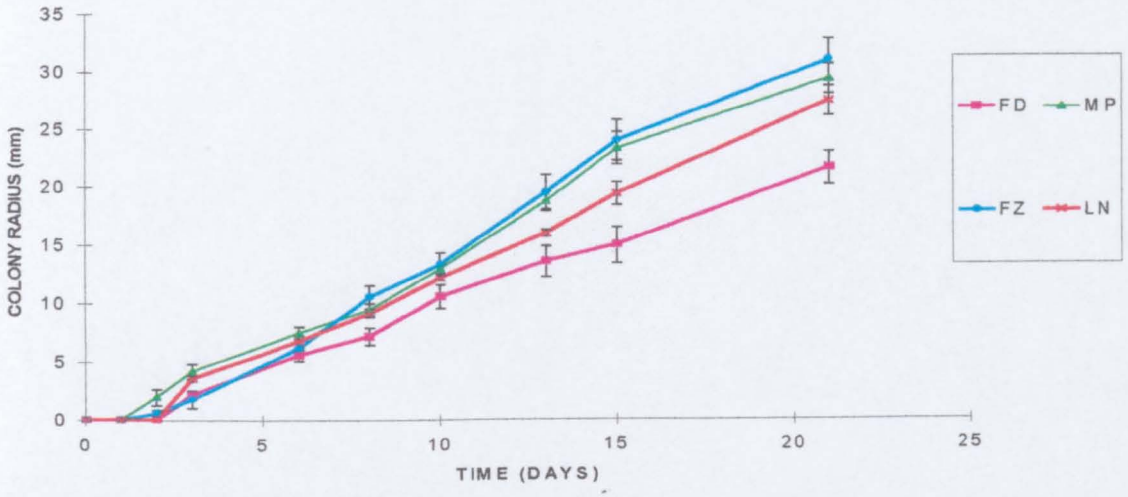
The difference in mean radial growth rates between preservation treatments was highly significant in isolates M1 ($P<0.005$) and M2 ($P<0.005$) (Fig. 8A). As seen at other testing times there was a lag period before growth initiated in replicates that had been lyophilised. However, initial growth of replicates stored as mycelial plugs in water was faster. Even after a recovery period there were significant differences in radial growth rate for replicates of isolates M1 and M2 (Fig. 8B) ($P<0.005$). The mean radial growth rates for replicates stored lyophilised and in liquid nitrogen of both isolates M1 and M2 increased, whereas the growth rate decreased when preserved by other methods (Table 1).

Table 1: Growth rates (mm day^{-1}) of *Metarhizium anisopliae* isolates M1 and M2 after 2 years of preservation. Maintained on SDA at 25°C.

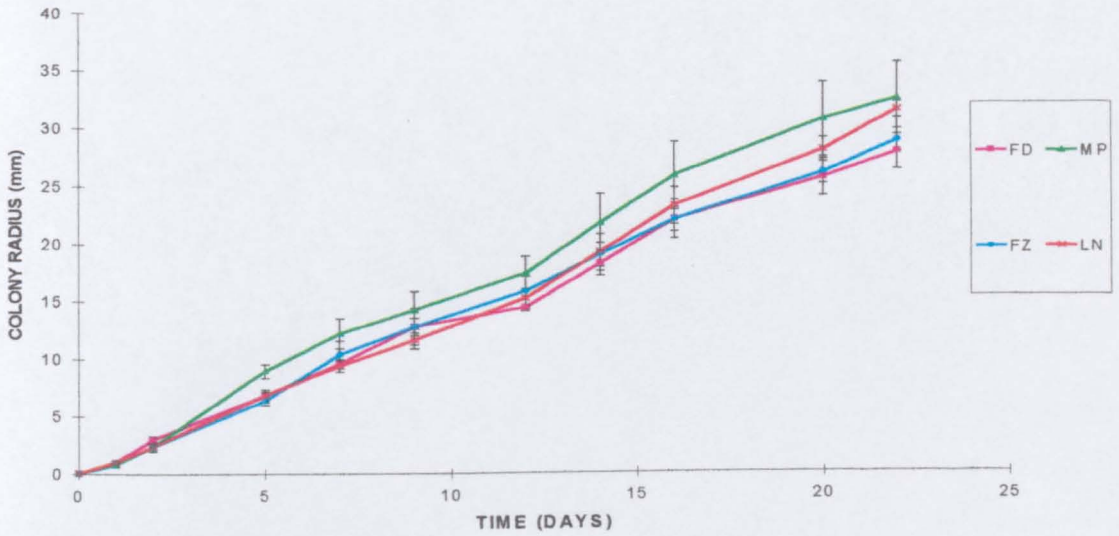
Preservation treatment	**M1**	**M1 (after recovery)**	**M2**	**M2 (after recovery)**
Lyophilisation	1.5	1.7	1.1	1.3
Mycelial Plugs in water	1.8	1.6	1.5	1.5
Freezing at –20°C	-	-	1.7	1.3
Cryopreservation	1.4	1.7	1.3	1.5

Fig 8: A. Radial growth of *Metarhizium anisopliae* isolate M2 after 2 years of preservation and B. after 2 years of preservation and a recovery period Maintained on SDA at 25°C

A.



B.



Key: CS, continual sub-culture; FD, lyophilisation; MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation in liquid nitrogen.

Conidial production

After 1 week of preservation

There was a significant increase ($P<0.05$) in the number of conidia produced by replicates of isolate M4 that had been preserved (Fig 9A). Replicates that had been lyophilised produced more conidia than replicates of any other treatment. Replicates that had been maintained by continual sub-culture produced the least conidia. There was increased variation between replicates within treatments that had been stored at –20°C (S.E. 5.2×10^5 conidia per mm^2 of culture area) and cryopreserved (S.E. 4.5×10^5 conidia per mm^2 ofculture area).

After 16 weeks of preservation

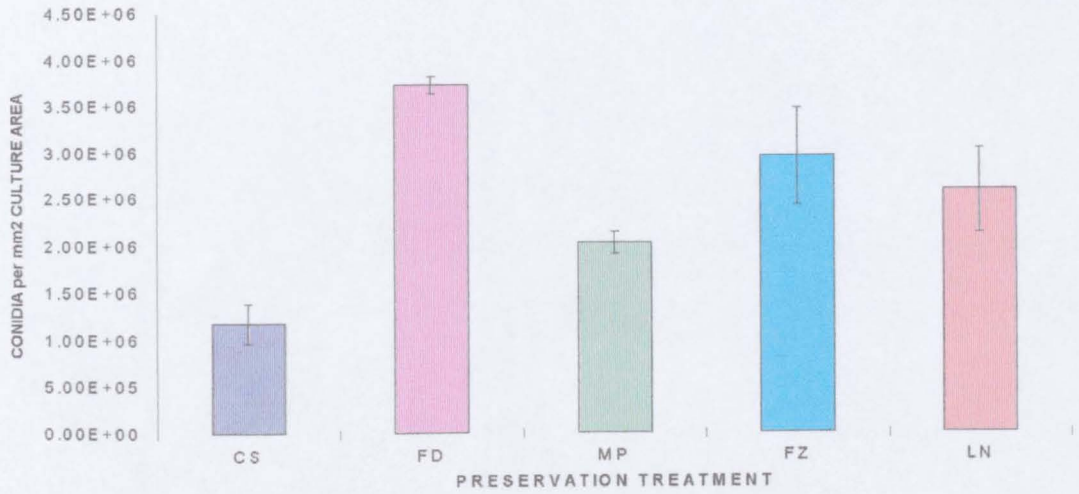
There was a significant difference in conidial production between preservation treatments ($P<0.005$) in replicates of isolate M4. Within preservation treatments there was considerable variation (S.E. 4.2×10^5 conidia per mm^2 of culture area) between replicates that had been cryopreserved. Replicates that had been lyophilised produced more conidia than the other treatments. Replicates that had been maintained by continual sub-culture and mycelial plugs in water produced the least number of conidia.

After 1 year of preservation

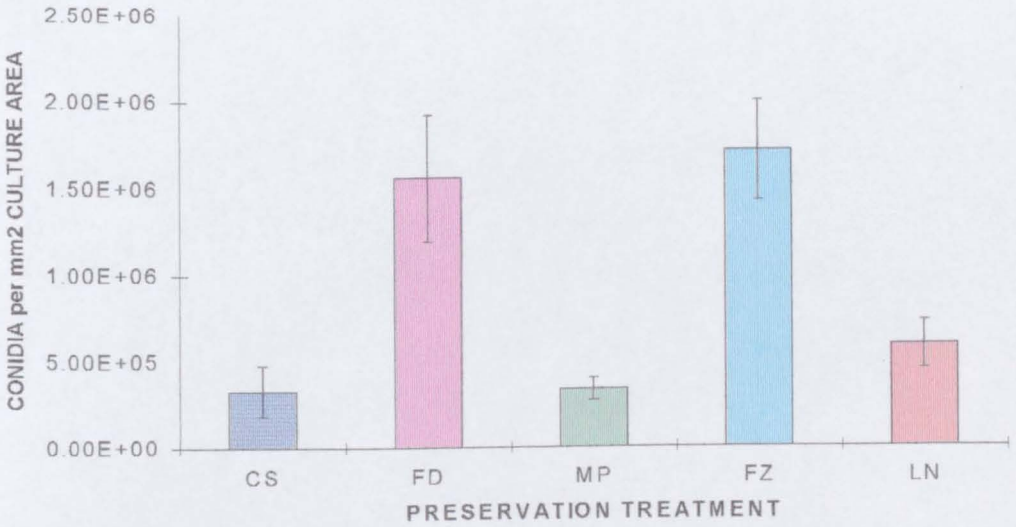
There was a highly significant difference in conidial production between preservation treatments ($P<0.005$) in replicates of isolate M2 (Fig 9B). Replicates stored as mycelial plugs in water produced less conidia than replicates of other treatments. The most conidia were produced by replicates that had been lyophilised and stored at -20°C, but there was considerable variation between replicates (S.E. 3.6 $\times 10^5$ conidia per mm^2 of culture area) that had been lyophilised There was a highly significant difference in conidial production between preservation treatments ($P<0.005$) in replicates of isolate M1.

Fig. 9: A. Conidial production by *Metarhizium* isolate M4 after 1 week of preservation and B. Conidial production by *Metarhizium anisopliae* isolate M2 after 1 year of preservation. Maintained for 21 days on SDA at 25°C.

A.



B.



Key: CS, continual sub-culture; FD, lyophilisation; MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation in liquid nitrogen.

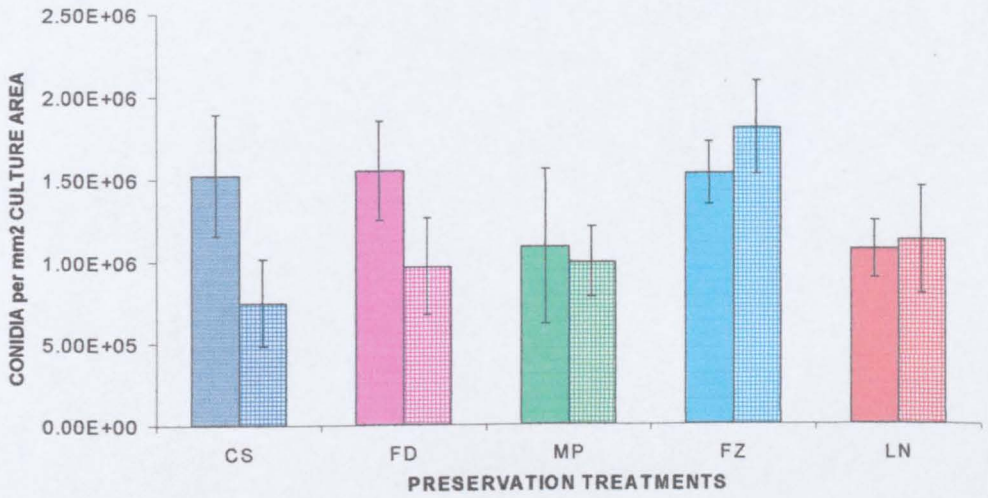
Replicates that had been stored as mycelial plugs in water produced 8 times less conidia than any other treatment ($P<0.05$). There was no significant difference in conidial production between preservation treatments ($P>0.05$) in replicates of isolate M4. However, there was high variation within all treatments (Fig 15). Variation ranged from S.E. $1.7x10^5$ conidia per mm^2 culture area for replicates that had been cryopreserved to S.E. $4.7x10^5$ conidia per mm^2 of culture area for replicates that had been stored as mycelial plugs in water. After a recovery period, the number of conidia produced by replicates that had been maintained by continual sub-culture and stored lyophilised decreased, but the high level of variation remained.

After 2 years of preservation

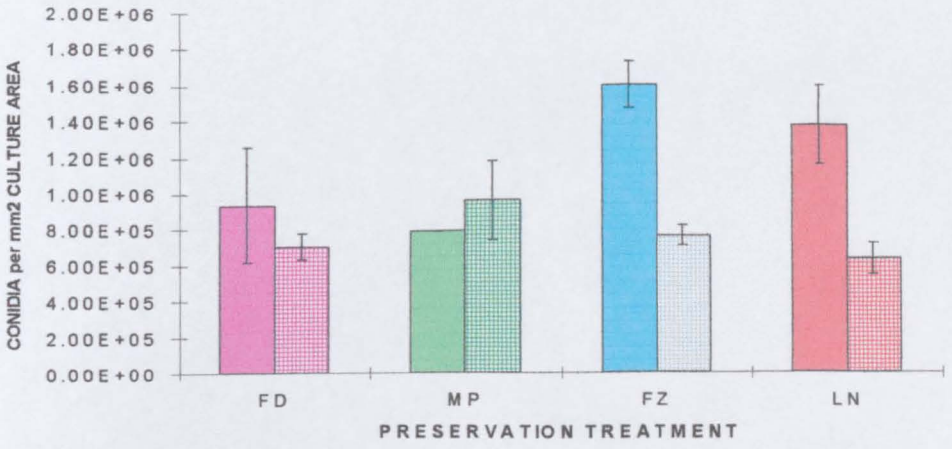
There was no significant difference ($P>0.05$) between the three remaining treatments of isolate M1 (Fig 16). However, there was large variation within replicates that had been stored as mycelial plugs in water (S.E $2.4x10^5$ conidia per mm^2 of culture area) and cryopreserved (S.E. $3.2x10^5$ conidia per mm^2 of culture area). After a recovery period, the variation of replicates within treatments decreased. There was no significant difference ($P>0.05$ but $P<0.10$) between the four remaining treatments of isolate M2. Replicates stored as mycelial plugs in water produced the least numbers of conidia. Replicates stored at –20°C produced the most numbers of conidia. There was high variation within all preservation treatments. For example, the variation between replicates that had been lyophilised was S.E $3.0x10^5$ conidia per mm^2 of culture area. After the recovery period, the variation between replicates within treatments had decreased and there was no significant difference between preservation treatments ($P>0.05$)

Fig. 10: A. Conidial production by *Metarhizium* isolate M4 after 1 year of preservation (dark) and a recovery period (hatched) and B. Conidial production by *Metarhizium anisopliae* isolate M2 after 2 years of preservation (dark) and a recovery period (hatched). Maintained for 21 days on SDA at 25°C.

A.



B.



Key: CS, continual sub-culture; FD, lyophilisation; MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation in liquid nitrogen.

3.3.3 *Fusarium oxysporum*

Viability

All replicates of *Fusarium oxysporum* were viable after treatment by all preservation methods and at each testing time after storage.

Culture morphology

Before preservation isolates of *Fusarium oxysporum* were cottony (floccose) with regular culture margins. Pigmentation was variable and of a red to purple colour. After resuscitation from preservation, some *Fusarium* replicates degenerated from the cottony (wild-type) cultural morphology to the ropey (mycelial) (Fig 11A) or slimy (pionnotal) states (Fig 11B). Some replicates degenerated to an intermediate state between two morphological states. A summary of the cultural morphologies exhibited by each replicate at each testing time is given in Table 2.

Fig. 11: A. Slimy cultural state, from a replicate of *Fusarium oxysporum* stored for two years as a mycelial plug in water B. Ropey cultural state, from a replicate of *Fusaium oxysporum* isolate F2 stored for two years at –20°C. Both maintained on PSA for 21 days at 20°C.

A). B).

After 1 day and 1 week of preservation

All replicates retained the cottony morphological state and exhibited the culture characteristics seen before preservation.

After 16 weeks of storage.

Six replicates of isolate F3 degenerated to an intermediary cottony/ropey state, 1 replicate stored as a mycelial plug in water deteriorated further to a ropey/slimy intermediary morphological state.

After 1 year in storage.

All replicates of isolate F2 remained cottony. Five replicates of isolate F1 (1 stored by lyophilisation, 3 stored at –20°C, 1 stored by cryopreservation) degenerated to the slimy cultural state. No sectors were detected after 1 year of preservation in isolates F1 and F2. Twelve of 25 replicates of isolate F3 had degenerative culture morphology after 1 year of storage. Three replicates stored by continual sub-culture, 1 stored by lyophilisation, 2 stored as mycelial plugs in water and 3 stored at –20°C had degenerated to the intermediary cottony/ropey state. One replicate that had been cryopreserved had degenerated to the ropey state and 2 replicates (1 stored as a mycelial plug and 1 stored at –20°C) had further degenerated to the intermediate ropey/slimy state. Four replicates of isolate F3 sectored, 2 of the replicates stored as mycelial plugs in water, 1 of the replicates maintained by continual sub-culture and 1 of the replicates that had been stored at –20°C.

After 2 years of storage.

All 5 replicates of isolates F1 maintained by continual sub-culture had degenerated to the slimy cultural state. Of the remaining replicates, only the lyophilised, 3 of those stored at –20°C and 2 of the 5 stored in liquid nitrogen retained cottony morphology. Of the replicates of isolate F2, only 1 of the 5 replicates maintained by continual sub-culture retained the original cottony state. All of the replicates stored lyophilised and cryopreserved retained the cottony state and only 1 each of the replicates stored as a mycelial plug in water and at –20°C had degenerated (to the ropey state).

Table 2: Degeneration of culture morphology in *Fusarium* isolates after resuscitaion from storage. Maintained for 28 days on PSA at 20°C.

Testing Time-		Day 1	Day1	Wk1	Wk16	YR1	YR1	YR1	YR2	YR2
ISOLATE-		**F1**	**F2**	**F3**	**F3**	**F1**	**F2**	**F3**	**F1**	**F2**
Rep	Method									
A1	CS	C	C	C	C	C	C	C	S	S
A2	CS	C	C	C	C	C	C	C	S	C
A3	CS	C	C	C	C/R	C	C	C/R	S	C/S
A4	CS	C	C	C	C/R	C	C	C/R	S	S
A5	CS	C	C	C	C/R	C	C	C/R	S	R/S
B1	FD	C	C	C	C	S	C	C	C	C
B2	FD	C	C	C	C	C	C	C	C	C
B3	FD	C	C	C	C	C	C	C/R	C	C
B4	FD	-	-	C	C/R	-	-	C	C	C
B5	FD	-	-	C	C	-	-	C	C	C
C1	MP	C	C	C	C	C	C	C/R	S	C
C2	MP	C	C	C	C	C	C	C	S	C
C3	MP	C	C	C	C/R	C	C	R/S	R/S	C
C4	MP	C	C	C	C	C	C	C/R	S	C
C5	MP	C	C	C	R/S	C	C	C	R/S	R
D1	FZ	C	C	C	C	S	C	R/S	R/S	C
D2	FZ	C	C	C	C/R	S	C	C/R	C	C
D3	FZ	C	C	C	C	S	C	C	C	C
D4	FZ	-	-	C	C	-	-	C/R	C	C
D5	FZ	-	-	C	C	-	-	C/R	C	R
E1	LN	C	C	C	C	S	C	R	C	C
E2	LN	C	C	C	C	C	C	C	C	C
E3	LN	C	C	R	C	C	C	C	S	C
E4	LN	-	-	C	C	-	-	C	S	C
E5	LN	-	-	C	C	-	-	C	R/S	C

CS=Continual Sub-culture, FD=Lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at –20°C, LN= Cryopreservation,

C= Cottony, R=Ropey, S=Slimy, / = intermediate stage, – = not examined

Two replicates of isolate F2 (1 that had been maintained by continual sub-culture and 1 that had been stored at –20°C) and 2 replicates of isolate F1 (1 of which had been stored in liquid nitrogen and 1 maintained by continual sub-culture) sectored. Sector morphology was similar to the areas from which sectors arose, except in a replicate of isolate F2 stored for two years at –20°C (Fig 12) where sectors degenerated to the slimy morphological state. In all 3 isolates, pigmentation (red to purple-vineaceous in colour) was variable in hue and intensity within replicates of the same treatment, over all testing times. Culture margins were generally smooth and regular, although faster growing cultures were often irregular.

Fig. 12: Sectorisation in *Fusarium oxysporum* isolate F2, stored for two years at –20°C and maintained on PSA for 28 days at 20°C.

Effect of a recovery period after preservation

Some replicates were more likely to display cottony morphology following a recovery period of maintenance on SNA following preservation. For example, 12 replicates of *Fusarium oxysporum* isolate F1 stored for 2 years, immediately inoculated after preservation onto PSA produced slimy cultures. When maintained on SNA for 28 days and then sub-cultured onto PSA all were cottony. However, some replicates showed degenerative morphology after the recovery period. They had previously exhibited cottony morphology immediately following resuscitation.

Radial growth

After 1 day of storage

Fungal preservation method affected radial growth in *Fusarium oxysporum.* The difference in mean radial growth rates between the preservation treatments was highly significant in isolates F1 ($P<0.005$) (Fig. 13A) and F2 ($P<0.005$). The mean growth rates of replicates of isolate F1 ranged from 3.6 mm day^{-1} for replicates stored at –20°C to 4.0 mm day^{-1} for replicates stored in liquid nitrogen. The mean growth rates of replicates of isolate F2 ranged from 4.9 mm day^{-1} for replicates stored lyophilised to 5.3 mm day^{-1} for replicates stored at –20°C. There was a 1 to 2 day delay in the onset of growth for replicates of both isolates that had been lyophilised and faster initial growth of replicates stored as mycelial plugs in water.

After 1 week of storage

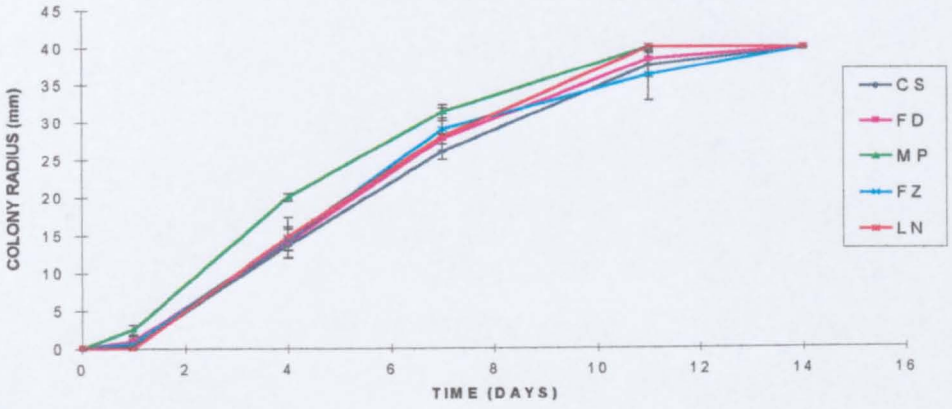
There was a significant difference in mean radial growth rates between preservation treatments of replicates of isolate F3 ($P<0.05$) (Fig 13B). Mean growth rates of replicates from preserved lines were lower than that maintained by continual sub-culture (5.3 mm day^{-1}). Of the remaining treatments, mean growth rates ranged from 4.9 mm day^{-1} for replicates that had been cryopreserved, 5.1 mm day^{-1} for replicates stored at –20°C and had been lyophilised and 5.3 mm day^{-1} for replicates stored as mycelial plugs in water. Initial growth of replicates stored by cryopreservation was slow and there was a 1-day delay in the onset of growth of replicates that had been lyophilised. After a recovery period (Fig 13C) there was no observable difference in the onset of growth of replicates of any treatment and there was no significant difference between mean radial growth rates ($P>0.05$). However, there was an increase in growth rates for replicates from all preservation treatments. Mean radial growth rates ranged from 5.4 mm day^{-1} for replicates stored at -20°C to 5.6 mm day^{-1} for replicates that had been lyophilised.

After 16 weeks of storage

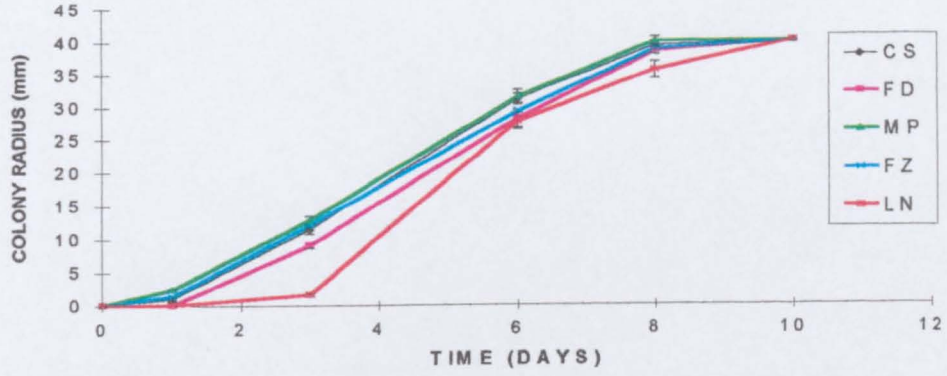
There was a significant difference in mean radial growth rates between preservation treatments of replicates of isolate F3 ($P<0.05$) (Fig 13). Mean growth rates ranged from 3.9 mm day^{-1} for replicates that had been cryopreserved, 4.0 mm day^{-1} for replicates stored at –20°C, 5.0 mm day^{-1} for lyophilised replicates, 5.4 mm day^{-1} for replicates maintained by continual sub-culture and 5.6 mm day^{-1} for replicates stored as mycelial plugs in water.

Fig. 13: A. Radial growth of *Fusarium oxysporum* isolate F1 after 1 day of preservation. B. *Fusarium oxysporum* isolate F3 after 1 week of preservation and C. *Fusarium oxysporum* isolate F3 after 1 week of preservation and a recovery period. Maintained on PSA at 20°C.

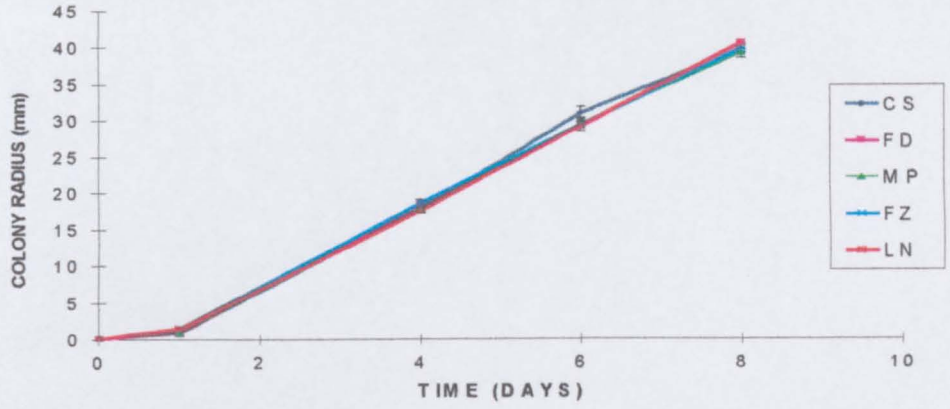
A.



B.



C.



Key: CS, continual sub-culture; FD, lyophilisation; MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation in liquid nitrogen.

There was a 1-day delay in the onset of growth of replicates that had been lyophilised and cryopreserved and a 2-day delay of replicates that had been stored at –20°C. After a recovery period there was no observable difference in the onset of growth of replicates of any treatment and there was no significant difference between mean radial growth rates ($P>0.05$). However, there was an increase in growth rates for replicates from all preservation treatments. Mean growth rates range from 5.8 mm day^{-1} for replicates stored lyophilised to 6.4 mm day^{-1} for replicates that had been cryopreserved.

After 1 year of storage

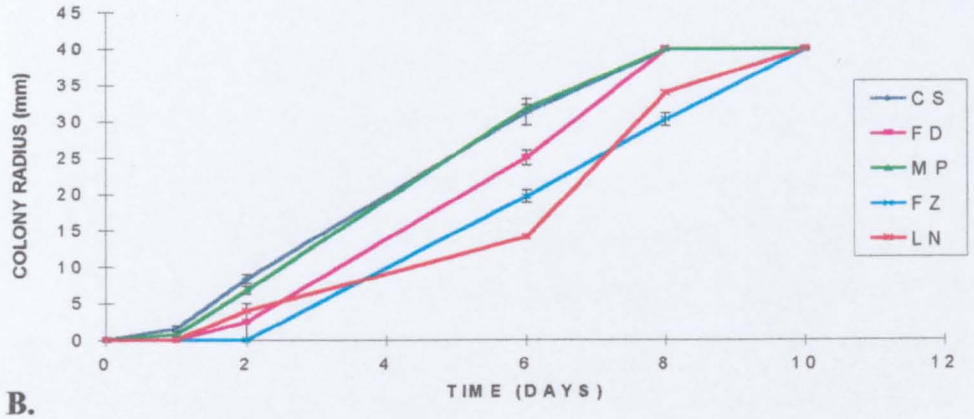
Mean radial growth rates differed between replicates from the different preservation treatments in all *Fusarium* isolates. The differences between mean radial growth rates of replicates of isolate F1 between the preservation treatments was significant ($P<0.05$) and ranged from 3.4 mm day^{-1} for replicates stored at –20°C through to 4.6 mm day^{-1} for replicates maintained by continual sub-culture. There was a 2 day delay in the onset of growth for replicates that had been lyophilised. After a recovery period, there was still a significant difference between preservation treatments ($P<0.05$). Growth rates for replicates stored by lyophilisation, storage at –20°C and stored in liquid nitrogen increased. The growth rates of replicates maintained by continual sub-culture and mycelial plugs in water decreased. There was no delay in the onset of growth for any replicates from any preservation treatment after the recovery period.

Table 3: Mean radial growth rates (mm day^{-1}) of *Fusarium* isolates after one year of preservation

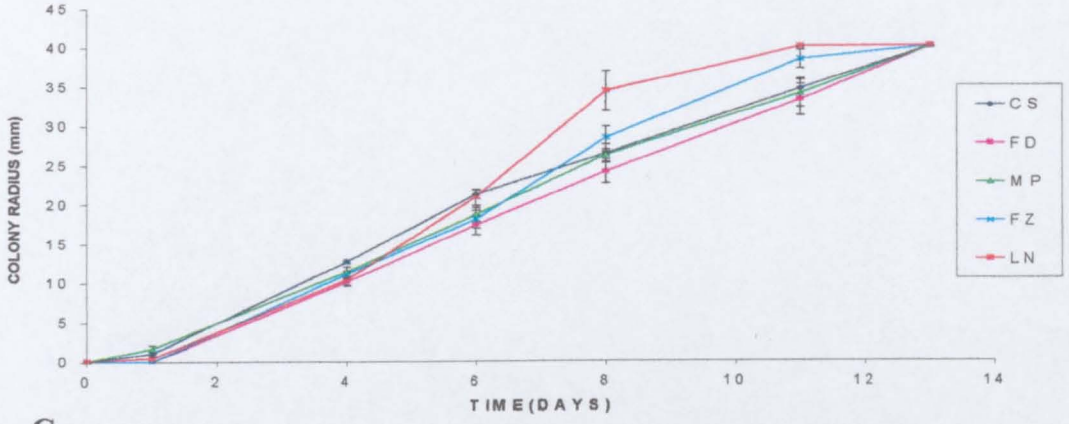
Preservation method	F1	F1 after recovery	F2	F2 after recovery	F3	F3 after recovery
Continual sub-culture	4.6	3.7	3.9	4.0	3.4	3.9
Lyophilisation	3.7	3.8	3.8	4.0	3.3	3.8
Mycelial plugs in water	4.3	3.3	3.9	4.2	3.3	3.7
Storage at –20°C	3.4	3.8	3.6	4.2	3.9	3.9
Cryopreservation	3.5	4.0	3.7	4.1	4.2	4.0

Fig. 14: A. Radial growth of *Fusarium oxysporum* isolate F3 after 16 weeks of preservation, B. after 1 year of preservation, C. after 1 year of preservation and a recovery period. Maintained on PSA at 20°C.

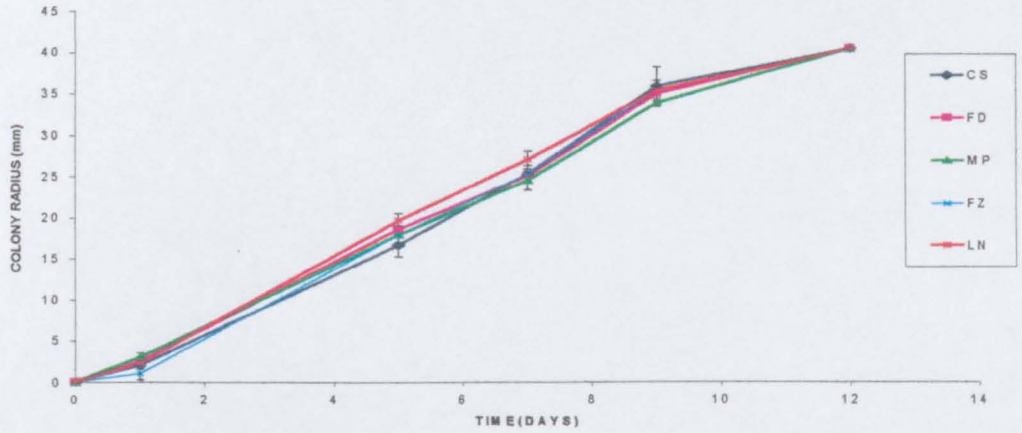
A.



B.



C.



Key: CS, continual sub-culture; FD, lyophilisation; MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation in liquid nitrogen.

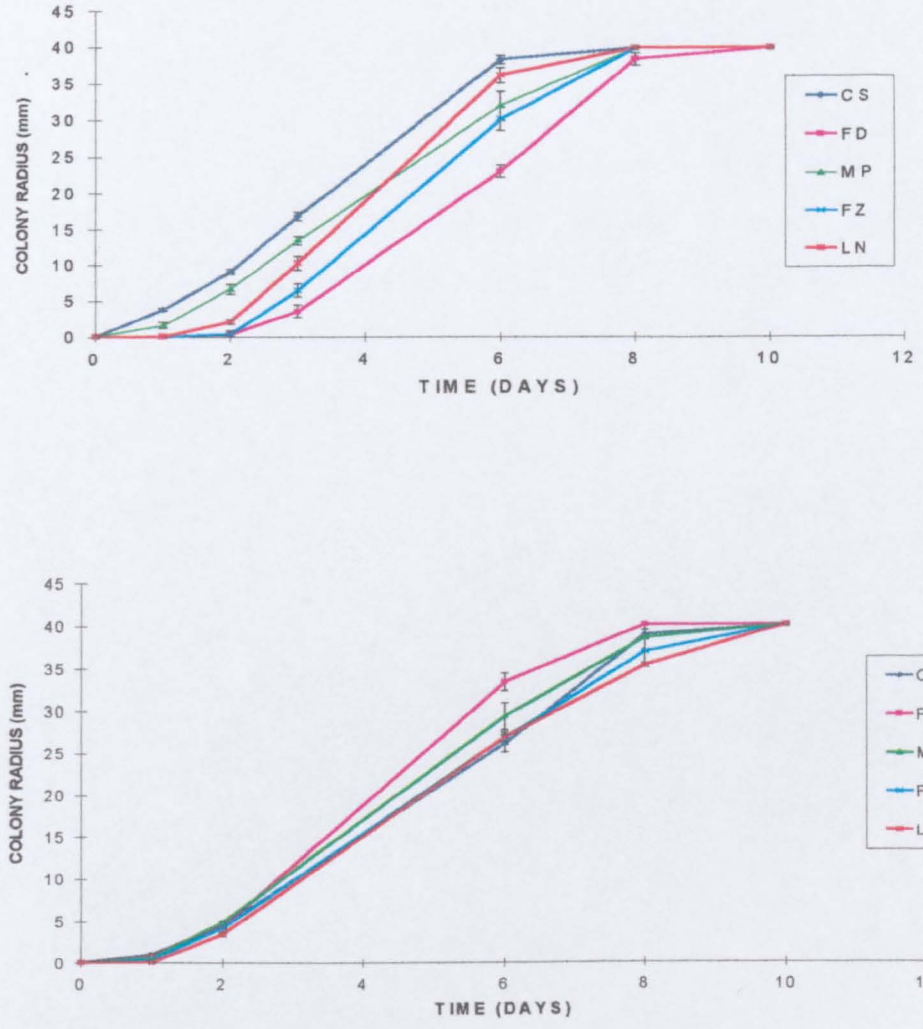
The difference between mean radial growth rates of replicates of isolate F2 from the different preservation treatments was significant ($P<0.05$) and ranged from 3.7 mm day^{-1} for replicates that had been cryopreserved to 4.0 mm day^{-1} for replicates maintained by continual sub-culture (Table 3). There was a two-day delay in the onset of growth for replicates that had been lyophilised. After a recovery period the mean radial growth rates increased for replicates from all of the preservation treatments and there was less of a difference between growth rates. However, the difference between the preservation treatments remained significant ($P<0.05$). The difference between mean radial growth rates of treatments of isolate F3 (Fig 14B) was significant ($P<0.05$). Rates ranged from 3.3 mm day^{-1} for replicates stored as mycelial plugs in water to 4.2 mm day^{-1} for replicates stored by cryopreservation. After a recovery period (Fig 14C) there was no significant difference in growth rate between preservation treatments ($P>0.05$), radial growth rates ranging from 3.7 mm day^{-1} for replicates stored as mycelial plugs in water to 4.0 mm day^{-1} for replicates stored by cryopreservation.

After 2 years of storage

There were greater differences in mean radial growth rates between replicates from the different preservation treatments in *Fusarium* isolates F1 and F2 after 2 years of storage. The differences between mean radial growth rates of replicates of isolate F1 was highly significant ($P<0.0005$). Rates ranged from 4.8 mm day^{-1} for replicates stored as mycelial plugs in water, 5.3 mm day^{-1} for replicates maintained by continual sub-culture, 5.4 mm day^{-1} for replicates stored in liquid nitrogen, 5.6 mm day^{-1} for replicates that had been lyophilised and 6.2 mm day^{-1} for replicates stored at –20°C. The differences in mean radial growth rates of replicates of isolate F2 was highly significant ($P<0.005$) (Fig 25). Rates ranged from 4.0 mm day^{-1} for replicates that had been lyophilised, 5.3 mm day^{-1} for replicates stored at –20°C, 5.6 mm day^{-1} for replicates stored as mycelial plugs in water, 6.4 mm day^{-1} for replicates stored in liquid nitrogen and 6.6 mm day^{-1} for replicates maintained by continual sub-culture. There was a delay in the onset of growth for replicates of isolates F1 and F2 that had been lyophilised and stored at –20°C. After the recovery period, the difference in mean radial growth rates between the preservation treatments was not significant ($P>0.05$) in isolates F1 and F2 (Fig 15B). The mean growth rates of treatments of both isolates decreased except for replicates of isolate F1 stored by

mycelial plugs in water and replicates of isolate F2 stored lyophilised, which increased after the recovery period.

Fig. 15: A. Radial growth of *Fusarium oxysporum* isolate F2 after 2 years of preservation, and B. after 2 years of preservation and a recovery period. Maintained on PSA at 20°C.



Key: CS, continual sub-culture; FD, lyophilisation; MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation in liquid nitrogen.

Conidial production

After 1 week of storage

There was a significant decrease ($P<0.05$) in the number of microconidia produced by replicates of isolate F3 that underwent preservation compared to the continual sub-culture replicates that had not been preserved (Fig 15A). There was no significant difference ($P>0.05$) between the replicates that had been lyophilised, cryopreserved, stored at –20°C and as mycelial plugs in water. Production of macroconidia was influenced by preservation treatment ($P<0.05$), with replicates of treatments stored lyophilised and cryopreserved producing less conidia than replicates preserved by other methods.

After 16 weeks of storage

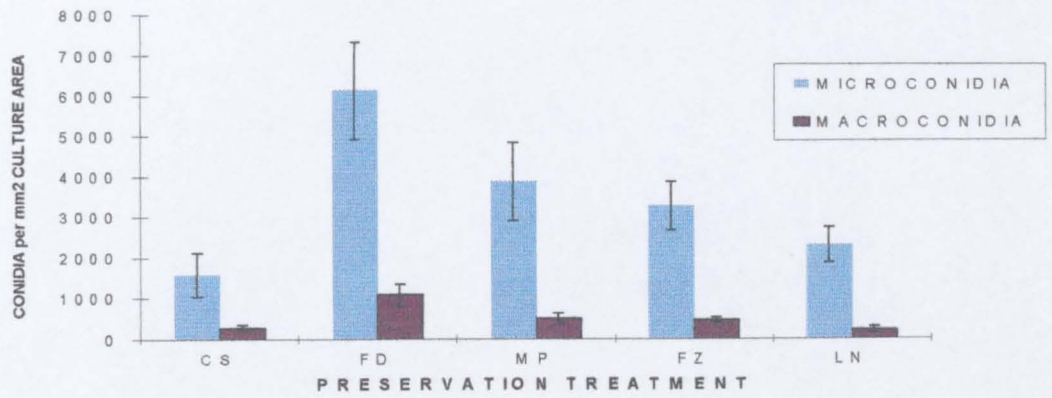
There was a highly significant difference ($P<0.005$) in microconidial production between replicates stored by all preservation treatments (Fig 15B). The ratio of microconidia to macroconidia was low (compared to the week 1 testing time) for all preservation treatments. Within treatments, there was a big difference between replicates stored as mycelial plugs in water (S.E. 3866 conidia per mm^2 of culture area). However, after a recovery period there was no significant difference ($P>0.05$) in conidial production between treatments (Fig 15C) and the difference between replicates stored as mycelial plugs in water had decreased (S.E. 1664 conidia per mm^2 of culture area). There was no significant difference in macroconidial production after sixteen weeks of preservation either before or after the recovery period.

After 1 year of storage

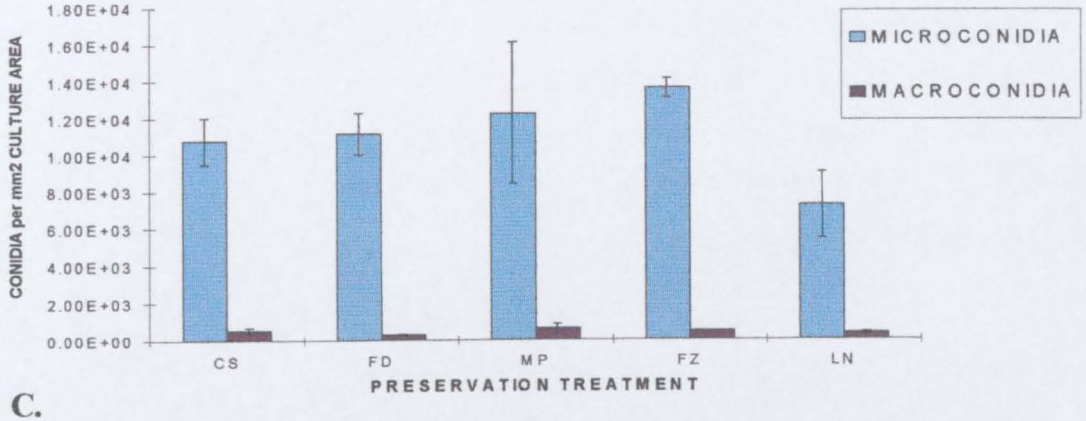
There was a significant difference between microconidial production between different preservation treatments in isolate F1 ($P<0.05$). The difference between replicates maintained by continual sub culture (S.E. 822 conidia per mm^2 of culture area) and lyophilisation (S.E. 691 conidia per mm^2 of culture area) was large compared to replicates of other treatments i.e. those stored at –20°C (S.E. 226.700 conidia per mm^2 of culture area). The difference in macroconidial production between treatments was highly significant ($P<0.005$). After a recovery period there was no significant difference between preservation treatments in either micro- or macroconidial production ($P>0.05$).

Fig. 16: A. Conidial production by *Fusarium oxysporum* Isolate F3, after 1 week of preservation. B. after 16 weeks of preservation and C. after 16 weeks of preservation and a recovery period. Maintained on SNA for 14 days at 20°C.

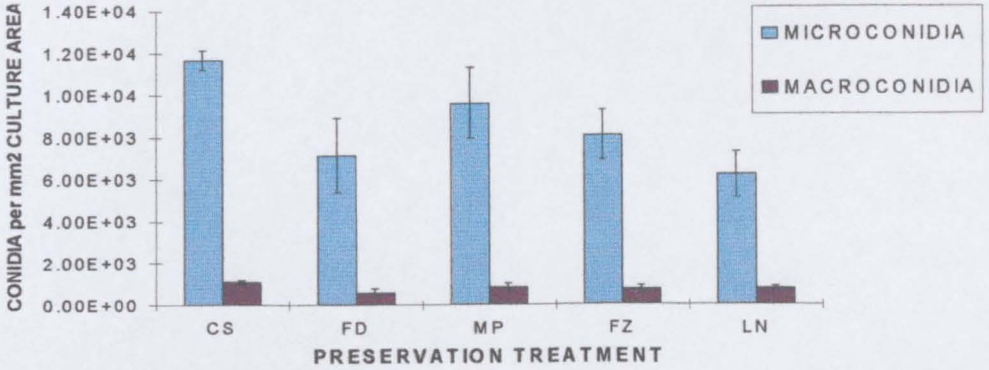
A.



B.



C.



Key: CS, continual sub-culture; FD, lyophilisation; MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation in liquid nitrogen.

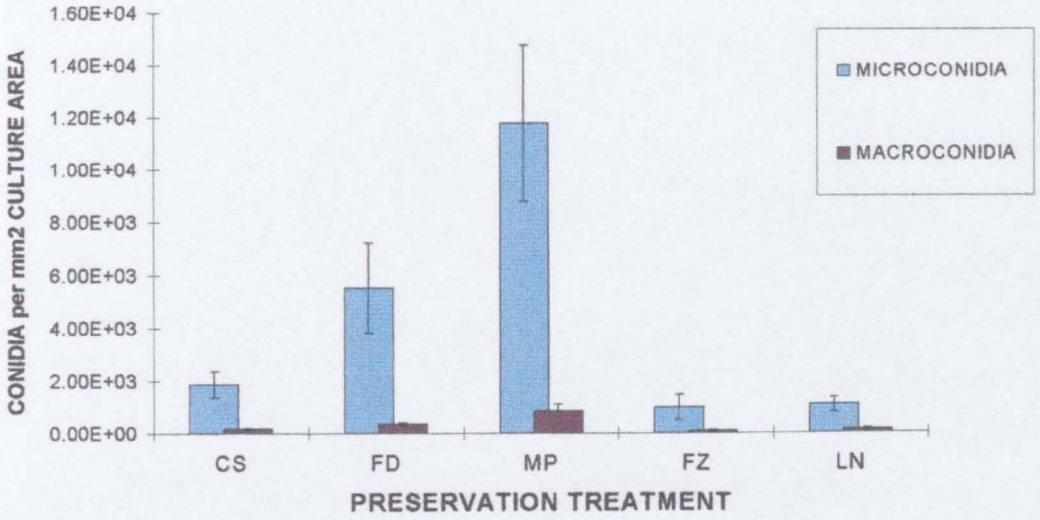
In isolate F2 (Fig 17a), the difference in microconidial production between preservation treatments was highly significant ($P<0.005$). Replicates stored as mycelial plugs in water produced twice as many conidia than any other treatment and the difference in conidial production between replicates was very high (S.E. 11775 conidia per mm^2 of culture area). The difference between treatments in macroconidial production was highly significant ($P<0.05$). After the recovery period there remained a significant difference ($P<0.05$) in microconidial production (Fig 31) but not in macroconidial production ($P>0.05$). In isolate F3, there was no significant difference ($P>0.05$) in either micro- or macroconidial production between replicates from the different preservation treatments after 1 year of storage, but the variances within treatments for microconidial production was higher than that seen at previous testing times. For example, S.E. 1322 conidia per mm^2 of culture area, for replicates maintained by continual sub-culture, S.E 925 conidia per mm^2 of culture area for replicates stored by mycelial plugs in water. The ratio of microconidia to macroconidia was significantly lower ($P<0.05$) than after 1 week for all treatments. After the recovery period there was less difference between preservation treatments for microconidial ($P>0.5$) and macroconidial production ($P>0.05$). However, within treatments the variance decreased except for replicates stored as mycelial plugs in water where the large variance remained (S.E. 1387 conidia per mm^2 of culture area)

<u>After 2 years of storage</u>

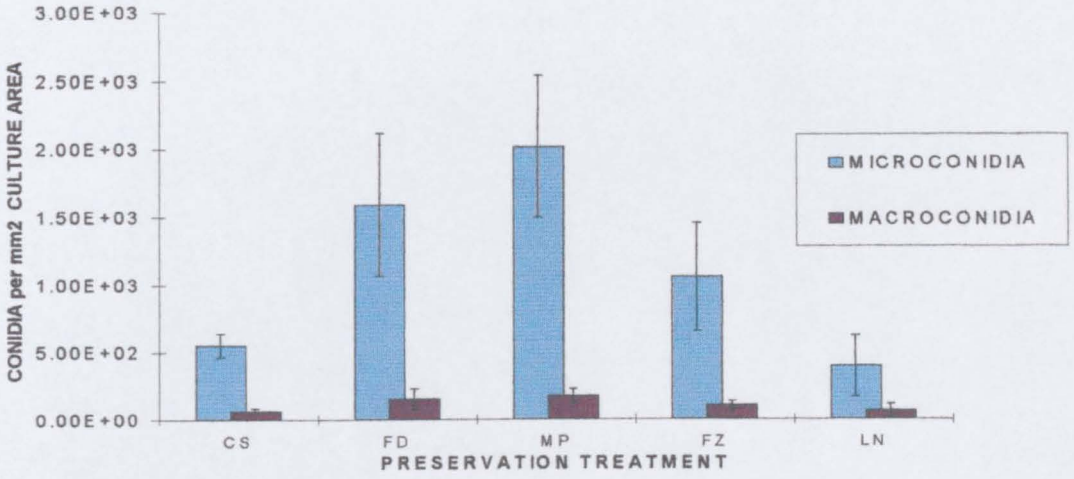
There were significant differences in microconidial ($P<0.05$) and macroconidial ($P<0.05$) production between preservation treatments in replicates of isolate F2 (Fig 18A). Within preservation treatments, less variation in microconidial production was seen in replicates that had been lyophilised (S.E 285 conidia per mm^2 of culture area) and cryopreserved (S.E 392 conidia per mm^2 of culture area) compared to replicates stored as mycelial plugs in water which had the largest variation (S.E 1139 conidia per mm^2 culture area). The ratio of microconidia to macroconidia was significantly lower ($P<0.05$) than after 1 year of storage for all treatments except for replicates stored cryopreserved which rose. After the recovery period, the significant difference between preservation treatments remained for both micro- ($P<0.05$) and macroconidial production ($P<0.05$) (Fig 18B). There was no decrease in the variation within any treatment. The mean number of microconidia produced decreased for all treatments except for those that had been lyophilised.

Fig. 17: A. Conidial production by *Fusarium oxysporum* Isolate F2, after 1 year of preservation and B. after 1 year of preservation and a recovery period. Maintained on SNA for 14 days. at –20°C.

A.



B.



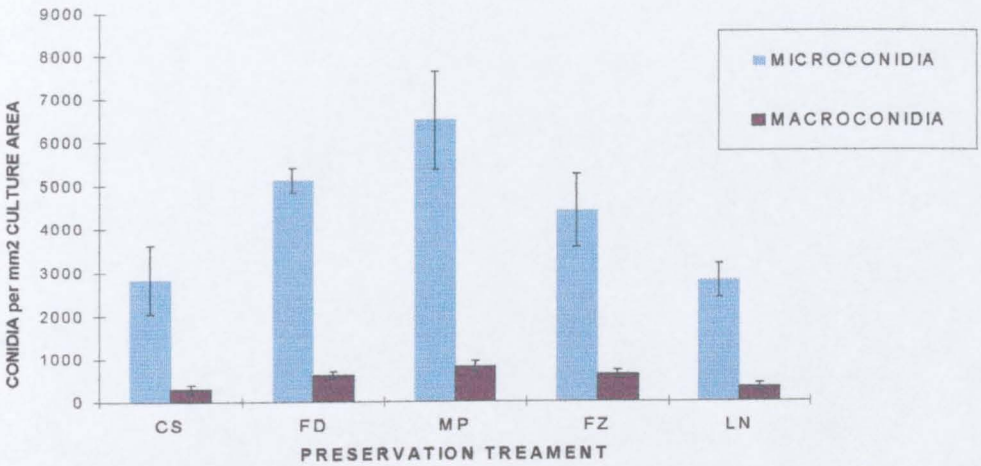
Key: CS, continual sub-culture; FD, lyophilisation; MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation in liquid nitrogen.

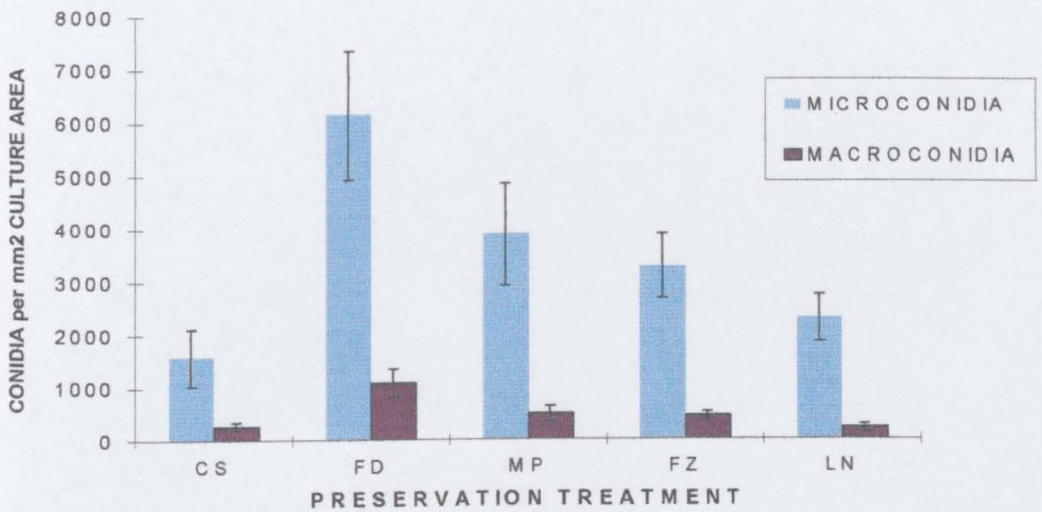
After 2 years of storage there was no significant differences in microconidial production ($P>0.05$, $P=0.0850$) in replicates of isolate F1. However, there was a significant difference in macroconidial ($P<0.05$) production between preservation treatments. The mean number of microconidia produced by replicates stored as mycelial plugs in water (1135 conidia per mm^2 of culture area), was significantly lower ($P<0.05$) than the combined mean number of microconidia produced by the remaining treatments (2601 conidia per mm^2 of culture area). The ratio of microconidia to macroconidia was significantly lower ($P<0.05$) than after 1 year of storage for all treatments. However, the ratio of microconidia to macroconidia (Table 4) produced by replicates stored as mycelial plugs in water was lower (1.8:1) than those of the other treatments (mean 3.8:1). After the recovery period there was no significant difference in macroconidial production ($P>0.05$) between preservation treatments. The mean number of microconidia produced decreased for all treatments. However, there was no recovery in microconidial production between preservation treatments ($P= 0.069$), and the variation within replicates remained.

Table 4: Mean microconidial to macroconidial ratios (to 1) of replicates of *Fusarium oxysporum* isolate F1.

	PRESERVATION TREATMENT				
Testing Time	Continual sub-culture	Lyophilisation	Mycelial plugs in water	Freezing at –20°C	Cryopres-ervation
Year 1	10.1	8.0	6.4	12.7	12.5
Year 1 + recovery period	7.6	14.4	7.9	12.0	37.1
Year 2	2.5	3.0	1.8	4.0	5.7
Year 2 + recovery period	4.0	3.5	1.4	1.6	2.8

Fig. 18: A. Conidial production by *Fusarium oxysporum* Isolate F2, after 2 years of preservation and B. after 2 years of preservation and a recovery period. Maintained on SNA for 14 days at 20°C.





Key: CS, continual sub-culture; FD, lyophilisation; MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation in liquid nitrogen.

3.4 Discussion

3.4.1 Viability

No isolates of *Serpula lacrymans* survived lyophilisation. Non-sporulating fungi are extremely difficult to lyophilise, as the hyphae have high water content and do not have thick walls, which would otherwise provide protection during the lyophilisation process. Without substantial walls, hyphae are susceptible to ice damage (Tan *et al.* 1991) and solution effects (Merryman *et al.* 1977) which may be irreversible. Very few workers have reported successful lyophilisation of mycelial formulations of basidiomycetes (Tan 1991). Possible improvements in methodology are discussed in Chapter 8. Initial viability of replicates stored as mycelial plugs in water was good, although viability decreased quickly over a relatively short period of storage. The high water potentials encountered could be damaging, as *Serpula* is reported to be capable of survival at extremely low water potentials (Jennings 1991). Alternatively, cellular metabolism may not be adequately suppressed, resulting in growth under stressed conditions that may be harmful as the length of time in storage increases. Maintenance by continual sub-culture was the best method for ensuring viability of cultures. However, problems were experienced with this technique. A number of replicates became contaminated with *Penicillium* spp., and even with hyphal tip transfer, the contaminant was very hard to eradicate, therefore strict aseptic technique must be observed when sub-culturing *Serpula.* The initial viability of replicates stored by freezing at -20°C was good. However, viability rapidly decreased with longer storage periods. Doi (1988) reported that *Serpula* could withstand freezing for 48hrs at –5°C. After 16 weeks of preservation, just 2 out of 10 replicates of isolate S3 were viable and after 1 year all *Serpula* isolates were dead. Ice damage, dehydration & uncontrolled cooling rates are factors that could account for poor viability. Viability following cryopreservation in liquid nitrogen was strain-dependent. Replicates of isolate S3 were viable at all testing times, but viability was reduced in isolates S1 and S2 as the investigation progressed. Longer storage periods should not affect viability of cryopreserved specimens (Smith pers.comm). However, deterioration in viability could be accounted for by changes in internal LN refrigerator conditions as a response to disruption of the liquid nitrogen supply or minor alterations in thawing procedure. Different responses to cryopreservation at the species level have been reported previously. Smith and Thomas (1998) found that two strains of *Aspergillus amstelodami* responded differently to cooling. Strain-

specific cooling rates could be needed for different isolates of *Serpula* to reduce the effects of intracellular ice damage and solution effects. Preservation regime did not affect viability of *Fusarium* isolates. All replicates of *Metarhizium* preserved by cryopreservation, lyophilisation and at –20°C were viable throughout the investigation. However, viability of replicates maintained by mycelial plugs in water was not guaranteed. Replicates of 2 of the isolates were viable at all testing times but there was a 40% decrease in viability after one year of storage in replicates of isolate M4. The results indicate that the viability of replicates after storage in water is strain-dependent. The use of mycelial plugs in water to store cultures of *Metarhizium* is not recommended if viability after long storage periods is required. Continual sub-culture was not a good way of ensuring viability in *Metarhizium*.

3.4.2 Culture morphology

The culture morphology of *Metarhizium* replicates deteriorated throughout the investigation. Replicates of isolates that had been cryopreserved tended to produce areas of floccose mycelium. Although mainly from the inoculation site, floccose mycelium could also arise from actively growing regions of the mycelium. Areas of floccose mycelium were generally unable to sporulate, an observation also noted in an isolate *Metarhizium anisopilae* var. *acridum* (formerly identified as *M.flavoviride*) (C. Prior pers.comm.). The possibility that floccose hyphae are produced as a result of high concentrations of conidia in the inoculum can be discounted. Floccose mycelium was also produced in replicates from treatments that had been stored at –20°C and as mycelial plugs in water where the inoculum was different. After a recovery period, floccose mycelium was less likely to be produced. This suggests that its production is characteristic of the initial phase of growth following recovery from preservation. The culture morphology of replicates stored as mycelial plugs in water deteriorated throughout the investigation. These cultures appeared to sporulate poorly with the mycelium turning yellow. This may be indicative of stress. Many of the *Metarhizium* cultures sectored; only replicates that had been stored by continual sub-culture and lyophilised did not sector. Replicates of isolate M4 had an increased tendency to sector, suggesting that sectorisation was strain-specific. Sector morphology differed from the parent culture. Sporulation was reduced or absent and the mycelium was often different from which it arose, with limited floccose hyphae. An investigation into sectorisation is included in Chapter 7.

After 2 years of storage, only the replicates that had been lyophilised produced cultures that all exhibited typical culture morphology. These results suggests that lyophilisation is the most suitable protocol available to preserve the culture morphology of *Metarhizium* isolates. However, the variable cultural stability of *Metarhizium* would suggest that great care should be taken when sub-culturing. Atypical cultural morphology could be inadvertently transferred.

Cultural degeneration in *Fusarium*, from the cottony to the ropey and slimy morphological states has been well documented (Booth 1971, Wing *et al.* 1995). In replicates that had been maintained by continual sub-culture, degeneration occurred after only 16 weeks. After 2 years of storage only 1 of 10 replicates had retained the cottony state. Less degeneration was seen in replicates that were stored at –20°C after 2 years of preservation. Booth (1971) reported that *Fusarium* cultures were extremely labile, and that culture media could stimulate adaptations, which may change morphology and cultural characteristics. To compensate he suggested that preservation methods that induced dormancy, for example, lyophilisation and cryopreservation should be applied. In this investigation, replicates of 2 isolates that had been lyophilised, showed no evidence of culture degeneration at any testing time. However, 3 replicates of an isolate that had been cryopreserved had degenerated to the slimy morphological state after 2 years of preservation. The results suggest that intraspecific variation may account for the different degrees of degeneration in the response of isolates to specific preservation and storage regimes. The process of preservation is the equivalent of a single sub-culture transfer, which suggests that the process of preservation can induce degeneration. Analysis of culture morphology of the continual sub-culture replicates, suggests that isolates withstand more transfers before degeneration is induced. When replicates were inoculated onto a maintenance media on resuscitation and then subbed onto PSA after a recovery period, cultures were less likely to exhibit degeneration. However, some cultures were likely to degenerate further. Once a culture had degenerated to a slimy cultural state it was unlikely to revert to any other cultural state. Wing *et al.* (1995) working with *Fusarium compactum* and *Fusarium acuminatum* also reported that cultures were unlikely to recover once they had degenerated. Pigmentation was not a suitable criterion for assessing culture characteristics. Even before preservation pigmentation was variable and remained so throughout the investigation. Culture margins were

generally smooth and regular, faster growing replicates tended to have less regular culture margins which were often associated with less dense growth. Despite the labile nature of *Fusarium*, sectorisation was rare. After 2 years, only 2 out of 25 replicates sectored. Sector morphology was generally similar to the parent colony, except in a replicate where the sector exhibited degenerative culture morphology. Although strain-specific, the choice of preservation method that would best conserve culture morphology in *Fusarium* would appear to be lyophilisation.

3.4.3 Radial growth

In isolates of *Fusarium* and *Metarhizium*, radial growth was affected after just one day and one week of storage. However, after the recovery period there was no significant difference between preservation treatments, which illustrates that radial growth can return to pre-preservation rates after an extended recovery time. *Metarhizium* isolates showed a gradual increase in differences in radial growth rates between preservation treatments as the investigation progressed. There was no recovery in the difference in radial growth rate between preservation treatments in replicates of *Metarhizium* isolate M4 after one year of storage and a recovery period and in replicates of isolates M1 and M2 after two years of preservation and a recovery period. The results suggest that with increasing storage time, radial growth will become increasingly changed from that exhibited by the original isolate. Some isolates appear more resistant to the stresses encountered during preservation and storage suggesting a strain-specific response. For example, compared to replicates of isolates M1 and M2, growth rates were changed more by preservation in replicates of isolate M4 after one year of storage. The variance between replicates within treatments increased over time, especially in replicates that were stored by mycelial plugs in water. The differences are possibly as a result of poor suppression of dormancy during storage in water.

Isolates of *Fusarium* showed increased the differences in radial growth rate between replicates from the different preservation treatments over the two year testing period. However, there was no significant difference between preservation treatments after the recovery period following two years of storage, which suggests that although preservation regime can induce changes in radial growth rates on resuscitation, there is no long-term effect on radial growth. Variation within

replicates from the different preservation regimes did not increase over the testing period, and any variation decreased after the recovery period. Growth rates of replicates stored as mycelial plugs in water or continual sub-culture were generally faster than the rates of any other preservation treatments throughout the investigation. This could be because the level of dormancy attained during storage was less than that of other treatments; subsequently recovery and growth could be quicker on resuscitation from storage. There was also no evidence of strain-specific response between isolates of *Fusarium*. Replicates of *Fusarium* and *Metarhizium* that had been lyophilised had a one to two day delay in the onset of growth throughout the investigation. The delay could be due to the time taken by spores to rehydrate and then regain turgor pressure and biochemical stability after resuscitation.

3.4.4 Conidial density

Conidial production was affected by preservation regime in isolates of *Metarhizium* and *Fusarium*. The differences in the number of conidia produced after preservation and storage was strain-specific. For example, after 2 years of preservation, the mean number of conidia produced by replicates of isolate F1 stored as mycelial plugs in water was less than the number produced by any other treatment. However, the mean number of conidia produced by replicates of isolate F2 stored by mycelial plugs in water was greater than the number produced by any other treatment. In isolates of *Metarhizium*, preservation initially promoted the production of more conidia. Many fungi react to conditions that do not promote optimal vegetative growth by producing conidia. For example, taxonomists use nutrient agars that have low carbohydrate sources to promote sporulation. On resuscitation, the recovering fungus may be "shocked" into a strategy that encourages conidial production after the stresses encountered during preservation and storage. In isolates of *Fusarium*, preservation initially reduced conidial production. A hypothesis to explain reduced sporulation following preservation is that poor growth and development may inhibit the pathways associated with conidial production. Adams (1995) concluded that conidial development is the result of an intricate series of tightly regulated events and that before sporulation, cells must undergo a defined period of growth and be developmentally competent. Many genes are implicated in the process of sporulation (Timberlake 1980). If the genetic and biochemical pathways are not activated, as a result of poor growth or physical disruption then

sporulation may be reduced or inhibited. The preservation protocols could also induce mutants that may be deficient in their capability to sporulate. This is discussed in later chapters. In replicates of *Fusarium* and *Metarhizium* the variance between replicates within preservation treatments increased with time, most notably in replicates stored as mycelial plugs in water but also in replicates that had been maintained by continual sub-culture and stored at –20°C. Longer storage times, appear to induce instability and affect the ability of these replicates to produce conidia. The variance between replicates that had been lyophilised and cryopreserved, although initially larger than that produced by other treatments remained relatively constant throughout the investigation. The initial difference between replicates may be due to the processes involved during preservation and would suggest that the length of storage had no further effect on conidial production. In isolates of *Fusarium oxysporum*, the ratio of microconidial to macroconidial production was initially high (31:1 for unpreserved replicates of isolate F3) but as the investigation progressed the ratio decreased. For example, the ratio for replicates of isolate F1 stored as mycelial plugs in water for one year was 7.9:1, but after two years, this had decreased to 1.4:1. Microconidia are normally produced rather than macroconidia. However, as the number of microconidia produced declined, the number of macroconidia produced remained constant, hence the ratio also declined. In isolates of *Metarhizium*, the differences in mean conidial production between preservation treatments was not significant after the 28-day recovery period. The result suggests that conidial production can recover post-preservation. However, there was still considerable variation between replicates within treatments. In two isolates of *Fusarium* (F1 and F2) there was no significant recovery after the recovery period after one and two years of preservation. Hence, changes in conidial production induced by preservation regime may be irreversible. However, there was recovery between treatments of isolate F3 after 16-weeks of preservation.

3.4.5 Suitability of methods.

The methods employed were suitable for the purpose of assessing the culture characteristics of a fungus. However, some methods could be improved. Pigmentation was variable throughout the investigation and difficult to record. The use of computerised image analysis could provide an accurate measure of the hue and intensity of pigmentation. Image analysis could also be used to assess the

density, rate and pattern of mycelial growth (Donnelly *et al.* 1999). Radial growth was an adequate method for assessing the state of a fungus after resuscitation from preservation but is not necessarily indicative of the health of the fungus as no quantification of the density of growth was taken. Measurement of extension of individual hyphae could also be undertaken and the method improved by more frequent measurements. The results obtained from counts of conidial density provided some good data and the method was reproducible. An assessment of viable conidia, of the inoculum following cryopreservation or lyophilisation and the conidia produced post-preservation could provide a more accurate assessment of the state of a fungus. Sporulation could also be assessed at more regular time intervals following inoculation.

3.4.6 Summary

Culture characteristics are indicative of strain stability following preservation and storage. Response to preservation regime is strain-specific. Culture characteristics are critical factors that are used for taxonomic and teaching purposes and in the registration of patents and environmental agents. Therefore, as any preservation regime can induce changes in the culture characteristics of an isolate, mycologists should preserve type strains, and isolates for which the stability of cultural characteristics is essential, by a variety of preservation regimes. The results from this investigation suggest that lyophilisation was the most suitable protocol for the test fungi. For *Serpula lacrymans*, continual sub-culture was the best protocol. As a quality control measure, the culture characteristics of a fungus should be determined on defined media under a set of controlled parameters, before preservation and at least 28 days following preservation.

CHAPTER 4: THE EFFECT OF PRESERVATION AND STORAGE ON SECONDARY METABOLITE PRODUCTION

4.1 Introduction

Secondary metabolites are compounds that are principally produced after the primary phase of growth. Hence, secondary metabolites are not involved in primary fungal metabolism but are products of it. Secondary metabolism is not essential for, and plays no part in growth and occurs maximally under conditions of restricted growth (Singleton and Sainsbury 1993). Fungi produce a diverse range of secondary metabolites (Garraway and Evans 1991) and types of secondary metabolite produced are usually distinct for specific groups of fungi (Martin and Demain 1978). Some workers have used metabolite profiles to classify fungi. For example, Svendsen and Frisvad (1994) demonstrated that chemosystematics of secondary metabolite profiles could be applied to separate 279 terverticillate *Penicillium* spp. using cluster analysis. Similarly, Larsen and Frisvad (1995) used volatile metabolite analysis to chemosystematically separate 132 terverticillate *Penicillium* spp. to the species level. Many fungal secondary metabolites are biologically active and some are used commercially in the production of antibiotic drugs (e.g. griseofulvin from *Penicillium griseofulvum*), other pharmacologically active compounds (e.g. cyclosporin from *Tolypocladium inflatum*), compounds for food and industry and as dyes (e.g. orchil from *Roccella* spp.). However, some secondary metabolites are harmful to living organisms (mycotoxins). These include the carcinogenic aflatoxins produced by *Aspergillus* spp. and patulin produced by *Penicillium expansum.* Secondary metabolites are synthesised from a variety of precursors and complex biochemical pathways derived from primary metabolism. AcetylCoA is an important precursor (Garraway and Evans 1991) but others precursors include amides, amino acids and additional metabolic intermediates. A wide variety of metabolites (e.g. carotenoids, sterols) belong to the terpene group of compounds, which are synthesised via the mevalonic acid pathway. Other metabolites are derived from the polyketide pathway (e.g. cytochalasins, penicillic acid), the shikimate-chorosimate pathway (e.g. aromatic compounds) and those derived from non-aromatic amino acids (e.g. cephalosporins). In the industrial manufacture of fungal secondary metabolite products, it is important for isolates to retain metabolic stability during the fermentation stages. However, fungi can cease to produce specific products (B.Lane pers.comm.), so it is vital that replicates of each fungus are successfully preserved and stored in culture collections as a back-up in case of difficulty with the

manufacturing strains. Metabolic stability is also important for isolates used in chemotaxonomy, biological control and in bioassays. Svendsen and Frisvad (1994) found that two strains of *Penicillium camembertii* that were reported by Bridge *et al.* (1989) to produce citrinin, did not produce citrinin in their investigation. Strain degradation may be correlated with changes in secondary metabolite production (Bu'lock *et al.* 1986, Abraham *et al.* 1941, Kale *et al.* 1994,).

Chromatographic methods are commonly used to separate and identify secondary metabolites. Thin layer chromatography (TLC) is a relatively cheap and simple method. The agar plug TLC technique, developed by Paterson (1996) is suitable for fungi grown on agar and can be used to separate intracellular and extracellular secondary metabolites. Metabolites can be characterised and identified by a visual/UV identification system using Rf values that are compared to data obtained from previously analysed standards. Gradient High Performance Liquid Chromatography (HPLC) is a highly automated system that allows improved detection and separation of metabolites (Paterson and Kemmelmeier 1989). Detection of metabolites is achieved using UV wavelength or Diode Array Detection. Alkylphenones can be used to standardise HPLC systems and to calculate retention indices (Paterson and Kemmelmeier 1990). Known metabolite standards can be used to identify metabolites. However, to confirm the identity of metabolites or unknowns mass spectrometry can be used. The fungi used in this project are known to produce a range of secondary metabolites. In common with many other mitosporic fungi, *Metarhizium* synthesises a vast array of secondary metabolites. Depsipeptide entomotoxins (destruxins) are thought to cause pathogenesis in the insect host. Destruxins, which are natural insecticides, can be identified on TLC using a variety of different solvent systems (Tamura and Takahashi 1991). However, destruxins A, B, C & D can only be separated by HPLC as they have the same Rf value on TLC solvent systems. *Fusarium oxysporum* produces a large and diverse range of secondary metabolites. Knowledge of the biosynthesis of these metabolites has provided an insight into their economic importance both as mycotoxins, antibiotics or other useful metabolites (Moss 1984). Fermentation studies have established optimal growth conditions for *Fusarium* spp. in liquid culture, which allows specific metabolites to be produced commercially (Anderson and Solomons 1984). Many isolates of *F.oxysporum* produce the polyketide, bikaverin (Bu`lock

1984), the secondary metabolite that gives the deep purple colour that is characteristic of most *Fusarium* isolates in plate culture. *Fusarium oxysporum* isolates can also produce zearalenone (another polyketide), a mycotoxin that may have a role as an anabolic agent, depsipeptides (nitrogen compounds) and helvolic acid (a terpanoid) (Bu'lock 1984). Other secondary metabolites synthesised by *Fusarium* are phytotoxic, some of which are reported to be host-specific e.g. phytonivein (Hiroe & Nishmura 1956). Unlike some other *Fusarium* species, *F.oxysporum* does not produce gibberellins.

The aim of this chapter was to assess the effects of preservation and storage on the stability of secondary metabolite production in *Metarhizium anisopliae* and *Fusarium oxysporum*.

4.2 Material and methods

Materials and methods are described in section 2.4.4

4.3 Results

4.3.1 *Metarhizium anisopliae*

4.3.1.1 Thin layer chromatography

Metarhizium anisopliae isolate M1

Both intra- and extracellular secondary metabolite profiles of replicates of isolate M1 were changed by preservation and storage. Four extracellular secondary metabolites (Table 1) and 6 intracellular secondary metabolites were detected (Table 5) that were representative of the typical secondary metabolite profile of isolate M1.

Table 1: Extracellular secondary metabolite profile of *Metarhizium anisopliae* isolate M1

Metabolite	Properties	Mean $Rf_{(x100)}$ +/- S.E.
M1 A	Y L/UV	9.7 +/- 0.2
M1 B	W S/UV	15.1 +/- 0.2
M1 C	Y/O L/UV	24.7 +/- 0.3
MI D	Y L/UV	34.9 +/- 0.6

W= white, Y= yellow, O= orange, L/UV= long-wave ultraviolet light, S/UV= Short-wave ultraviolet light.

After 1 year of preservation the characteristic extracellular secondary metabolite profile of isolate M1 was only exhibited in 6 replicates. A single metabolite was not detected in the profiles of the remaining 10 replicates, 9 of which all behaved similarly in that single metabolite (M1B) that appeared in the original profile was not detected (Table 2). After the recovery period (Table 3), only 3 replicates that had been stored as mycelial plugs in water did not exhibit an extracellular secondary metabolite profile typical of the original isolate. The recovery is illustrated in Fig. 1.

Table 2: Summary of replicates of *Metarhizium anisopliae* isolate M1 producing extracellular secondary metabolite profiles, different from the original isolate. After 1 year of storage. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at –20°C, LN= Cryopreservation)

Metabolite	*	CS1	CS2	CS3	CS5	FD1	MP2	MP3	MP4	MP5	LN2
M1 A	●	●	●	●	●	●	●	●	●	●	●
M1 B	●	X	X	X	X	X	X	X	X	X	●
M1 C	●	●	●	●	●	●	●	●	●	●	X
MI D	●	●	●	●	●	●	●	●	●	●	●

*Original Profile (see Table 1 for properties). ● Metabolite detected, X metabolite not detected

Replicates CS4, FD2, FD3, MP1, LN 1, LN3 produced extracellular secondary metabolite profiles characteristic of the original isolate (Table 1)

Table 3. Summary of replicates of *Metarhizium anisopliae* isolate M1 producing extracellular secondary metabolite profiles, different from the original isolate. After 1 year of storage and a recovery period (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Metabolite	*	MP 2	MP 4	MP 5
M1 A	●	●	●	●
M1 B	●	X	X	X
M1 C	●	X	●	●
MI D	●	●	●	●

* Original Profile (see Table 1 for properties).

● Metabolite detected, X metabolite not detected

Replicates CS 1,2,3,4,5; FD 1,2,3; MP1, 3, LN 1,2,3 produced extracellular secondary metabolite profiles characteristic of the original isolate (Table 1).

After 2 years of storage, 3 out of the 11 replicates analysed did not exhibit the characteristic extracellular secondary metabolite profile of isolate M1. Two replicates stored as mycelial plugs in water and a replicate that had been lyophilised each lost a single metabolite that had appeared in the original profile. After the recovery period only a single replicate that had been lyophilised failed to exhibit the extracellular secondary metabolite profile typical of the original isolate.

Table 4. Summary of replicates of *Metarhizium anisopliae* isolate M1 producing extracellular secondary metabolite profiles, different from the original isolate. After 2 year of storage (a) and a recovery period (b). (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

		--------(a)----------			---(b)---
Metabolite	*	FD4	MP 3	MP5	FD4
M1 A	●	●	●	●	●
M1 B	●	●	●	●	●
M1 C	●	X	X	X	X
MI D	●	●	●	●	●

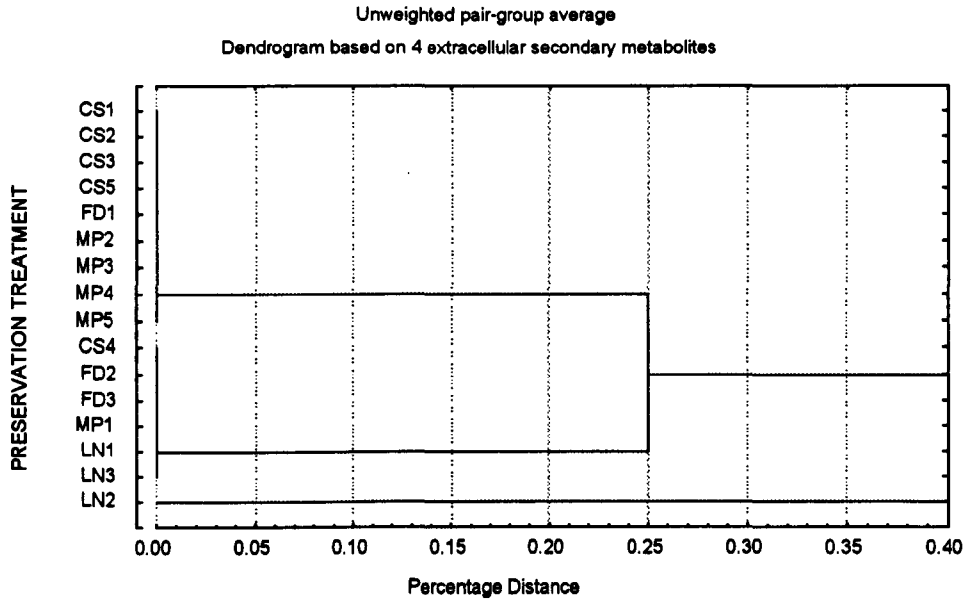
* Original Profile (see Table 1 for properties).

● Metabolite detected, X metabolite not detected

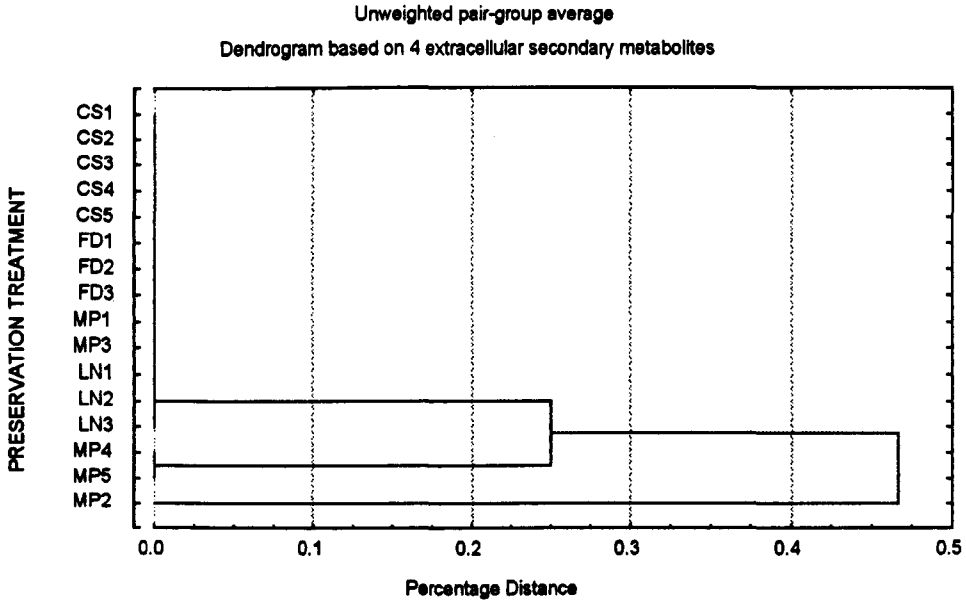
(a) Replicates FD 1,2,3,5; MP1, 3, LN 2,4,5 produced extracellular secondary metabolite profiles characteristic of the original isolate (Table 1). (b) All replicates (except FD4) produced extracellular secondary metabolite profiles characteristic of the original isolate after the recovery period.

Fig. 1: A. Dendrogram produced from extracellular secondary metabolite profile of *Metarhizium anisopliae* isolate M1 after 1 year of storage and B. after 1 year of storage and a recovery period.

A.



B.



Key: CS, continual sub-culture; FD, lyophilised; MP, mycelial plugs in water; LN, cryopreserved.

After 1 year of storage, only replicates that had been cryopreserved exhibited an intracellular secondary metabolite profile characteristic typical of the original isolate (Table 5). Up to 4 intracellular secondary metabolites that appeared in the original profile were not detected in the profiles of the remaining replicates (Table 7), which grouped according to preservation method on the dendrogram (Fig 2A).

Table 5: Intracellular secondary metabolite profile of *Metarhizium anisopliae* isolate M1

Metabolite	Properties	Mean Rf (x100) +/- S.E.
M1 E	Y L/UV	9.3 +/-0.5
M1 F	W S/UV	14.9 +/-0.9
M1 G	Y VIS / BR L/UV	42.6 +/-0.2
M1 H	Y/W L/UV	49.5 +/-0.6
M1 I	Y VIS / BR L/UV	73.2 +/-0.6
MI J	Y/W L/UV	87.9 +/-0.4

W= white, Y= yellow, BR= brown, L/UV= long-wave ultraviolet light, S/UV= Short-wave ultraviolet light, VIS= visible under white light.

Metabolite M, a "streaked" intracellular secondary metabolite (brown, long wave UV) detected in the profile of the original isolate was detected in replicates throughout the investigation (Table 6). Metabolite M was not detected in the intracellular metabolite profiles of a number of replicates, but was regained in many replicates after a recovery period.

Table 6: Detection of "metabolite M" in *Metarhizium anisopliae* isolate M1

	PRESERVATION TREATMENT (replicate number)															
TT	CS 1	CS 2	CS 3	CS 4	CS 5	FD 1	FD 2	FD 5	MP 1	MP 2	MP 3	MP 4	MP 5	LN 1	LN 2	LN 3
Y1	+	+	+	-	+	+	-	+	-	-	-	-	-	+	+	+
Y1r	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
				FD 1	FD 2	FD 3	FD 4	FD 5	MP 1	MP 2	MP 3	LN 1	LN 2	LN 3		
Y2				+	-	+	-	+	-	-	-	+	+	+		
Y2r				+	-	+	-	+	+	+	+	+	+	+		

Key: TT, testing time; Y, year; r, recovery period; +, metabolite M detected; -, metabolite M not detected; CS, continual sub-culture; FD, lyophilised; MP, mycelial plugs in water; LN, cryopreserved.

After the recovery period (Fig 2B), all 6 intracellular secondary metabolites were detected in 7 out 16 replicates (44%). However, only 1 of the replicates that had been maintained by continual sub-culture and 1 of the replicates stored as a mycelial plugs in water recovered to exhibit an intracellular secondary metabolite profile typical of the original isolate.

Table 7: Summary of replicates of *Metarhizium anisopliae* isolate M1 producing intracellular secondary metabolite profiles, different from the original isolate. **A.** After 1 year of storage and **B.** After 1 year of storage and a recovery period. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

A.

Metabolite	*	CS 1	CS 2	CS 3	CS 4	CS 5	FD 1	FD 2	FD 3	MP 1	MP 2	MP 3	MP 4	MP 5
M1 E	●	●	●	●	●	●	●	X	●	●	●	●	●	●
M1 F	●	X	●	X	X	X	●	●	●	X	X	X	X	●
M1 G	●	X	X	X	X	X	X	X	X	X	X	X	X	X
M1 H	●	X	X	X	X	X	X	X	X	●	X	X	X	X
M1 I	●	●	●	●	X	●	●	X	●	X	X	X	X	X
MI J	●	●	●	●	●	●	●	●	●	●	●	●	●	●

*Original Profile (see Table 5 for properties).
● Metabolite detected, X metabolite not detected

Replicates LN 1,2,3 produced intracellular secondary metabolite profiles characteristic of the original isolate (Table 5).

B.

Metabolite	*	CS 1	CS 2	CS 3	CS 4	FD 2	MP 2	MP 3	MP 4	MP 5
M1 E	●	X	●	●	●	●	●	●	●	●
M1 F	●	X	X	X	X	●	X	X	X	●
M1 G	●	●	●	●	●	●	●	●	●	X
M1 H	●	●	●	●	●	●	●	●	●	●
M1 I	●	●	●	●	X	X	X	X	X	X
MI J	●	●	●	●	●	●	●	●	●	●

* Original Profile (see Table 5 for properties).
● Metabolite detected, X metabolite not detected

Replicates CS 5; FD1,3; MP1; LN 1,2,3 produced intracellular secondary metabolite profiles characteristic of the original isolate (Table 5).

After 2 years of storage (Table 8), all 6 intracellular metabolites were only detected in a single replicate that had been stored as a mycelial plug in water. Up to 3 metabolites that appeared in the original profile were not detected in the replicates that did not exhibit the original intracellular secondary metabolite profile. After the recovery period, only 6 out of 11 replicates exhibited the characteristic profile of isolate M1 (Table 8).

Table 8 Summary of replicates of *Metarhizium anisopliae* isolate M1 producing intracellular secondary metabolite profiles, different from the original isolate. **A.** After 2 years of storage and **B.** After 2 years of storage and a recovery period. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

A.

Metabolite	*	FD 1	FD 2	FD 3	FD 4	FD 5	MP 3	MP 4	LN 2	LN 4	LN 5
M1 E	●	●	●	X	●	X	●	●	X	X	X
M1 F	●	X	X	●	●	●	●	●	●	●	●
M1 G	●	●	X	●	X	●	●	X	X	●	●
M1 H	●	●	●	●	●	●	●	●	●	●	●
M1 I	●	X	●	X	●	X	X	X	X	X	X
MI J	●	●	●	●	●	●	●	●	●	●	●

* Original Profile (see Table 5 for properties).
● Metabolite detected, X metabolite not detected

Replicate MP 1 produced an intracellular secondary metabolite profiles characteristic of the original isolate (Table 5).

B.

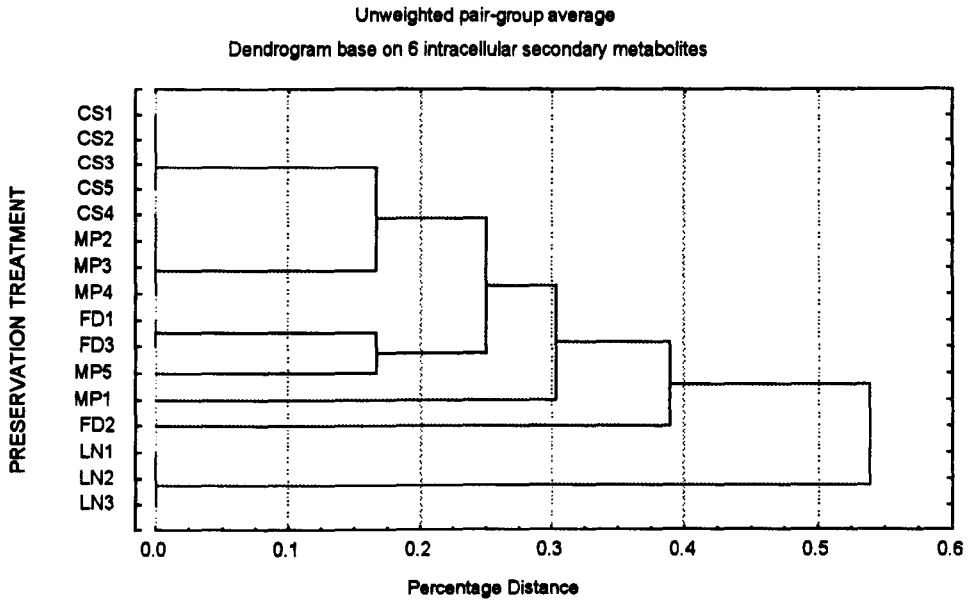
Metabolite	*	FD 2	FD 4	LN 2	LN 4	LN 5
M1 E	●	1	1	1	0	1
M1 F	●	1	1	1	1	1
M1 G	●	0	0	1	1	1
M1 H	●	1	1	1	1	1
M1 I	●	1	1	0	0	0
MI J	●	1	1	1	1	1

* Original Profile (see Table 5 for properties).
● Metabolite detected, X metabolite not detected

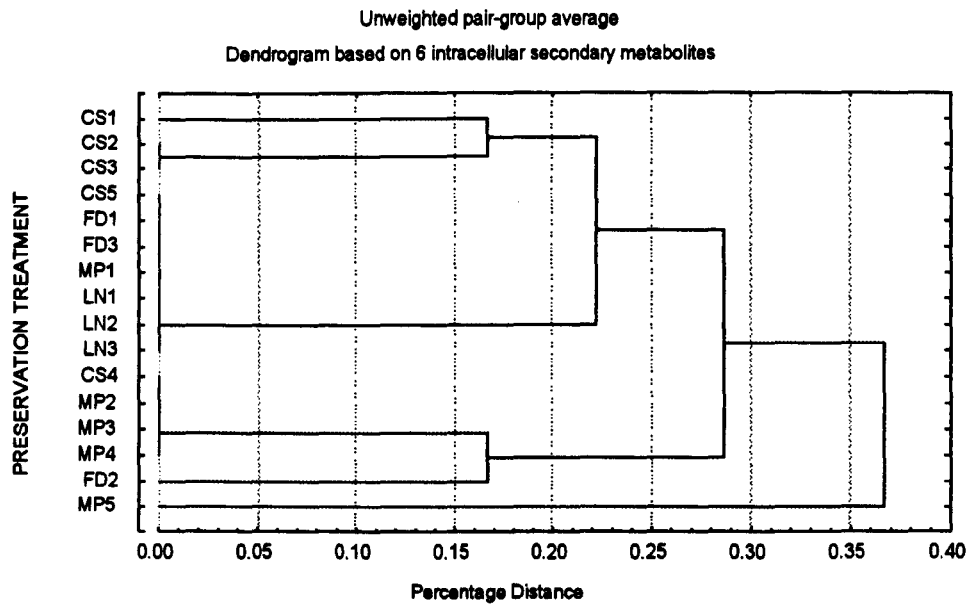
Replicates MP 1,3,4; FD 1,3,5; produced an intracellular secondary metabolite profiles characteristic of the original isolate (Table 5)

Fig. 2A: Dendrogram produced from intracellular secondary metabolite profile of *Metarhizium anisopliae* isolate M1 after 1 year of storage and B. after 1 year of storage and a recovery period.

A.



B.



Key: CS, continual sub-culture; FD, lyophilised; MP, mycelial plugs in water; LN, cryopreserved.

Metarhizium anisopliae isolate M2

Preservation and storage changed both intra- and extracellular secondary metabolite profiles of replicates of isolate M2. Five extracellular secondary metabolites (Table 11) and 7 intracellular secondary metabolites were detected (Table X) that were representative of the secondary metabolite profile of isolate M2.

Table 11: Extracellular secondary metabolite profile of *Metarhizium anisopliae* isolate M2

Metabolite	Properties	Mean Rf (x100) +/- S.E.
M2 A	W/Y L/UV	8.4 +/- 0.1
M2 B	Y L/UV	16.8 +/- 0.3
M2 C	Y L/UV	23.5 +/- 0.2
M2 D	O/Y L/UV	33.1 +/- 0.2
M2 E	W/Y S/UV	43.3 +/- 0.3

W= white, Y= yellow, L/UV= long-wave ultraviolet light, S/UV= Short-wave ultraviolet light,

Table 12: Summary of replicates of *Metarhizium anisopliae* isolate M2 producing extracellular secondary metabolite profiles, different from the original isolate. After 1 year of storage. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Metabolite	*	CS 1	CS 2	CS 3	CS 4	CS 5	MP 5	FZ 1	LN 3
M2 A	●	●	●	●	●	●	●	●	●
M2 B	●	●	●	●	●	●	●	●	X
M2 C	●	X	X	X	X	X	X	●	●
M2 D	●	●	●	●	●	●	●	X	X
M2 E	●	●	●	●	●	●	●	●	●

* Original Profile (see Table 11 for properties).
● Metabolite detected, X metabolite not detected

Replicates MP 1,2,3,4; FD 1,2,5; FZ 2,3; LN 1,5 produced an extracellular secondary metabolite profiles characteristic of the original isolate (Table 11).

After 1 year of storage, 11 out of 19 replicates (58%) exhibited the typical extracellular secondary metabolite profile that was characteristic of isolate M2 (Fig 3A). Profiles were changed by preservation in all 5 replicates that had been

maintained by continual sub-culture, in one replicate stored as a mycelial plug in water, in a single replicate stored at –20°C and in a single replicate that had been cryopreserved (Table 12). After a recovery period (Fig 3B), only a single replicate that had been stored as a mycelial plug in water, failed to exhibit the characteristic extracellular secondary metabolite profile of isolate M2. Extracellular secondary metabolites M2 C, M2 D, M2E were not detected in this replicate. After 2 years of storage, all of the replicates stored lyophilised, cryopreserved and at –20°C exhibited similar extracellular secondary metabolite profiles characteristic of isolate M2 (Table 11). A single metabolite that appeared in the original profile (M2 B) was not detected in 2 replicates that had been stored as mycelial plugs in water. The other 3 replicates that had been stored as mycelial plugs in water exhibited the extracellular metabolite profile characteristic to isolate M2. After the recovery period (Fig 3B), 15 out of 19 replicates exhibited the extracellular secondary metabolite profile characteristic of isolate M2. Only 1 of the replicates that had been stored as a mycelial plug in water recovered to exhibit the extracellular secondary metabolite profile characteristic to isolate M2. Three replicates that had initially exhibited the typical extracellular profile, did not after the recovery period

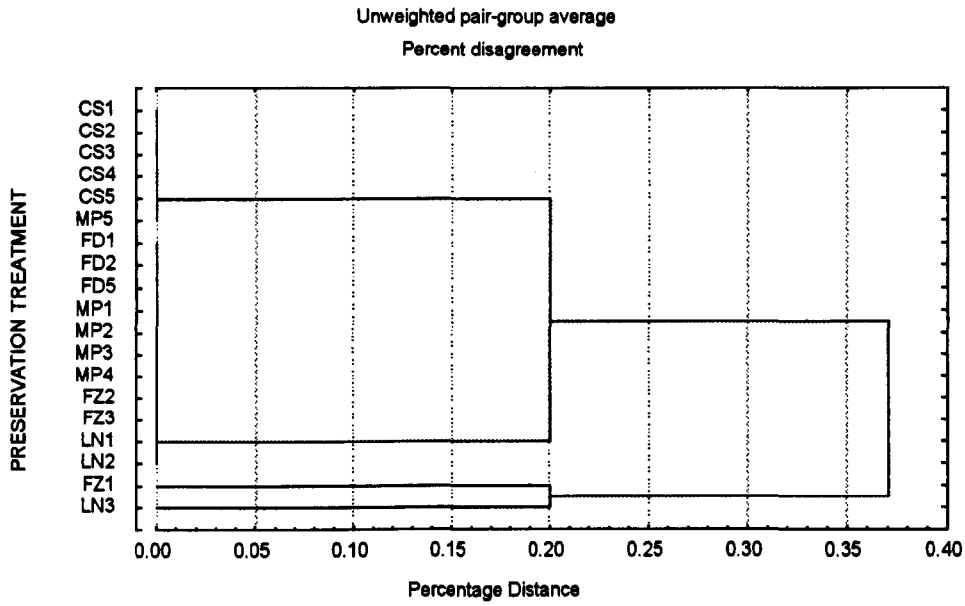
Table 13: Summary of replicates of *Metarhizium anisopliae* isolate M2 producing extracellular secondary metabolite profiles, different from the original isolate. After 2 years of storage and a recovery period. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at –20°C, LN= Cryopreservation)

Metabolite	*	FD 1	FD 2	MP 2	MP 4
M2 A	●	●	●	●	●
M2 B	●	●	●	X	X
M2 C	●	●	●	●	●
M2 D	●	●	●	●	●
M2 E	●	X	X	●	●

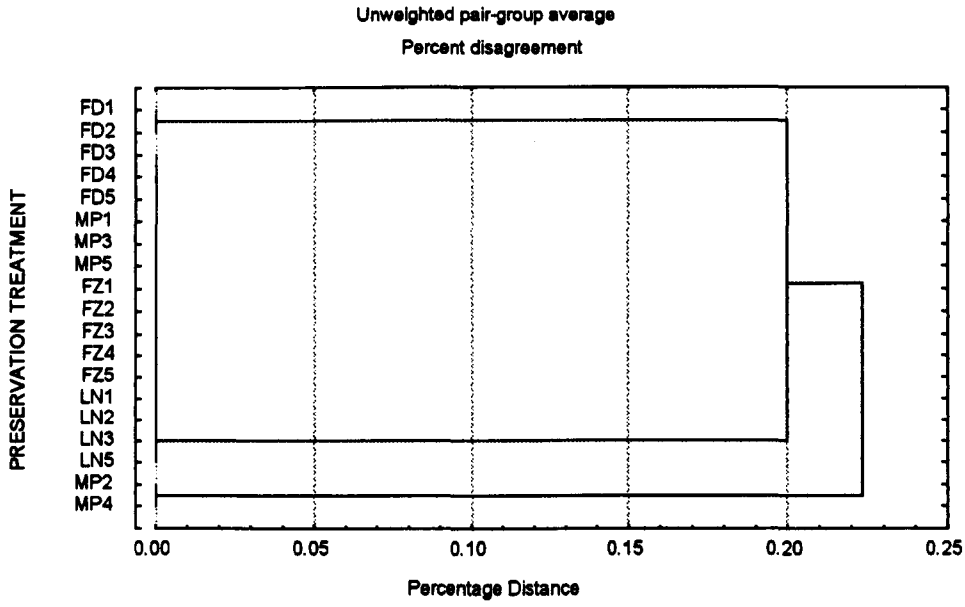
* Original Profile (see Table 11 for properties).
● Metabolite detected, X metabolite not detected

Replicates MP 1,2,3,4; FD 1,2,5; FZ 2,3; LN 1,5 produced an extracellular secondary metabolite profiles characteristic of the original isolate (Table 11).

Fig. 3: A. Dendrogram produced from extracellular secondary metabolite profile of *Metarhizium anisopliae* isolate M2 after 1 year of storage. B. after 2 years of storage and a recovery period.



B.



Key: CS, continual sub-culture; FD, lyophilised; MP, mycelial plugs in water; LN, cryopreserved.

After 1 year of storage (Table 15), 13 out of 18 replicates (72%) exhibited the intracellular secondary metabolite profile of 7 metabolites characteristic of the original isolate (Fig 4A).

Table 14: Intracellular secondary metabolite profile of *Metarhizium anisopliae* isolate M2

Metabolite	Properties	Mean $Rf_{(x100)}$ +/- S.E.
M2 F	Y/W L/UV	6.44 +/- 0.26
M2 G	Y VIS BR L/UV	36.76 +/- 0.31
M2 H	Y VIS BR L/UV	45.05 +/- 0.42
M2 I	Y/W S/UV	65.35 +/- 0.55
M2 J	O L/UV	79.65 +/- 1.28
M2 K	BR L/UV	89.32 +/- 0.40
M2 L	T L/UV	98.59 +/- 0.08

W= white, Y= yellow, O= orange, T= turquoise, BR= brown, L/UV= long wave ultraviolet, S/UV= short wave ultraviolet, VIS = Visible under white light

Table 15: Summary of replicates of *Metarhizium anisopliae* isolate M2 producing intracellular secondary metabolite profiles, different from the original isolate. After 1 years of storage. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Metabolite	*	CS 2	CS 2	CS 3	CS 4	MP 4
M2 F	●	●	●	X	●	●
M2 G	●	X	X	●	X	●
M2 H	●	X	●	●	●	●
M2 I	●	●	●	●	●	●
M2 J	●	●	●	●	●	●
M2 K	●	●	●	●	●	X
M2 L	●	●	●	●	●	●

* Original Profile (see Table 14 for properties).
● Metabolite detected, X metabolite not detected

Replicates CS 1; MP 1,2,3; FD 1,2,5; FZ 1,2,3; LN 1,2,5 produced an intracellular secondary metabolite profiles characteristic of the original isolate (Table 15).

"Metabolite M" was detected in all of the replicates that had been cryopreserved, 2 out of the 3 replicates that had been lyophilised, 2 out of the 3 replicates stored at –20°C, 1 out of the 5 replicates as mycelial plugs in water and 1 out of the 5 replicates maintained by continual sub-culture. After the recovery period (Fig 4B), 3 replicates did not exhibit the secondary metabolite profile that was typical of the original isolate. Replicate FD1, which had initially produced an intracellular secondary metabolite profile typical of the original isolate did not produce metabolites M2 F and M2 G, M2 I. Replicates MP4 and MP5 did not produce metabolite M2 H. "Metabolite M" was detected in all replicates, except a single replicate that was stored as a mycelial plug in water. After 2 years of storage, 9 out of 19 replicates (47.4%) exhibited the intracellular secondary metabolite profile characteristic of isolate M2 (Fig 4C). "Metabolite M" was detected in all of the replicates that exhibited the typical profile which included all of those that had been cryopreserved, 2 out of the 5 replicates that had been lyophilised, 2 out of the 5 replicates stored at –20°C and 2 out of the 5 replicates stored as mycelial plugs in water. After the recovery period, all replicates exhibited an intracellular secondary metabolite profile typical of the original isolate.

Table 16: Summary of replicates of *Metarhizium anisopliae* isolate M2 producing intracellular secondary metabolite profiles, different from the original isolate. After 2 years of storage. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at –20°C, LN= Cryopreservation)

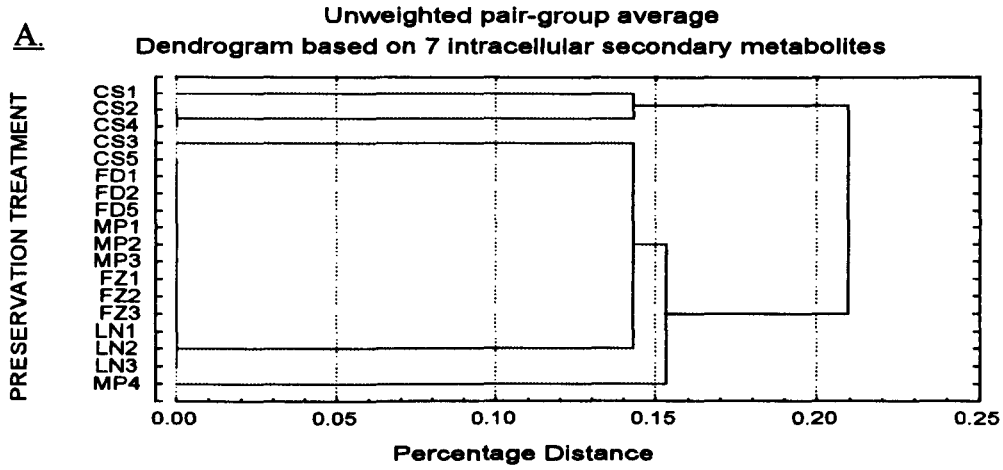
Metabolite	*	FD 1	FD 2	FD 3	FD 5	MP 2	MP 3	MP 4	FZ 1	FZ 2	FZ 5
M2 F	●	●	●	●	●	●	●	●	●	●	●
M2 G	●	●	●	●	●	●	●	●	●	●	●
M2 H	●	●	●	●	●	●	●	●	●	●	●
M2 I	●	●	●	●	●	●	●	●	●	●	●
M2 J	●	●	●	●	●	●	●	●	●	●	●
M2 K	●	●	●	●	●	●	●	●	●	●	●
M2 L	●	X	X	X	X	X	X	X	X	X	X

* Original Profile (see Table 14 for properties).
● Metabolite detected, X metabolite not detected

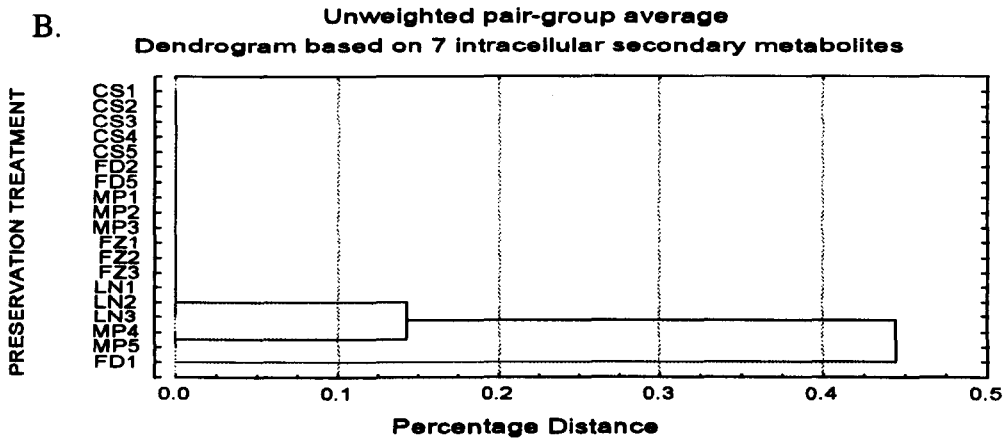
Replicates MP 1,5; FD 4; FZ 3,4; LN 1,2,3,4,5 produced an intracellular secondary metabolite profiles characteristic of the original isolate (Table 15).

Fig. 4: A. Dendrogram produced from intracellular secondary metabolite profile of *Metarhizium anisopliae* isolate M2 after 1 year of storage. B. after 1 year and a recovery period and C. after 2 years of storage.

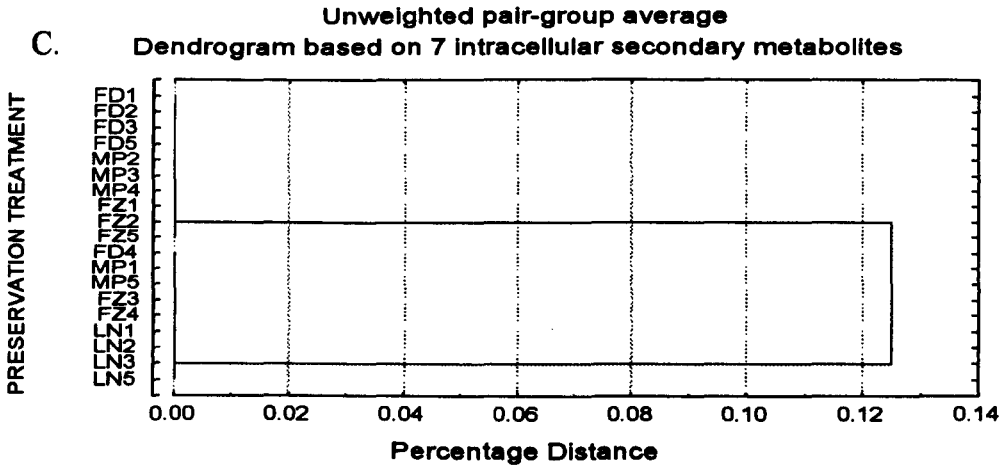
A.



B.



C.



Key: CS, continual sub-culture; FD, lyophilised; MP, mycelial plugs in water; FZ, frozen at -20°C; LN, cryopreserved

Metarhizium spp. isolate M4

Both intra- and extracellular secondary metabolite profiles of replicates of isolate M4 were changed by preservation and storage. Eight extracellular secondary metabolites (Table 17) and 9 intracellular secondary metabolites were detected (Table 22) that were representative of the typical secondary metabolite profile of isolate M4.

Table 17: Extracellular secondary metabolite profiles of *Metarhizium* spp. isolate M4

Metabolite	Properties	Mean Rf (x100) +/- S.E.
M4 A	W L/S UV P A/S	7.6 +/- 0.1
M4 B	Y/W L/UV	11.0 +/- 0.1
M4 C	W L/UV	16.2 +/- 0.2
M4 D	Y L U/V P A/S	30.3 +/- 0.3
M4 E	Y L/UV	39.5 +/- 0.3
M4 F	P L/UV	49.8 +/- 0.3
M4 G	P L/UV	55.7 +/- 0.5
M4 H	P A/S	96.5 +/- 1.6

W= white, Y= yellow, P= purple L/UV= long-wave ultraviolet light, S/UV= Short-wave ultraviolet light.

Table 18: Summary of replicates of *Metarhizium anisopliae* isolate M4 producing extracellular secondary metabolite profiles, different from the original isolate. After 1 week of storage. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Metabolite	*	FD 1	FD 4	MP 1	MP 5	FZ 1	FZ 3	LN 1
M4 A	●	●	X	●	●	●	●	●
M4 B	●	●	X	●	●	●	●	●
M4 C	●	●	X	X	●	●	●	●
M4 D	●	●	●	X	●	X	X	X
M4 E	●	●	X	●	●	●	●	●
M4 F	●	●	X	●	X	●	X	X
M4 G	●	X	X	●	X	●	X	X
M4 H	●	●	●	●	●	●	●	●

* Original Profile (see Table 17 for properties).

● Metabolite detected, X metabolite not detected

Replicates CS 1,2,3; MP 2; FD 2,3,5; FZ 2; LN 2,3,4 produced an extracellular secondary metabolite profiles characteristic of the original isolate (Table 17).

Table 19: Summary of replicates of *Metarhizium anisopliae* isolate M4 producing extracellular secondary metabolite profiles, different from the original isolate. After 1 week of storage and a recovery period. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Metabolite	*	CS2	FD1	FD4	MP4
M4 A	●	●	●	●	●
M4 B	●	●	●	●	●
M4 C	●	X	●	●	●
M4 D	●	●	●	●	●
M4 E	●	●	●	●	●
M4 F	●	●	●	X	●
M4 G	●	X	X	X	X
M4 H	●	●	●	●	●

* Original Profile (see Table 17 for properties).
● Metabolite detected, X metabolite not detected

Replicates CS 1,3,4; MP 1,2; FD 2,3; FZ 1,3,5; LN 1,3,4,5 produced an extracellular secondary metabolite profiles characteristic of the original isolate (Table 17).

Extracellular secondary metabolite profiles were affected by preservation after just 1 week of storage (Fig 5A). Only 11 of the 18 replicates (61%) retained the extracellular secondary metabolite profile of 8 metabolites that was characteristic of the original isolate (Table 18). After the recovery period (Fig 5B) 14 out of 18 replicates (78%) exhibited the extracellular secondary metabolite profile typical of the original isolate. Two metabolites that appeared in the original profile were still not detected in replicate that had been lyophilised, in which initially 6 were not detected (Table 19). Following the recovery period after 16 weeks of storage, only 3 out of 20 replicates exhibited extracellular secondary metabolite profiles that differed from the profile of the original isolate (Table 20). After 1 year of storage (Fig 6A), 5 replicates did not exhibit the extracellular secondary profile typical of the original isolate. After the recovery period (Fig 6B), 16 out of 21 replicates exhibited the typical extracellular secondary metabolite profile. One of the 4 replicates from each preservation treatment did not exhibit the extracellular secondary metabolite profile typical of the original isolate.

Table 20: Summary of replicates of *Metarhizium anisopliae* isolate M4 producing extracellular secondary metabolite profiles, different from the original isolate. After 16 weeks of storage and a recovery period. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Metabolite	*	CS1	FD1	MP1
M4 A	●	●	●	●
M4 B	●	●	●	●
M4 C	●	X	●	●
M4 D	●	X	X	●
M4 E	●	●	●	●
M4 F	●	●	X	●
M4 G	●	X	X	X
M4 H	●	●	●	X

* Original Profile (see Table 17 for properties).

● Metabolite detected, X metabolite not detected

Replicates CS 1,3,4; MP 1,2; FD 2,3; FZ 1,3,5; LN 1,3,4,5 produced an extracellular secondary metabolite profiles characteristic of the original isolate (Table 17).

Table 21: Summary of replicates of *Metarhizium anisopliae* isolate M4 producing extracellular secondary metabolite profiles, different from the original isolate. a) After 1 year of storage and b) after 1 year of storage and a recovery period. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

		----------a----------					----------b----------			
Metabolite	*	FD1	MP2	MP5	LN4	LN5	CS1	FD1	MP5	LN4
M4 A	●	●	●	●	●	●	●	●	●	●
M4 B	●	●	●	●	●	●	●	●	X	●
M4 C	●	●	●	●	●	●	●	●	●	●
M4 D	●	●	●	●	●	X	X	●	X	●
M4 E	●	●	●	●	●	●	●	●	●	●
M4 F	●	X	X	X	●	●	●	●	●	●
M4 G	●	X	X	●	●	●	●	X	●	●
M4 H	●	●	●	●	X	X	●	●	●	X

* Original Profile (see Table 17 for properties).

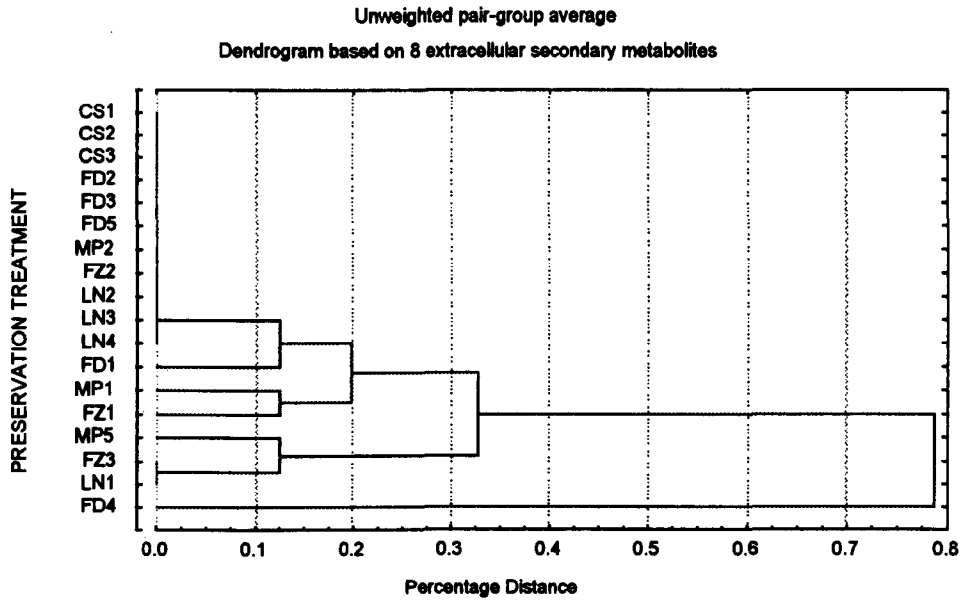
● Metabolite detected, X metabolite not detected

After 1 year of storage: Replicates CS 1,2,3,4,5; MP 2; FD 2,3,4,5; FZ 1,2,3,4,5; LN 1,2,3 produced an extracellular secondary metabolite profiles characteristic of the original isolate (Table 17).

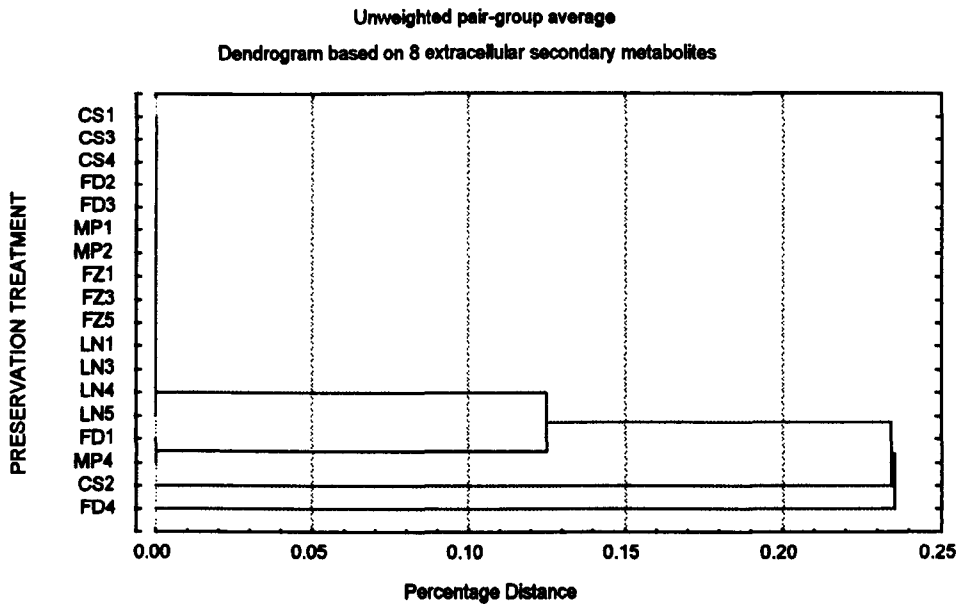
After 1 year of storage and a recovery period: Replicates CS 2,3,4,5; MP ,2; FD 2,3,4,5; FZ 1,2,3,4,5; LN 1,2,3,5 produced an extracellular secondary metabolite profiles characteristic of the original isolate (Table 17).

Fig. 5: A. Dendrogram produced from extracellular secondary metabolite profile of isolate M4 after 1 week of storage and B. after 1 week of storage and a recovery period

A.



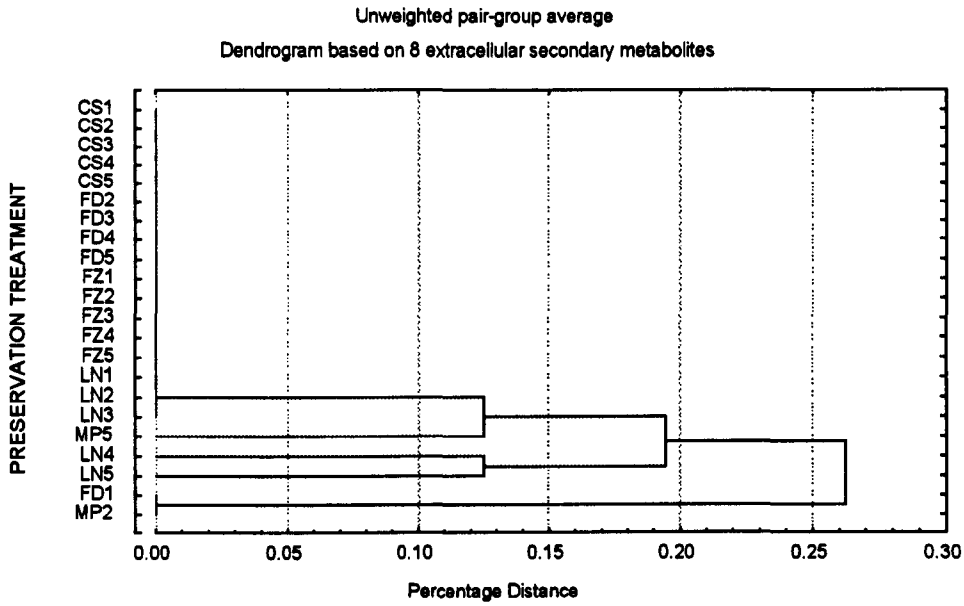
B.



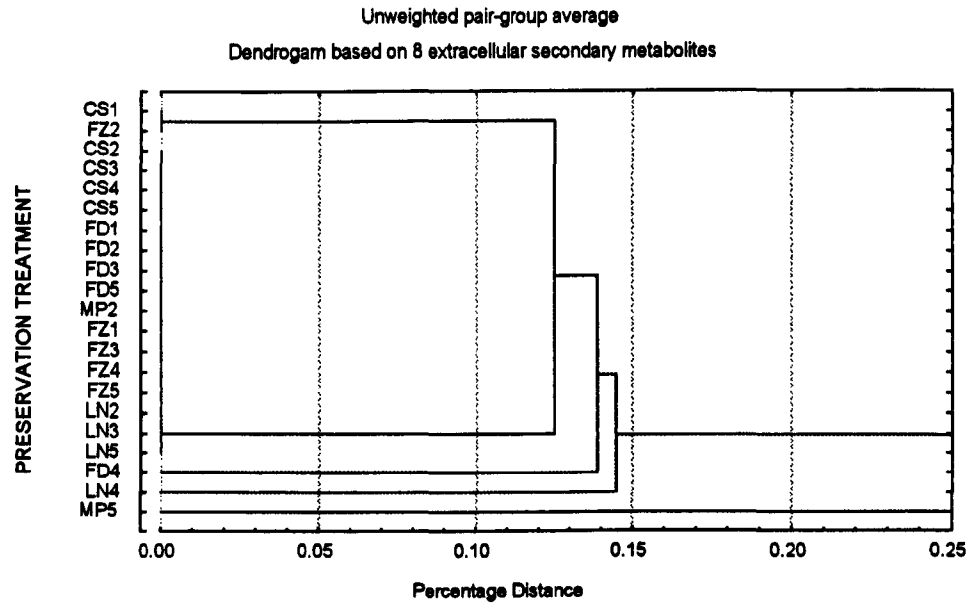
Key: CS, continual sub-culture; FD, lyophilised; MP, mycelial plugs in water; FZ, frozen at -20°C; LN, cryopreserved).

Fig. 6: A. Dendrogram produced from extracellular secondary metabolite profile of isolate M4 after 1 year of storage and B. after 1 year and a recovery period.

A.



B.



Key: CS, continual sub-culture; FD, lyophilised; MP, mycelial plugs in water; FZ, frozen at –20°C; LN, cryopreserved

After 1 week of storage, 10 out of 18 replicates (55.5%) exhibited the intracellular secondary metabolite profile typical of the original isolate (Fig 7A / Table 23). Metabolite M was not detected in isolate M4 at any time during the investigation.

Table 22: Intracellular secondary metabolite profile of *Metarhizium* spp. isolate M4

No.	Properties	Mean Rf (x100) +/- S.E.
M4 I	Y L/UV	4.6 +/- 0.1
M4 J	Y/W L/UV	7.2 +/- 0.1
M4 K	Y L/UV G/AS	10.2 +/- 0.1
M4 L	Y L/UV	32.8 +/- 0.4
M4 M	P L/UV	68.6 +/- 0.2
M4 N	P L/UV	74.7 +/- 0.3
M4 O	L/UV	84.2 +/- 0.2
M4 P	G A/S Y L/UV	90.6 +/- 0.1
M4 Q	T A/S	96.4 +/- 0.2

W= white, Y= yellow, P= purple, G=green L/UV= long-wave ultraviolet light, A/S=after spray,

Table 23: Summary of replicates of *Metarhizium anisopliae* isolate M4 producing intracellular secondary metabolite profiles, different from the original isolate. a) After 1 week of storage and b) after 1 week of storage and a recovery period. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

		----------(a)----------								---(b)---	
Metabolite	*	FD 1	FD 4	MP 2	MP 5	FZ 2	FZ 3	LN 2	LN 4	CS 2	MP 4
M4 I	●	●	●	●	●	●	●	●	●	●	●
M4 J	●	●	●	●	●	●	●	●	●	●	●
M4 K	●	●	●	●	●	●	●	●	●	●	●
M4 L	●	●	X	●	●	●	●	X	●	●	●
M4 M	●	●	X	●	●	●	●	X	●	X	X
M4 N	●	X	X	X	X	X	X	X	X	●	●
M4 O	●	●	X	●	●	●	●	●	●	●	●
M4 P	●	●	●	●	●	●	●	●	●	●	●
M4 Q	●	●	●	●	●	●	●	●	●	●	●

* Original Profile (see Table 22 for properties).

● Metabolite detected, X metabolite not detected

After 1 week of storage: Replicates CS 1,2,3; MP 1; FD 2,3,5; FZ 1; LN 3,5 produced an intracellular secondary metabolite profiles characteristic of the original isolate (Table 22).

After 1 week of storage and a recovery period: Replicates CS 1,3,4; MP 1,2; FD 3,4,5; FZ 3,4,5; LN 2,3,4,5 produced an intracellular secondary metabolite profiles characteristic of the original isolate (Table 22).

Table 24: Summary of replicates of *Metarhizium anisopliae* isolate M4 producing intracellular secondary metabolite profiles, different from the original isolate. a) After 1 year of storage. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Metabolite	*	CS3	CS4	CS5	FD1	FD2	FD3	FD4	MP2	MP5	FZ1	FZ3	FZ4	FZ5	LN1	LN4	LN5
M4 I	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
M4 J	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
M4 K	●	●	●	●	●	●	●	X	●	●	●	●	●	●	●	●	●
M4 L	●	X	X	X	X	X	X	X	X	X	X	X	X	●	●	X	X
M4 M	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
M4 N	●	●	●	●	●	●	●	●	X	X	X	●	●	X	X	●	●
M4 O	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
M4 P	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
M4 Q	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

* Original Profile (see Table 22 for properties).

● Metabolite detected, X metabolite not detected

After 1 year of storage: Replicates CS 1,2; FD 5; FZ 2; LN 2,3, produced an intracellular secondary metabolite profiles characteristic of the original isolate (Table 22).

Table 25: Summary of replicates of *Metarhizium anisopliae* isolate M4 producing intracellular secondary metabolite profiles, different from the original isolate. a) After 1 year of storage and a recovery period. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Metabolite	*	CS5	FD4	LN2	LN3	LN4
M4 I	●	●	●	●	●	●
M4 J	●	●	●	●	●	●
M4 K	●	●	●	●	●	●
M4 L	●	●	●	●	●	●
M4 M	●	●	●	●	●	●
M4 N	●	●	●	●	●	●
M4 O	●	●	●	●	●	●
M4 P	●	X	X	X	X	X
M4 Q	●	●	X	●	●	●

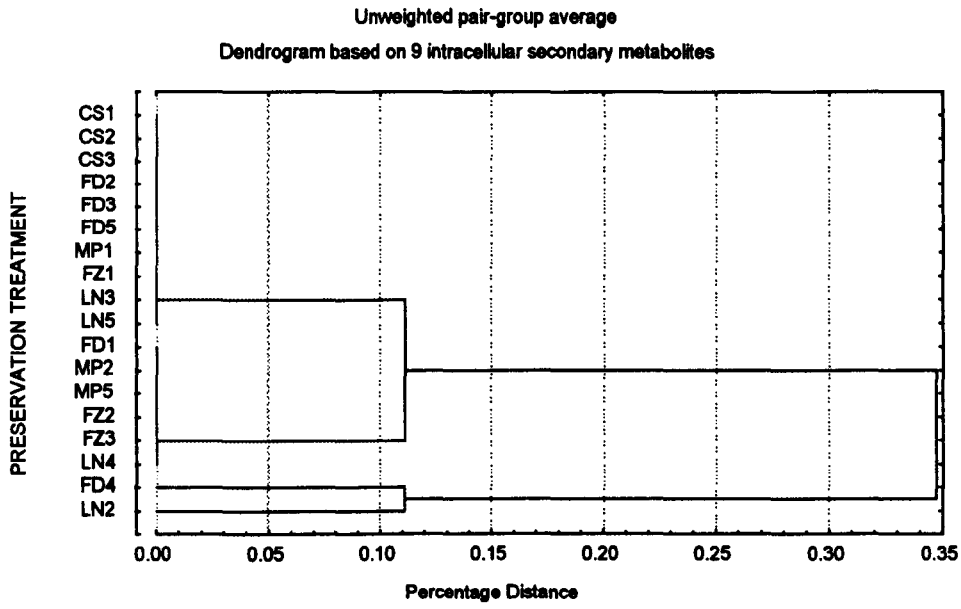
* Original Profile (see Table 22 for properties).

● Metabolite detected, X metabolite not detected

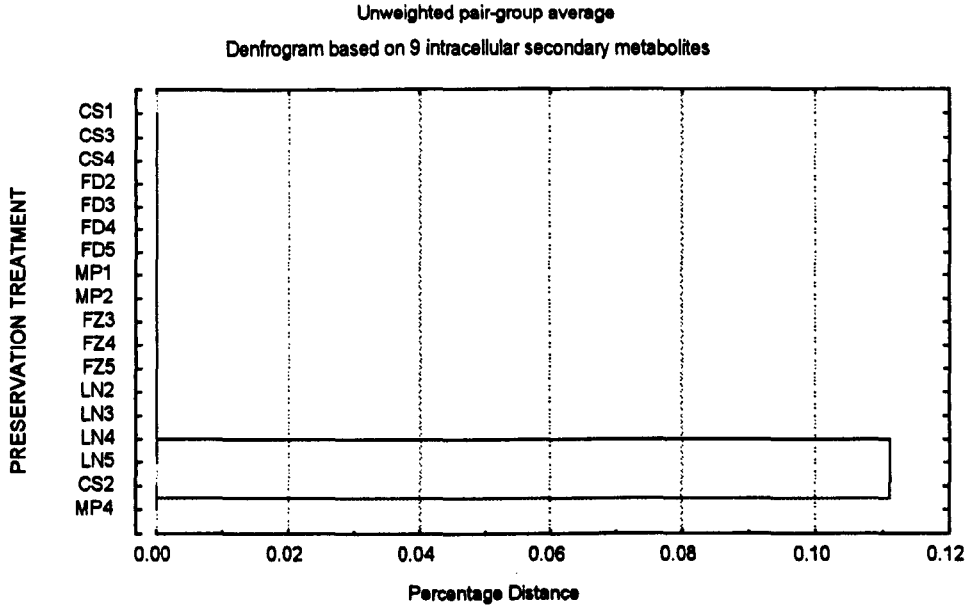
After 1 year of storage: Replicates CS 1,2,3,4; FD 1,2,3,5; FZ 1,2,3,4,5; LN 5, produced an intracellular secondary metabolite profiles characteristic of the original isolate (Table 22).

Fig. 7: A. Dendrogram produced from intracellular secondary metabolite profile of isolate M4 after 1 week of storage and B. after 1 week of storage and a recovery period

A.



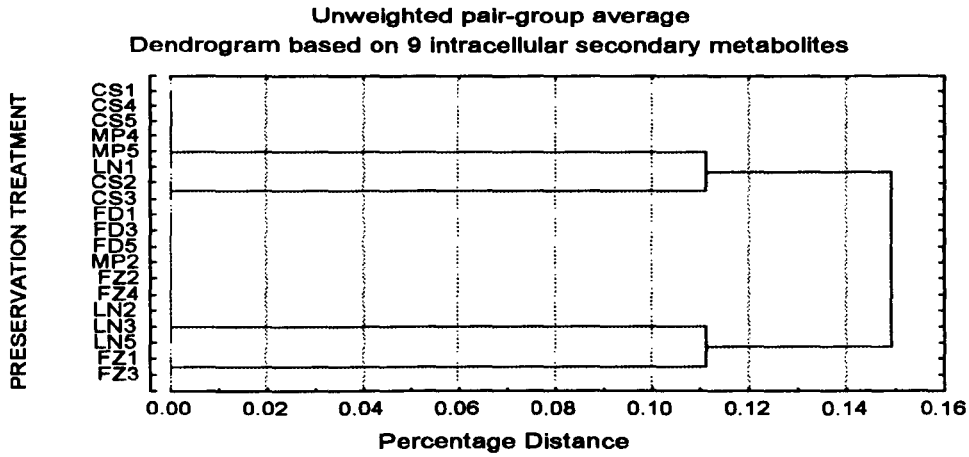
B.



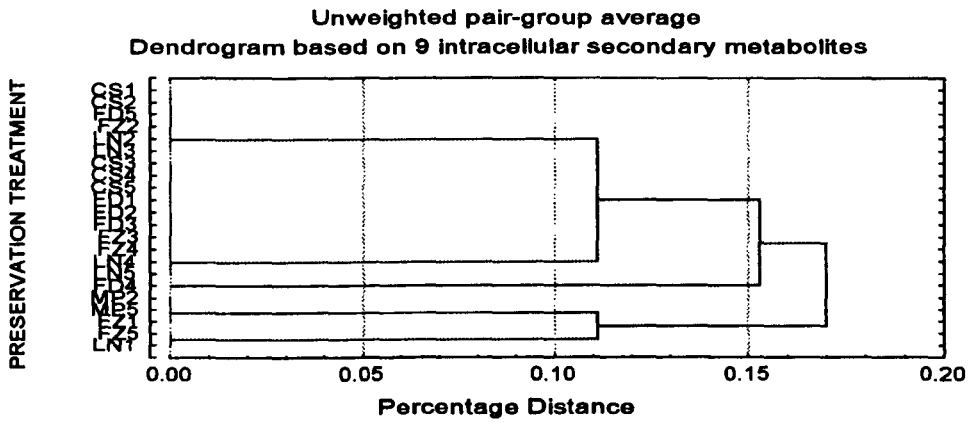
Key: CS, continual sub-culture; FD, lyophilised; MP, mycelial plugs in water; FZ, frozen at –20°C; LN, cryopreserved.

Fig. 8: A. Dendrogram produced from intracellular secondary metabolite profile of isolate M4 after 16 weeks of storage, B. after 1 year of storage and C. after 1 year of storage and a recovery period.

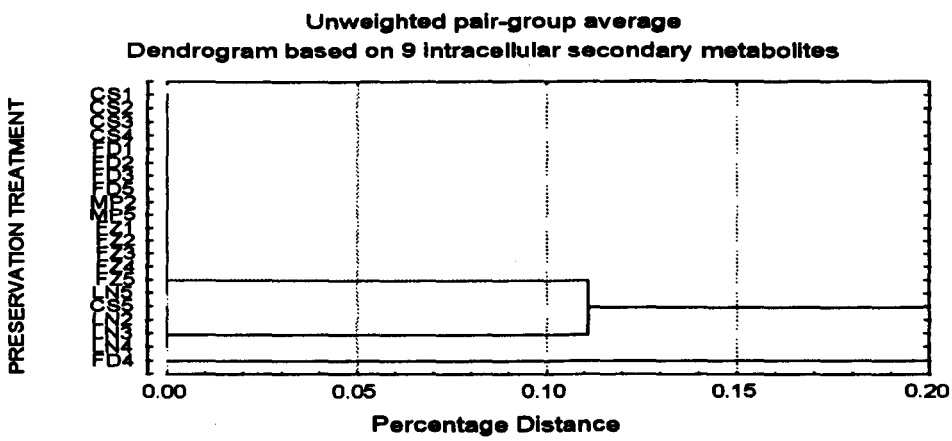
A.



B.



C.



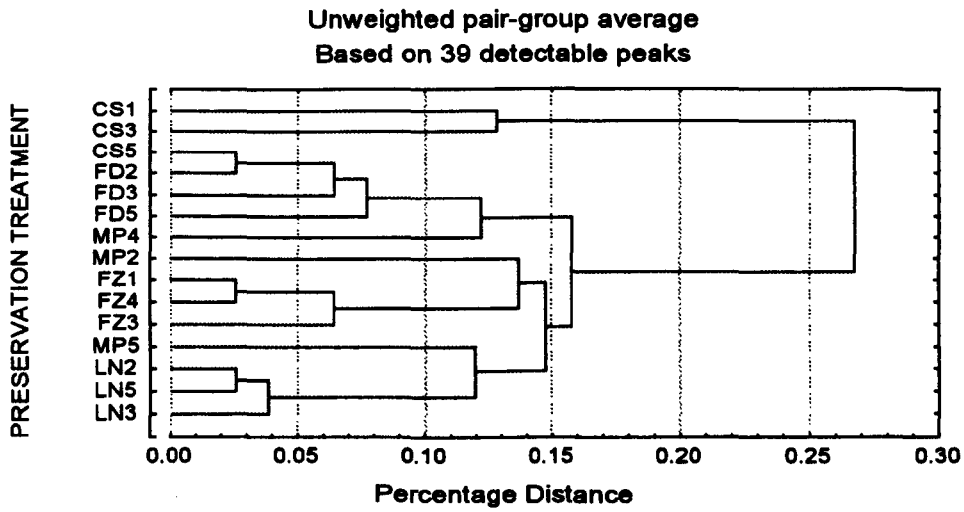
Key: CS, continual sub-culture; FD, lyophilised; MP, mycelial plugs in water; FZ, frozen at -20°C; LN, cryopreserved.

After the recovery period (Table 23), a metabolite that appeared in the original profile was not detected in a replicate that had been stored as a mycelial plug in water and a replicate maintained by continual sub-culture (Fig 7B). After 16 weeks of storage, 9 out of 19 replicates (47.4%) retained the intracellular secondary metabolite profile typical of the original isolate (Fig 8A). After the recovery period, 2 replicates did not exhibit the intracellular secondary metabolite profile characteristic of the original isolate. After 1 year of preservation (Table 24), only 6 replicates retained the original intracellular secondary metabolite profile (Fig 8B). After the recovery period (Table 25), 16 out of 21 replicates (76.2%) exhibited an intracellular secondary metabolite profile typical of the original isolate (Fig 8C).

4.3.1.2 High Performance Liquid Chromatography
Metarhizium isolate M4 after 16 weeks of storage

Analysis of the secondary metabolite profiles obtained by HPLC analysis of replicates of *Metarhizium anisopliae* isolate M4 yielded 39 peaks. The replicates of 4 preservation treatments grouped together on the dendrogram (Fig 9). The most homology between the profiles within preservation treatments was amongst those that had been stored by cryopreservation, the least homology was exhibited by replicates that had been stored as mycelial plugs in water and by replicates maintained by continual sub-culture.

Fig 9: Dendrogram compiled from HPLC profiles of *Metarhizium anisopliae* isolate M4 after 16 weeks of storage.



Key: CS, continual sub-culture; FD, lyophilisation, MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation.

4.3.2 *Fusarium oxysporum*

4.3.2.1 Thin Layer Chromatography

Fusarium oxysporum isolate F1

Both extra- and intracellular secondary metabolite profiles were changed by preservation. Ten extracellular secondary metabolites were detected (Table 26) and 8 intracellular secondary metabolites were detected (Table 27) that were representative of the typical secondary metabolite profile of isolate F1.

Table 26: Extracellular secondary metabolite profile of *Fusarium oxysporum* isolate F1

Metabolite	Properties	Mean Rf (x100) +/- S.E.
F1 A	W L/UV	7.6 +/- 0.2
F1 B	W L/UV, T A/S	11.1 +/- 0.2
F1 C	Y L/UV	16.9 +/- 0.6
F1 D	BL A/S	25.33 +/- 0.3
F1 E	BL A/S	32.6 +/- 0.1
F1 F	P A/S	39.0 +/- 0.1
F1 G	W L/UV	39.5 +/- 0.3
F1 H	W/Y L/UV	48.9 +/- 0.3
F1 I	Y L/UV	56.7 +/- 0.2
F1 J	P A/S	90.2 +/- 0.3

BL= blue, W= white, Y= yellow, T= Turquoise, P= purple, O = orange, L/UV= long wave ultraviolet light, A/S= after *p*-anisaldehyde spray

After 1 year of preservation, 17 out of 19 replicates (89%) exhibited an extracellular secondary metabolite profile typical of the original isolate. This included all of the replicates maintained by continual sub-culture and stored by lyophilisation. Replicate MP2 did not produce metabolite F1 E and replicate LN1 did not produce F1 J. Following a recovery period (after 28-days of growth on a maintenance media before transfer onto YES media), all replicates exhibited an extracellular secondary metabolite profile typical of the original isolate. After 2 years of preservation (Table 27), only 9 out of 20 replicates (45%) exhibited an extracellular secondary metabolite profile typical of the original isolate (Fig 10A). All of the replicates that had been cryopreserved and 2 replicates that had been lyophilised were amongst this group. After a recovery period (Table 25), 14 out of 20 replicates (70%) exhibited an extracellular secondary metabolite profile typical of the original isolate (Fig 10B). However, none of the replicates stored by mycelial plugs in water exhibited the original extracellular metabolite profile after the recovery period.

Table 27: Summary of replicates of *Fusarium oxysporum* isolate F1 producing extracellular secondary metabolite profiles, different from the original isolate. After 2 years of storage. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Metabolite	*	CS 3	CS 4	FD 1	FD 2	MP 2	MP 3	MP 4	FZ 2	FZ 3	FZ 4
F1 A	●	●	●	●	●	●	●	●	●	●	●
F1 B	●	●	●	●	●	●	●	●	●	●	●
F1 C	●	X	●	●	●	●	●	●	●	●	●
F1 D	●	●	●	●	●	●	X	●	X	●	●
F1 E	●	●	●	●	●	●	●	●	●	●	●
F1 F	●	●	●	●	●	●	●	●	●	●	●
F1 G	●	X	X	●	●	X	X	X	X	●	●
F1 H	●	●	●	●	●	●	●	●	●	●	●
F1 I	●	●	●	●	●	●	●	●	●	●	●
F1 J	●	●	●	●	●	●	●	●	●	●	●
F1 S◆	X	X	X	●	●	X	X	X	X	●	●
F1 T◆	X	X	X	X	X	X	X	X	X	●	●

*Original Profile (see Table 26 for properties). ● Metabolite detected, X metabolite not detected. ◆ Secondary metabolites produced after preservation, that were not detected in the extracellular secondary metabolite profile of the original isolate.

Replicates CS 1,2; FD 3,4; MP1, FZ 1; LN 1,2,3,4 produced extracellular secondary metabolite profiles characteristic of the original isolate (Table 26)

Table 28: Summary of replicates of *Fusarium oxysporum* isolate F1 producing extracellular secondary metabolite profiles, different from the original isolate. After 2 years of storage and a recovery period. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Metabolite	*	CS4	FD4	MP1	MP2	MP3	MP4
F1 A	●	●	●	●	●	●	●
F1 B	●	●	●	●	●	●	●
F1 C	●	●	●	●	●	●	●
F1 D	●	●	●	X	X	X	X
F1 E	●	●	●	●	●	●	●
F1 F	●	●	●	●	●	●	●
F1 G	●	X	X	●	X	●	X
F1 H	●	●	●	●	●	●	●
F1 I	●	●	●	●	●	●	●
F1 J	●	●	●	●	●	●	●

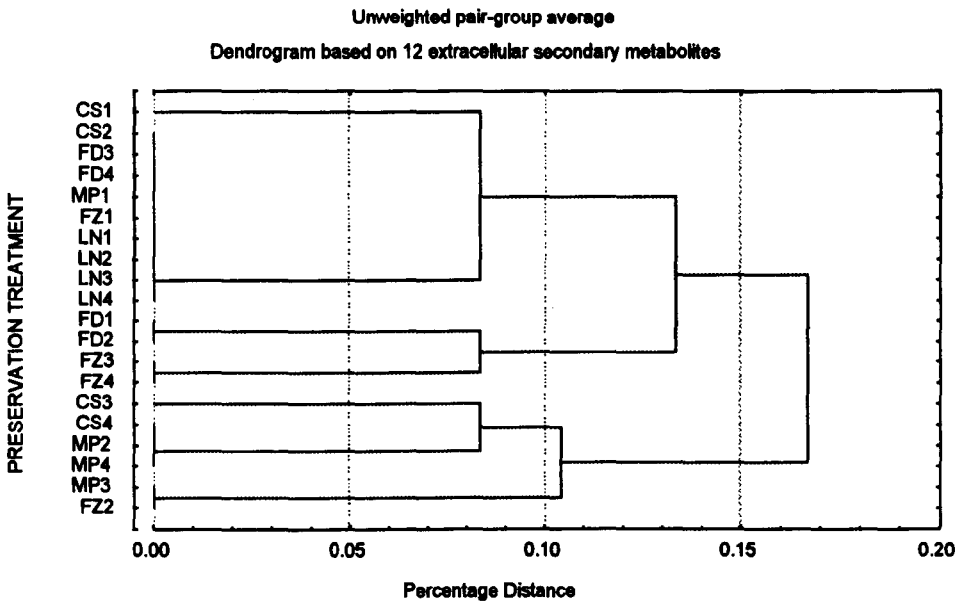
*Original Profile (see Table 26 for properties).

● Metabolite detected, X metabolite not detected

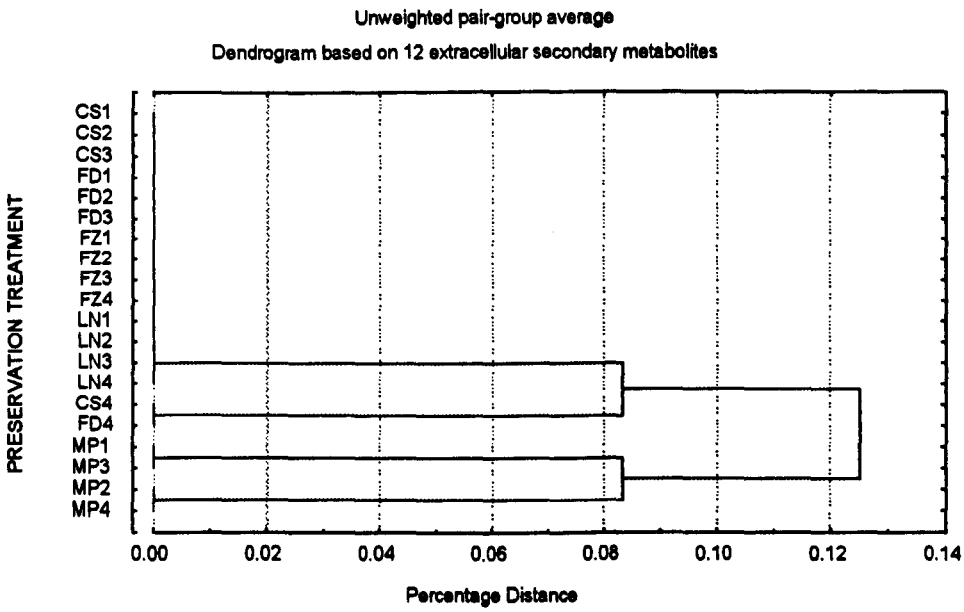
Replicates CS 1,2; FD 3,4; MP1, FZ 1; LN 1,2,3,4 produced extracellular secondary metabolite profiles characteristic of the original isolate (Table 26)

Fig. 10: A: Dendrogram produced from extracellular secondary metabolite profile of isolate F1 after 2 years of storage and B. after 2 years of storage and a recovery period. .

A.



B.



Key: CS, continual sub-culture; FD, lyophilisation, MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation).

Table 29: Intracellular secondary metabolite profile of *Fusarium oxysporum* isolate F1

Metabolite	Properties	Mean $Rf_{(x100)}$ +/- S.E.
F1 K	W/Y L/UV	4.6 +/- 0.1
F1 L	W/Y L/UV	6.5 +/- 0.1
F1 M	Y L/UV	9.7 +/- 0.2
F1 N	Y L/UV	17.2 +/- 0.2
F1 P	Y L/UV	21.0 +/- 0.3
F1 P	Y L/UV	38.0 +/- 0.2
F1 Q	Y L/UV	62.0 +/- 0.6
F1 R	Y L/UV	82.25 +/- 0.8

W= white, Y= yellow, L/UV= long wave ultraviolet light

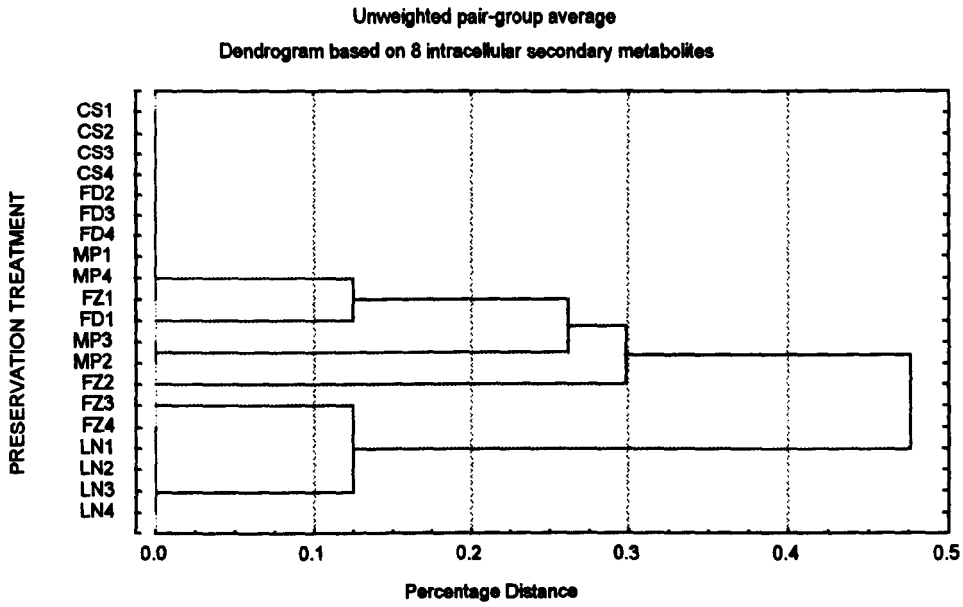
The intracellular secondary metabolite profiles of isolate F1, fell into 2 groups after both the 1 and 2 year testing times. One group comprised of all the replicates that had been stored at -20°C and stored cryopreserved. These produced metabolite profiles characteristic of the original isolate. The second group contained replicates that had lost metabolites from the profile of the original isolate. After 2 years of preservation no replicate exhibited the intracellular secondary metabolite profile characteristic of the original isolate (Table 30/Fig 11A). Following the recovery period after 2 years of preservation (Fig 11B), no replicates exhibited the secondary metabolite profile characteristic of the original isolate.

Table 30: Summary of replicates of Fusarium *oxysporum* isolate F1 producing intracellular secondary metabolite profiles, different from the original isolate. After 2 years of storage. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)● Metabolite detected, X metabolite not detected. M, metabolite. *Original profile (see table 29)

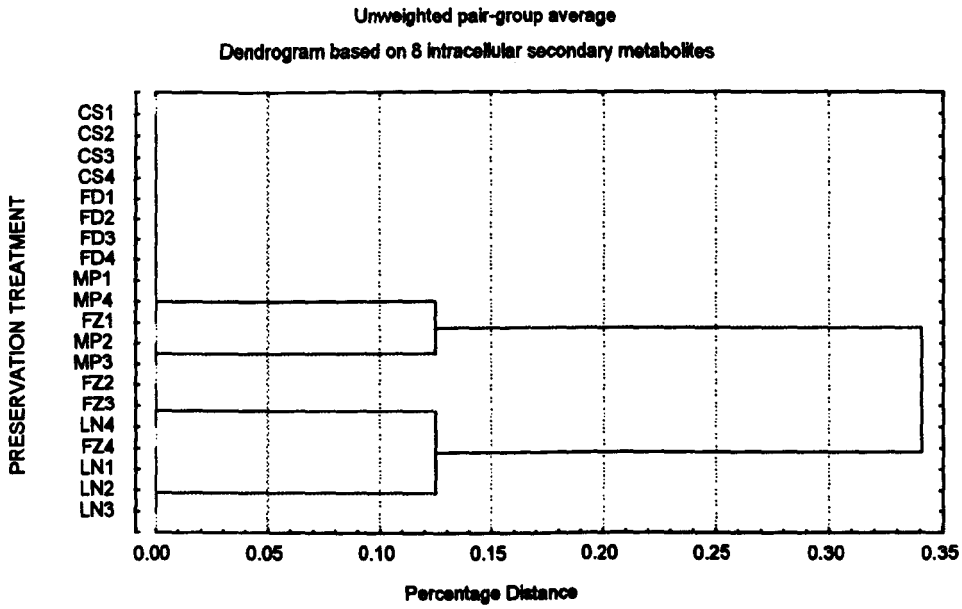
M	*	CS1	CS2	CS3	CS4	FD1	FD2	FD3	FD4	MP1	MP2	MP3	MP4	FZ1	FZ2	FZ3	FZ4	LN1	LN2	LN3	LN4
F1 K	●	●	●	●	●	●	●	●	●	●	●	●	●	●	X	●	●	●	●	●	●
F1 L	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	X	X	X	X	X	X
F1 M	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F1 N	●	●	●	●	●	●	●	●	●	X	X	●	●	●	●	●	●	●	●	●	●
F1 P	●	X	X	X	X	X	X	X	X	X	X	X	X	X	X	●	●	●	●	●	●
F1 P	●	X	X	X	X	X	X	X	X	●	●	X	X	X	X	X	●	●	●	●	●
F1 Q	●	X	X	X	X	X	X	X	X	X	X	X	X	X	●	●	●	●	●	●	●
F1 R	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

Fig. 11: A. Dendrogram produced from intracellular secondary metabolite profile of isolate F1 after 2 years of storage and B. after 2 years of storage and a recovery period.

A.



B.



Key: CS, continual sub-culture; FD, lyophilisation, MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation.

Table 31: Summary of replicates of *Fusarium oxysporum* isolate F1 producing intracellular secondary metabolite profiles, different from the original isolate. After 2 years of storage and a recovery period. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation) ● Metabolite detected, X metabolite not detected. M, metabolite. *Original profile (see table 29)

M	*	CS1	CS2	CS3	CS4	FD1	FD2	FD3	FD4	MP1	MP2	MP3	MP4	FZ1	FZ2	FZ3	FZ4	LN1	LN2	LN3	LN4
F1 K	●	●	●	●	●	●	●	●	●	●	●	●	●	●	X	●	●	●	●	●	●
F1 L	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	X	X	X	X	X	X
F1 M	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F1 N	●	●	●	●	●	●	●	●	●	X	X	●	●	●	●	●	●	●	●	●	●
F1 P	●	X	X	X	X	X	X	X	X	X	X	X	X	X	X	●	●	●	●	●	●
F1 P	●	X	X	X	X	X	X	X	X	●	●	X	X	X	X	X	●	●	●	●	●
F1 Q	●	X	X	X	X	X	X	X	X	X	X	X	X	X	●	●	●	●	●	●	●
F1 R	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

Fusarium oxysporum isolate F2

Both extra- and intracellular secondary metabolite profiles were changed by preservation. Eight extracellular secondary metabolites were detected (Table 32) and 5 intracellular secondary metabolites were detected (Table 33) that were representative of the typical secondary metabolite profile of isolate F2.

Table 32: Extracellular secondary metabolite profile of *Fusarium oxysporum* isolate F2:

Metabolite	Properties	Mean $Rf_{(X100)}$ +/-S.E.
F2 A	W L/UV	6.1 +/- 0.1
F2 B	W L/UV	9.3 +/- 0.7
F2 C	Y L/UV	14.7 +/- 0.1
F2 D	BL A/S	21.6 +/- 0.5
F2 E	Y L/UV	34.1 +/- 0.3
F2 F	Y L U/V	51.3 +/- 0.2
F2 G	Y L/UV	61.8 +/- 0.3
F2 H	P A/S	95.3 +/- 0.5

BL= blue, W= white, Y= yellow, P= purple, O = orange, L/UV= long wave ultraviolet light, A/S= after *p*-anisaldehyde spray

Table 33: Summary of replicates of *Fusarium oxysporum* isolate F2 producing extracellular secondary metabolite profiles, different from the original isolate. (a) After 1 year of storage and (b) after 1 year and a recovery period (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

		----------(a)----------									----------(b)----------		
Metabolite	*	CS4	FD1	FD2	FD3	MP1	MP2	MP3	LN1	LN3	CS4	MP1	MP3
F2 A	●	X	●	●	●	●	●	●	●	●	●	●	●
F2 B	●	●	●	●	●	●	●	●	●	●	●	●	●
F2 C	●	●	●	●	●	●	●	●	●	●	●	●	●
F2 D	●	●	X	X	X	X	X	X	X	X	X	●	●
F2 E	●	●	●	X	●	●	●	●	●	●	●	●	●
F2 F	●	●	●	●	●	●	●	●	●	●	●	●	●
F2 G	●	●	●	●	●	●	●	●	●	●	●	●	●
F2 H	●	●	X	X	●	X	●	X	●	●	●	X	X

*Original Profile (see Table 32 for properties).

● Metabolite detected, X metabolite not detected

After 1 year of storage: Replicates CS 1,2,3,5; MP 4,5; FZ 1,2,3; LN 2 produced extracellular secondary metabolite profiles characteristic of the original isolate (Table 32)

After 1 year of storage and a recovery period: Replicates CS 1,2,3,5; FD1,2,3; MP 2,4,5; FZ 1,2,3; LN 1,2,2 produced extracellular secondary metabolite profiles characteristic of the original isolate (Table 29)

After 1 year of preservation, 10 out of 19 replicates (52.6%) exhibited an extracellular secondary metabolite profile typical of the original isolate (Fig 12A). Only 1 out of 3 replicates that had been cryopreserved and none of the replicates that had been lyophilised retained the characteristic extracellular metabolite profile. In particular, a metabolite (F2D) was lost from all of the replicates where the profile was changed. Three metabolites that appeared in the characteristic profile were not detected in a replicate that had been lyophilised. After 2 years of storage, only a single replicate (cryopreserved replicate LN2) retained the characteristic extracellular metabolite profile (Fig 12B). However, 8 out of the 20 replicates (40%) regained the

characteristic profile after a recovery period (Fig 12C) including all of the replicates that had been cryopreserved. After 1 year of preservation only 3 replicates (15%) did not exhibit the characteristic extracellular metabolite profile (2 replicates stored as mycelial plugs in water and 1 maintained by continual sub-culture) after the recovery period.

Table 31: Extracellular secondary metabolite profiles of all replicates of *Fusarium oxysporum* isolate F2 after 2 years of storage. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Metab -olite	*	CS1	CS2	CS3	CS4	FD1	FD2	FD3	FD4	MP1	MP2	MP3	MP4	FZ1	FZ2	FZ3	FZ4	LN1	LN2	LN3	LN4
F2 A	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F2 B	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F2 C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F2 D	●	X	X	●	●	●	X	X	X	●	X	X	●	X	X	X	●	●	X	X	X
F2 E	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F2 F	●	X	X	X	X	X	X	●	●	X	●	X	X	X	X	X	X	●	●	●	●
F2 G	●	X	●	X	●	X	X	●	●	X	●	●	●	X	X	X	X	●	●	●	●
F2 H	●	X	●	X	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

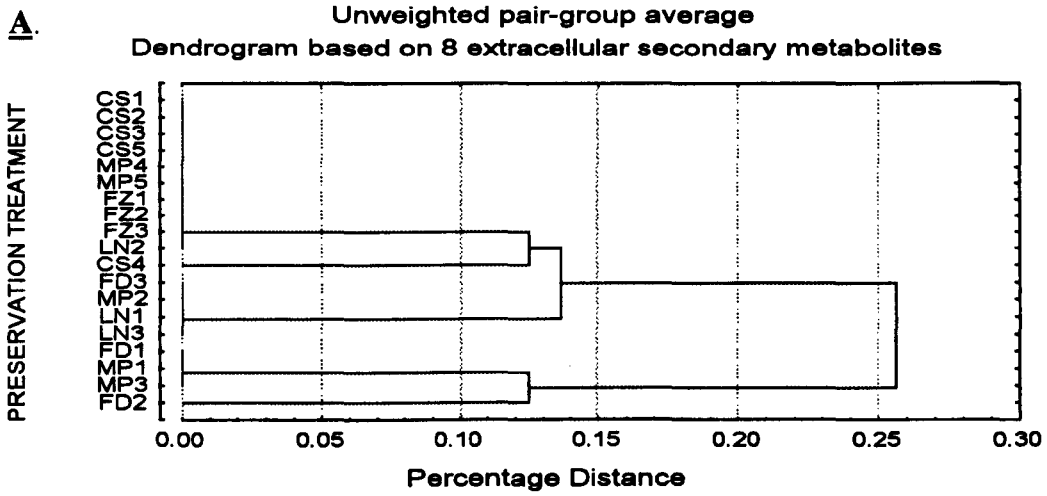
* profile of original isolate (see Table 29 for properties).
● Metabolite detected, X metabolite not detected

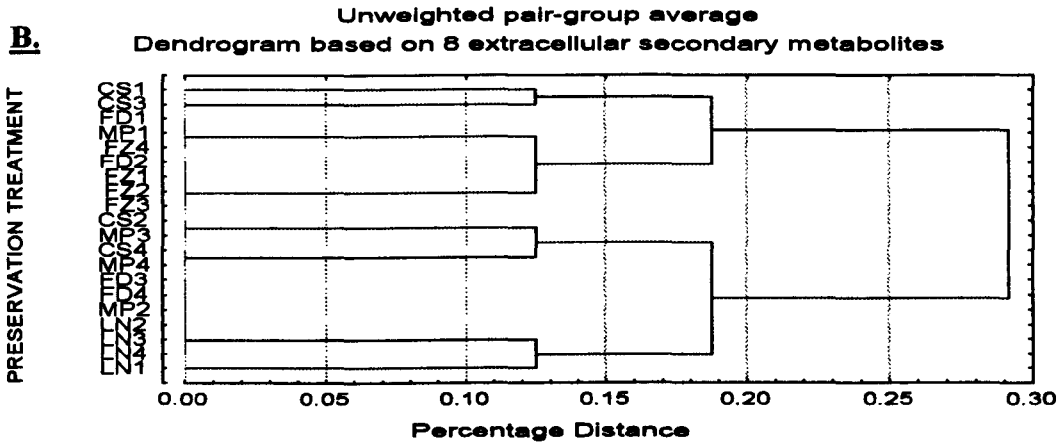
Table 32: Extracellular secondary metabolite profiles of all replicates of *Fusarium oxysporum* isolate F2 after 2 years of storage and a recovery period. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation

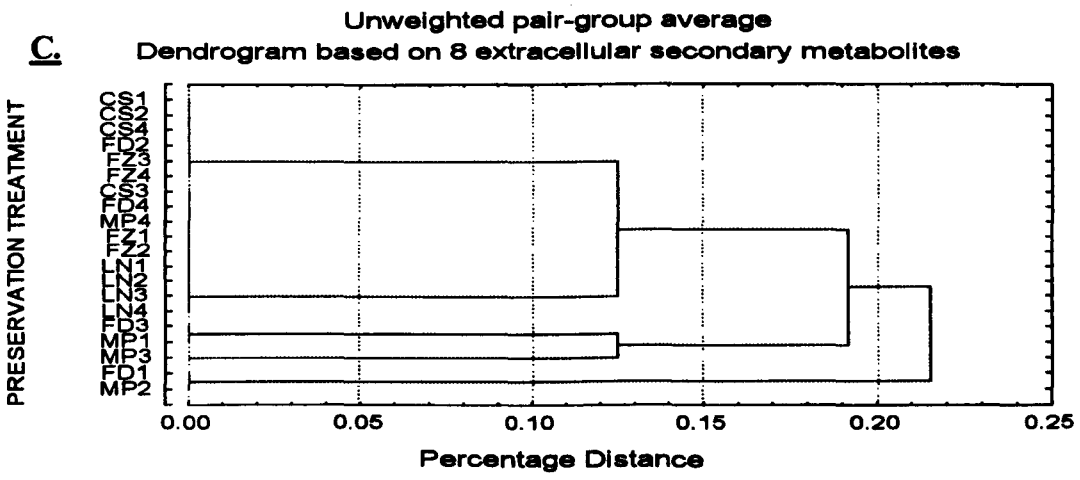
Metab -olite	*	CS1	CS2	CS3	CS4	FD1	FD2	FD3	FD4	MP1	MP2	MP3	MP4	FZ1	FZ2	FZ3	FZ4	LN1	LN2	LN3	LN4
F2 A	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F2 B	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F2 C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F2 D	●	●	●	●	●	●	●	X	●	X	●	X	●	●	●	●	●	●	●	●	●
F2 E	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F2 F	●	X	X	●	X	X	X	X	●	X	X	●	●	●	●	X	X	●	●	●	●
F2 G	●	●	●	●	●	X	●	●	●	●	X	●	●	●	●	●	●	●	●	●	●
F2 H	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

* profile of original isolate (see Table 29 for properties).
● Metabolite detected, X metabolite not detected

Fig. 12: A. Dendrogram produced from extracellular secondary metabolite profile of isolate F2 after 1 year of storage B. after 2 years of storage and C. after 2 years and a recovery period.







Key: CS, continual sub-culture; FD, lyophilisation, MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation.

Table 33: Intracellular secondary metabolite profile of *Fusarium oxysporum* isolate F2:

Metabolite	Properties	Mean Rf $_{(x100)}$ +/- S.E.
F2 I	Y L/UV	6.3 +/- 0.3
F2 J	W/Y L/UV	11.2 +/- 0.3
F2 K	BL/W S/UV	41.2 +/- 0.2
F2 L	Y L/UV	64.4 +/- 0.2
F2 M	Y L/UV	89.6 +/- 0.6

W= white, Y= yellow, BL= blue L/UV= long-wave ultraviolet light, S/UV= Short-wave ultraviolet light.

Table 34: Summary of replicates of *Fusarium oxysporum* isolate F2 producing intracellular secondary metabolite profiles, different from the original isolate. (a) After 1 year of storage and (b) after 1 year and a recovery period. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at –20°C, LN= Cryopreservation)

----------(a)---------- -----(b)------

Metabolite	*	CS1	CS3	CS4	CS5	FD1	MP1	MP3	MP4	MP5	FZ1	FZ2	FZ3	FD1	FD2	FD3
F2 I	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F2 J	●	●	●	X	●	●	●	●	X	X	●	X	X	X	X	X
F2 K	●	●	●	X	●	●	X	X	●	●	●	●	●	●	●	●
F2 L	●	●	●	X	●	X	●	●	●	●	●	●	●	●	●	●
F2 M	●	X	●	●	X	●	●	●	●	●	●	●	●	●	●	●

*Original Profile (see Table 33 for properties).

● Metabolite detected, X metabolite not detected

After 1 year of storage: Replicates CS 2; FD 2,3; MP 2; FZ 1; LN 1,2,3 produced intracellular secondary metabolite profiles characteristic of the original isolate (Table 33)

After 1 year of storage and a recovery period: Replicates CS 1,2,3,4,5; MP 1,2,3,4,5; FZ 1;2,3; LN 1,2,3 produced intracellular secondary metabolite profiles characteristic of the original isolate (Table 33)

After 1 year of preservation, 9 out of 19 replicates (47.4%) exhibited an intracellular secondary metabolite profile characteristic of the original isolate. (Fig 13A). All of the replicates stored by cryopreservation retained the original intracellular secondary metabolite profile. However, none of the replicates stored as mycelial plugs in water retained the typical secondary metabolite profile. Three out

of 5 metabolites were not detected in a replicate that had been maintained by continual sub-culture. After 2 years of preservation, 11 out of 20 replicates (55%) retained the intracellular secondary metabolite profile characteristic of isolate F2. These eleven were all of the replicates that had been cryopreserved, 3 maintained by continual sub-culture, 2 stored at –20°C, and a single replicate stored as a mycelial plug in water or stored lyophilised. After 1 year of storage and a recovery period, the intracellular secondary metabolite profile characteristic of isolate F2 was recovered in most replicates, except those that had been lyophilised. All 3 replicates lacked a metabolite (F2J). After 2 years of storage and a recovery period (Fig 13B), only 12 out of 20 replicates (60%) exhibited the intracellular secondary metabolite profile typical of the original isolate; of these only replicates stored at –20°C all recovered the characteristic profile. In 4 replicates, a second metabolite was not detected after the recovery period, including 2 that had been cryopreserved.

Table 35: Summary of replicates of *Fusarium oxysporum* isolate F2 producing intracellular secondary metabolite profiles, different from the original isolate. (a) After 2 years of storage and (b) after 2 years and a recovery period. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at –20°C, LN= Cryopreservation)

		----------(a)----------									----------(b)----------					
Metabolite	*	CS3	FD1	FD2	FD3	MP1	MP3	MP4	FZ2	FZ3	CS3	FD2	MP1	MP2	MP4	LN2	LN4
F2 I	●	●	●	●	●	●	●	●	●	●	●	X	●	●	●	●	●
F2 J	●	●	●	●	X	●	●	●	●	●	X	●	X	X	X	X	X
F2 K	●	●	●	●	●	●	X	X	●	●	●	●	●	●	●	●	●
F2 L	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F2 M	●	X	X	X	●	X	●	●	X	X	●	●	●	●	●	●	●

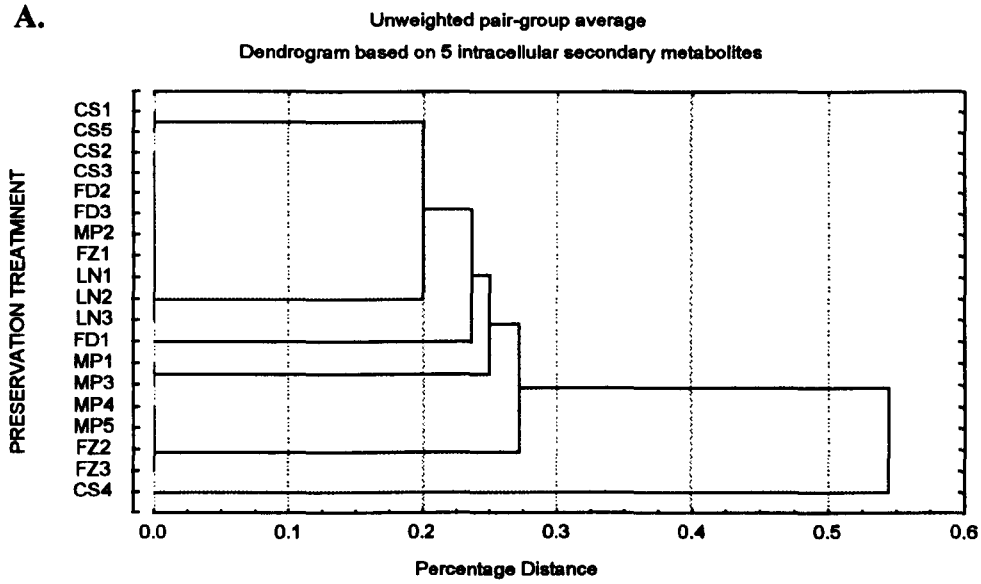
*Original Profile (see Table 33 for properties).

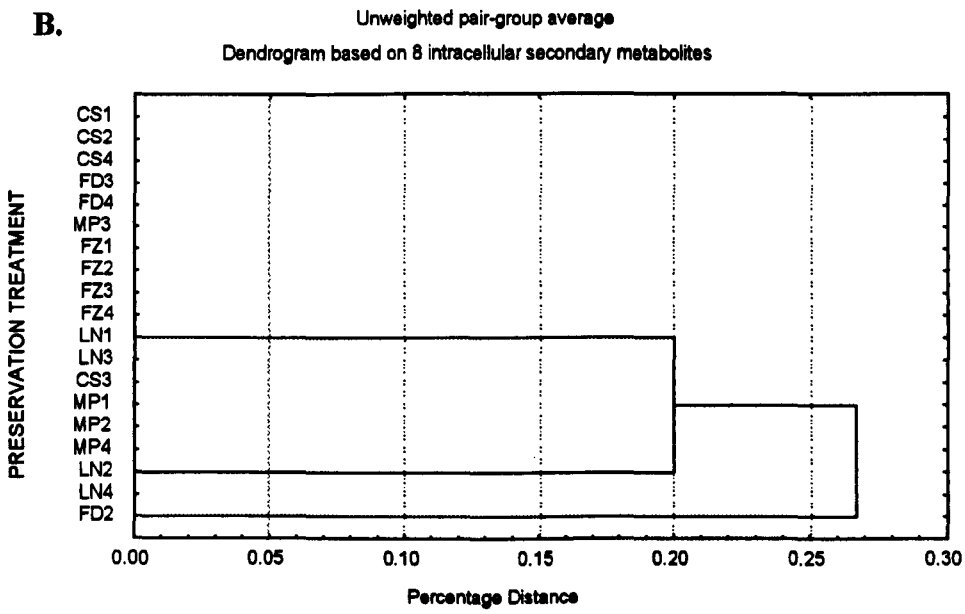
● Metabolite detected, X metabolite not detected

After 2 year of storage: Replicates CS 1,2,4; FD 4; MP 2; FZ 1,4; LN 1,2,3,4 produced intracellular secondary metabolite profiles characteristic of the original isolate (Table 33)

After 2 years of storage and a recovery period: Replicates CS 2,4; FD 3,4, MP 3; FZ 1;2,3,4; LN 1,3; produced intracellular secondary metabolite profiles characteristic of the original isolate (Table 33)

Fig. 13: A. Dendrogram produced from intracellular secondary metabolite profile of isolate F2 after 1 year of storage and B. after 2 years of storage and a recovery period.





Key, CS, continual sub-culture; FD, lyophilisation, MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation.

Fusarium oxysporum isolate F3

Both extra- and intracellular secondary metabolite profiles were changed by preservation. Eight extracellular secondary metabolites were detected (Table 36) and 9 intracellular secondary metabolites were detected (Table 37) that were representative of the typical secondary metabolite profile of isolate F3.

Table 36: Extracellular secondary metabolite profiles of *Fusarium oxysporum* isolate F3

Metabolite	Properties	Mean Rf(x100) +/- S.E.
F3 A	Y L/UV P A/S	6.56 +/- 0.20
F3 B	W S/UV-L/UV	9.05 +/- 0.20
F3 C	Y L/UV BL A/S	13.06 +/- 0.19
F3 D	FL P L/UV	20.05 +/- 0.15
F3 E	Y L/UV P/AS	27.53 +/- 0.33
F3 F	Y L/UV	36.20 +/- 0.20
F3 G	Y L/UV	40.65 +/- 0.42
F3 H	P A/S	96.29 +/- 0.23

W= white, Y= yellow, BR= brown, L/UV= long-wave ultraviolet light, S/UV= Short-wave ultraviolet light, VIS= visible, A/S= after *p*-anisaldehyde spray

Table 37: Summary of replicates of *Fusarium oxysporum* isolate F3 producing extracellular secondary metabolite profiles, different from the original isolate after 1 week of storage. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Metabolite	*	FD1	FD2	FD3	FD4	MP1	MP2	MP3	MP4	FZ1	FZ2	FZ3	FZ4	LN1	LN2	LN3	LN4
F3 A	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 B	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 D	●	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
F3 E	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 F	●	●	X	X	X	●	●	●	●	●	●	●	●	●	●	●	●
F3 G	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 H	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

*Original Profile (see Table 36 for properties). ● Metabolite detected, X metabolite not detected

After 1 week of storage: Only replicates CS 1,2,3 produced extracellular secondary metabolite profiles characteristic of the original isolate (Table 36)

After 1 week of storage (Table 37), all replicates that had undergone preservation exhibited a different extracellular secondary metabolite profile than the original (Fig 14A). A metabolite (F3 D, Rf $_{(x100)}$ 20.0, yellow under long wave UV light) was only detected in the replicates that had been maintained by continual sub-culture and was not detected in any other replicates. It was also not detected at any other testing-time during the investigation in any replicates. Three out of 4 replicates that had been lyophilised lost an additional metabolite (F3 F, Rf $_{(x100)}$ 36.2, under long-wave UV light). After the recovery period, all 20 replicates exhibited similar extracellular secondary metabolite profiles (ignoring metabolite F3 D). After 16 weeks of storage (Table 38), 15 out of 20 replicates (75%) exhibited an extracellular secondary metabolite profile typical of the original isolate (ignoring metabolite F3 D) (Fig 14B). After the recovery period all replicates exhibited identical metabolite profiles. After 1 year of preservation (Fig 15A/ Table 39), 13 out of 20 replicates (65%) exhibited an extracellular secondary metabolite profile typical of the original isolate (ignoring metabolite F3 D). After a recovery period (Fig 15B), 18 out of 20 replicates exhibited an extracellular secondary metabolite profile typical of the original isolate (ignoring metabolite F3 D). The profiles of 2 replicates that had been lyophilised did not recover after the recovery period.

Table 38: Summary of replicates of *Fusarium oxysporum* isolate F3 producing extracellular secondary metabolite profiles, different from the original isolate after 16 weeks of storage. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Metabolite	*	CS1	CS2	CS3	CS4	FD1	FD2	FD3	FD4	MP1	MP2	MP3	MP4	FZ1	FZ2	FZ3	FZ4	LN1	LN2	LN3	LN4
F3 A	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 B	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 D	●	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
F3 E	●	●	●	●	X	X	X	●	X	●	●	●	●	●	●	●	●	●	●	●	●
F3 F	●	●	●	●	●	●	●	●	●	●	●	X	●	●	●	●	●	●	●	●	●
F3 G	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 H	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

*Original Profile (see Table 36 for properties). ● Metabolite detected, X metabolite not detected

No replicates exhibited the profile characteristic of the original isolate.

Table 39: Summary of replicates of *Fusarium oxysporum* isolate F3 producing extracellular secondary metabolite profiles, different from the original isolate after 1 year of storage. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Metabolite	*	CS1	CS2	CS3	CS4	FD1	FD2	FD3	FD4	MP1	MP2	MP3	MP4	FZ1	FZ2	FZ3	FZ4	LN1	LN2	LN3	LN4
F3 A	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 B	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 D	●	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
F3 E	●	●	X	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 F	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 G	●	X	X	X	X	X	X	X	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 H	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

*Original Profile (see Table 36 for properties). ● Metabolite detected, X metabolite not detected

No replicates exhibited the profile characteristic of the original isolate.

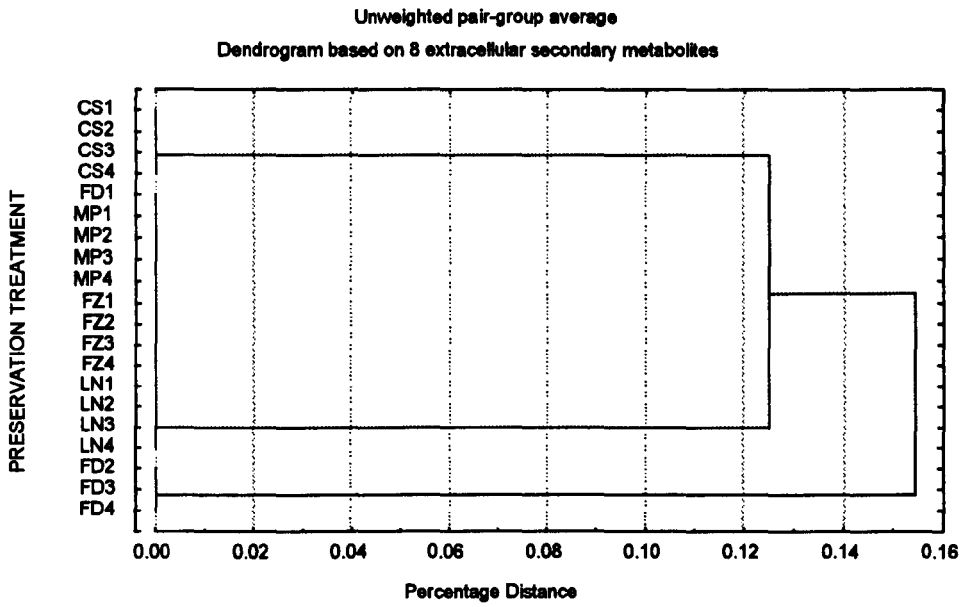
Table 40: Intracellular secondary metabolite profile of *Fusarium oxysporum* isolate F3:

Metabolite	Properties	Mean $Rf_{(x100)}$ +/- S.E.
F3 I	Y L/UV	5.92 +/- 0.11
F3 J	Y L/UV	11.20 +/- 0.10
F3 K	Y L/UV	16.05 +/- 0.16
F3 L	AS P	41.05 +/- 0.55
F3 M	AS P	46.55 +/- 0.44
F3 N	Y L/UV	66.34 +/- 0.46
F3 O	W/Y L/UV	91.83 +/- 0.25
F3 P	TQ A/S	94.70 +/- 0.29
F3 Q	AS P	97.46 +/- 0.17

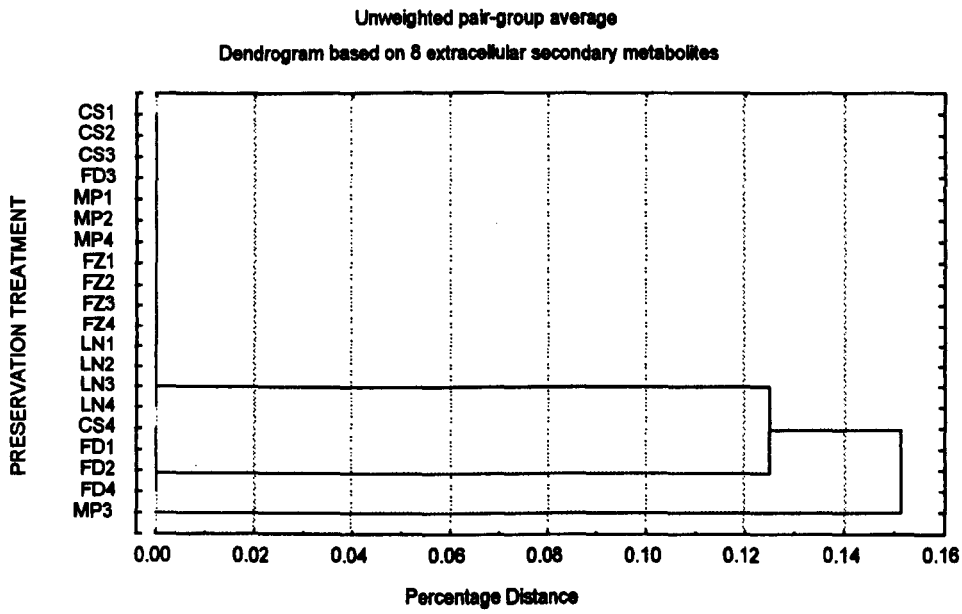
W= white, Y= yellow, P= purple, TQ= turquoise L/UV= long-wave ultraviolet light, A/S= after *p*-anisaldehyde

Fig. 14: A. Dendrogram produced from extracellular secondary metabolite profile of isolate F3 after 1 week of storage and B. after 16 weeks of storage .

A.

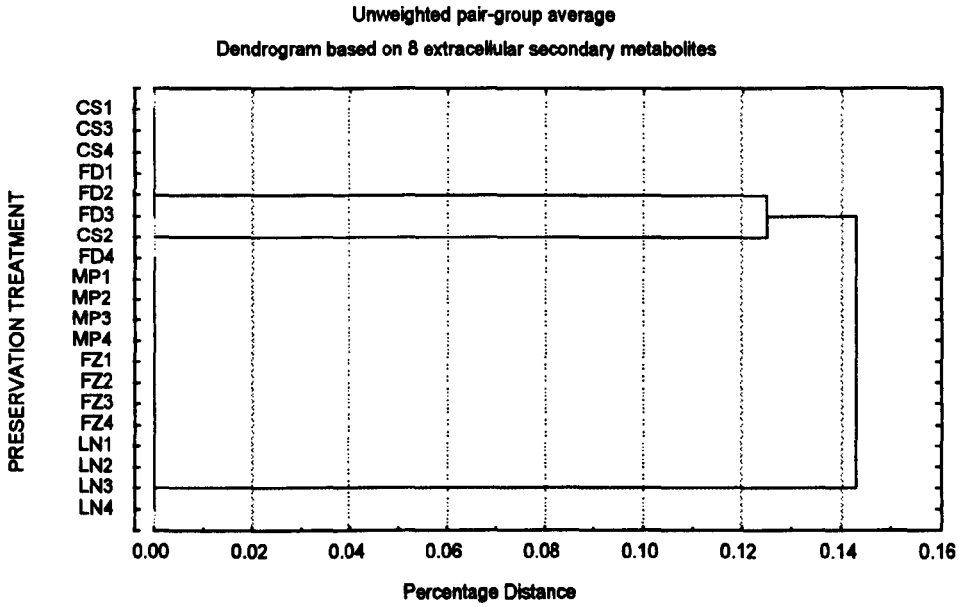


B.

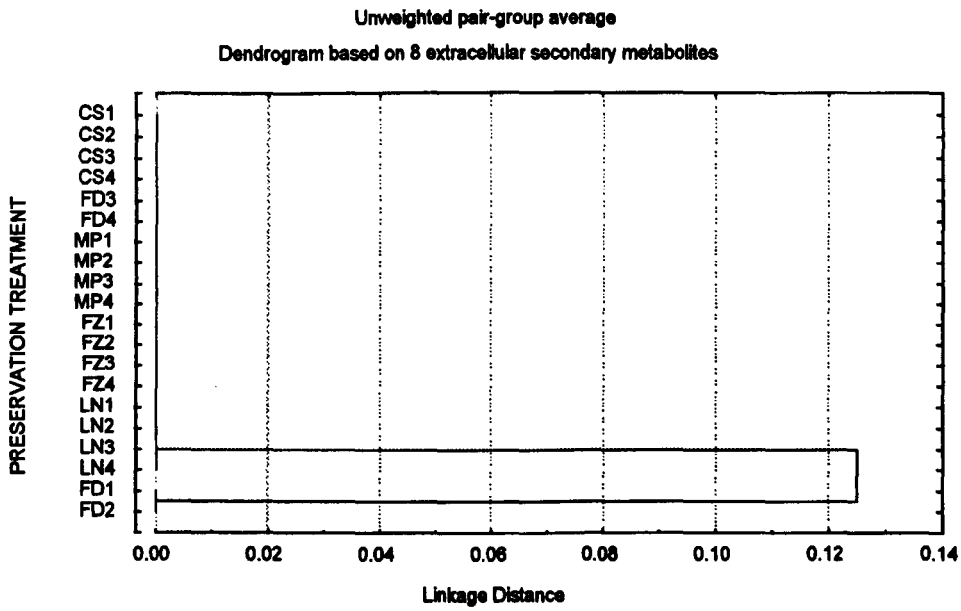


Key: CS, continual sub-culture; FD, lyophilisation, MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation

Fig. 15: A. Dendrogram produced from extracellular secondary metabolite profile of isolate F3 after 1 year of storage and B. after 1 year of storage and a recovery period.



B.



Key: CS, continual sub-culture; FD, lyophilisation, MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation.

After 1 week of storage, 13 out of 20 replicates (65%) exhibited an intracellular secondary metabolite profile typical of the original isolate (Fig 16A / Table 41). After the recovery period all replicates exhibited an intracellular secondary metabolite profile typical of the original isolate.

Table 41: Summary of replicates of *Fusarium oxysporum* isolate F3 producing intracellular secondary metabolite profiles, different from the original isolate after 1 week of storage. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Metabolite	*	CS4	MP3	MP4	FZ3	LN1	LN2	LN4
F3 I	●	●	●	●	●	●	●	●
F3 J	●	●	X	X	X	●	●	●
F3 K	●	●	●	●	●	●	●	●
F3 L	●	X	●	●	●	X	X	X
F3 M	●	X	●	●	●	X	X	X
F3 N	●	●	●	●	●	●	●	●
F3 O	●	●	●	●	●	●	●	●
F3 P	●	●	●	●	●	●	●	●
F3 Q	●	●	●	●	●	●	●	●

*Original Profile (see Table 40 for properties). ● Metabolite detected, X metabolite not detected

After 1 week of storage: Replicates CS 1,2,3, MP1,2; FD 1,2,3,4. FZ 1,2,4, LN3 produced extracellular secondary metabolite profiles characteristic of the original isolate (Table 40)

After 16 weeks of storage (Fig 16B), no replicates exhibited an intracellular secondary metabolite profile typical of the original isolate, as 2 metabolites ($Rf_{(x100)}$ 41, purple after spray and $Rf_{(x100)}$ 46.5, purple after *p*-anisaldehyde spray) were not detected. However, 15 out of 20 replicates (75%) exhibited the same profiles. After the recovery period, the metabolites (F3 L, $Rf_{(x100)}$ 41, purple after *p*-anisaldehyde spray and F3M, $Rf_{(x100)}$ 46.5, purple after *p*-anisaldehyde spray) were recovered in all replicates, which then exhibited the intracellular secondary metabolite profiles typical of the original isolate. After 1 year of storage (Fig 16C), 12 out of 20 replicates (60%) exhibited similar intracellular secondary metabolite profiles. However, 3 metabolites detected at the previous testing times were not detected in the profiles of any of the replicates. After the recovery period, the intracellular secondary metabolites that were not detected following resuscitation from storage

were recovered in all replicates which exhibited intracellular secondary metabolite profile characteristic of the original isolate.

Table 42: Intracellular secondary metabolite profiles of replicates of *Fusarium oxysporum* isolate F3 after 16 weeks of storage (A) and 1 year of storage (B). (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

A.

Metabolite	*	CS1	CS2	CS3	CS4	FD1	FD2	FD3	FD4	MP1	MP2	MP3	MP4	FZ1	FZ2	FZ3	FZ4	LN1	LN2	LN3	LN4
F3 I	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 J	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 K	●	●	●	●	●	●	●	●	X	●	●	●	●	X	X	X	X	●	●	●	●
F3 L	●	X	X	X	X	X	X	X	●	X	X	X	X	●	●	●	●	X	X	X	X
F3 M	●	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
F3 N	●	●	●	●	●	●	●	●	●	●	●	●	●	X	X	X	X	●	●	●	●
F3 O	●	●	●	●	●	●	●	●	●	●	●	●	●	X	X	X	X	●	●	●	●
F3 P	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 Q	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

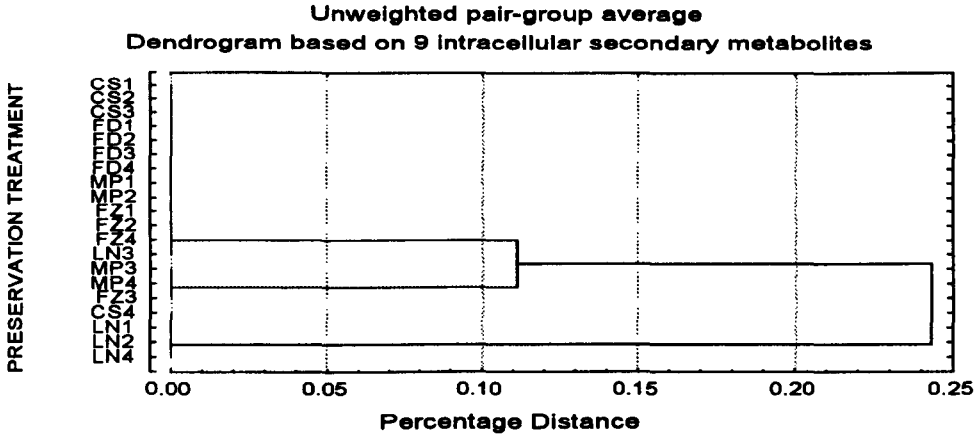
B.

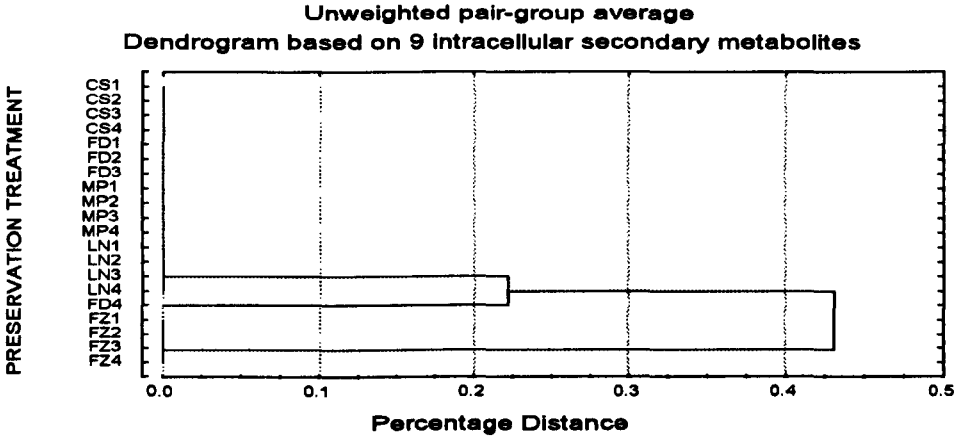
Metabolite	*	CS1	CS2	CS3	CS4	FD1	FD2	FD3	FD4	MP1	MP2	MP3	MP4	FZ1	FZ2	FZ3	FZ4	LN1	LN2	LN3	LN4
F3 I	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 J	●	X	X	X	X	X	X	X	X	X	X	X	X	X	X	●	X	●	●	●	●
F3 K	●	●	●	●	●	●	●	●	●	●	X	●	●	●	X	●	X	●	●	●	●
F3 L	●	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
F3 M	●	●	●	●	●	●	●	●	●	●	X	●	●	●	X	X	●	●	X	X	X
F3 N	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
F3 O	●	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
F3 P	●	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
F3 Q	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

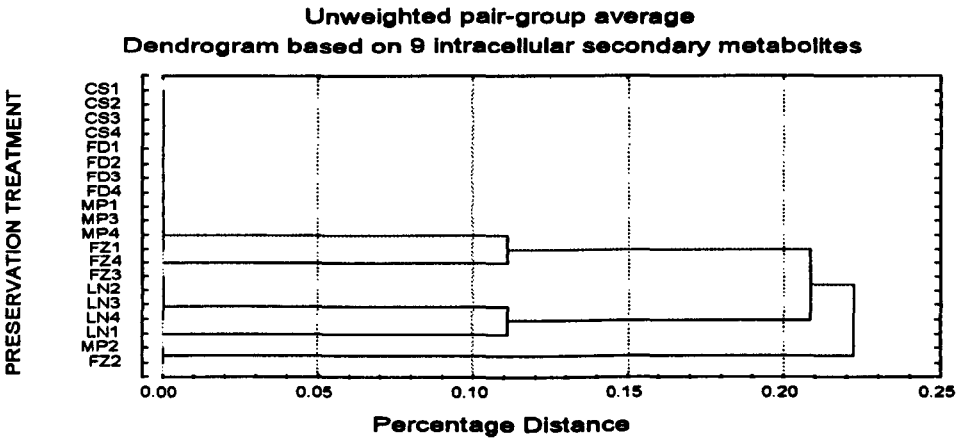
*Original Profile (see Table 40 for properties).

● Metabolite detected, X metabolite not detected

Fig. 15: A. Dendrogram produced from intracellular secondary metabolite profile of isolate F3 after 1 week of storage. B. after 16 weeks of storage and C. after 1 year of storage.







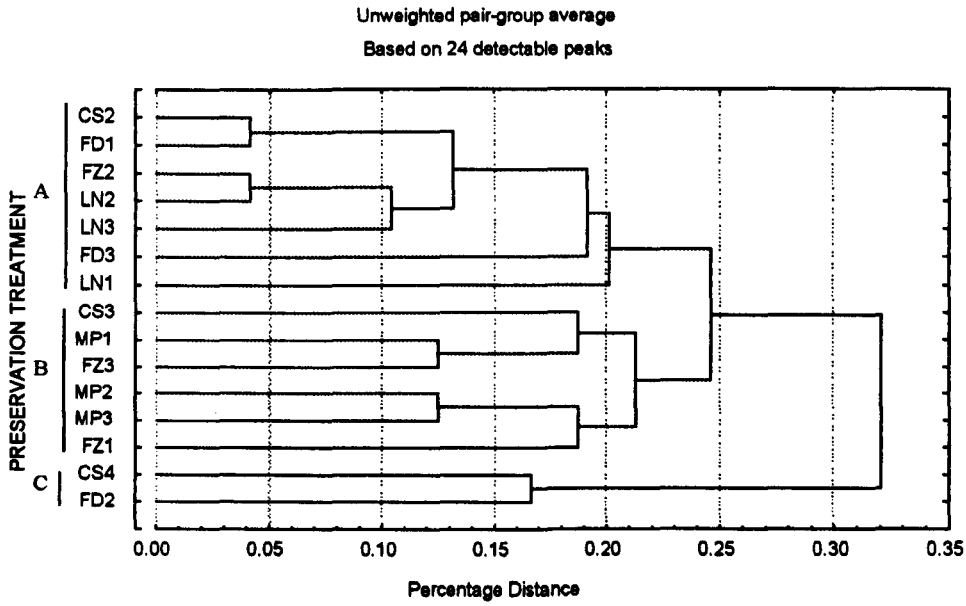
Key: CS, continual sub-culture; FD, lyophilisation, MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation.

4.3.2.2 High Performance Liquid Chromatography

Fusarium oxysporum isolate F3

Analysis of the secondary metabolite profiles obtained by HPLC analysis of replicates of *Fusarium oxysporum* isolate F3 after 16 weeks of storage yielded 24 detectable peaks. Replicates did not group on the dendrogram according to preservation method (Fig 17). However, replicates could be assigned into 3 groups (A, B and C). Group A consisted of all of the replicates that had been cryopreserved, 2 replicates that had been lyophilised, a replicate that had been stored at –20°C and a replicate that had been maintained by continual sub-culture. Group C consisted of 2 replicates (a replicate that had been lyophilised and a replicate maintained by continual sub-culture) that exhibited secondary metabolite profiles which differed substantially from the profiles of replicates that were assigned to groups A and B. Group B consisted of all of the remaining replicates and those that had been stored as mycelial plugs in water.

Fig 17. Dendrogram compiled from HPLC profiles of *Fusarium oxysporum* isolate F3 after 16 weeks of storage.



Key: CS, continual sub-culture; FD, lyophilisation, MP, mycelial plugs in water; FZ, freezing at –20°C; LN, cryopreservation.

4.4 Discussion

The extra- and intracellular secondary metabolite profiles of *Metarhizium anisopliae* and *Fusarium oxysporum* isolates were changed by preservation regime throughout the investigation. Secondary metabolism appears to be extremely sensitive in response to different preservation regimes. Even after relatively short storage times (<1 week), the profiles of many replicates preserved by different preservation treatments, were different from the profiles that were exhibited by the original isolates before preservation. The stability of secondary metabolite profiles decreased with time. Replicates that had been maintained by continual sub-culture, stored as mycelial plugs in water and stored at –20°C generally exhibited decreasing similarity as the length of storage increased. For example, after 1 year of storage, replicates of *Fusarium oxysporum* isolate F1 that had been maintained by continual sub-culture exhibited an extracellular secondary metabolite profile typical of the original isolate. After 2 years of storage, all of the replicates that had been maintained by continual sub-culture exhibited extracellular secondary metabolite profiles that had lost metabolites from the original profile. Intraspecific variability of the stability of secondary metabolite profiles in response to preservation and storage was evident in both *Fusarium* and *Metarhizium* isolates. Where all the replicates preserved by a specific preservation regime may have retained typical secondary metabolite profiles in one isolate, in another isolate of the same species replicates preserved by the same preservation regime may not have all exhibited typical secondary metabolite profiles. For example, the intracellular secondary metabolite profiles of replicates of isolate F1 stored by cryopreservation differed from the typical profile of the original isolate after 2 years of storage. However, replicates of isolate F2 that were stored by cryopreservation exhibited profiles that were similar to that exhibited by the original isolate. Similarly, after 1 year of preservation, 72% of the total number of replicates of *Metarhizium* isolate M2 preserved (irrespective of treatment) retained the intracellular secondary metabolite profile typical of the original isolate. However, only 27% of the replicates of *Metarhizium* isolate M4 retained the intracellular secondary metabolite typical of the original isolate. These results illustrate the need to consider strain-specific preservation protocols, as strains of the same species will react differently to preservation conditions. A protocol that retains the metabolite profiles in one strain may not necessarily confer stability in the metabolite profiles of replicates of other strains within the same species.

Instability in the profiles of all or some of the replicates that had been cryopreserved was evident in both intra- and extracellular secondary metabolite profiles of *Metarhizium* isolates M1 and M2, and the intracellular secondary metabolite profiles of *Fusarium* isolates F1, F2 and F3. There are no previous examples in the literature of physiological instability following cryopreservation in liquid nitrogen. However, cryopreservation induced changes in the secondary metabolite profiles of 5 out of the 6 isolates assessed which suggests that cryo-induced instability may be widespread. As the disruption in secondary metabolite profiles was seen after very short storage times, it is likely that it was the stresses encountered during the cooling and thawing processes of the protocol that induced instability rather than the length of storage *per se*. The possible mechanisms that may induce metabolic instability following cryopreservation are discussed in chapter 8. Following a recovery period, not all of the replicates exhibited secondary metabolite profiles typical of the original isolate. Replicates of *Fusarium* isolate F3 after 1 year of storage were an exception.

Despite the instability in the profiles of replicates that had been cryopreserved, "metabolite M" was detected in the profiles of all replicates that had been cryopreserved. "Metabolite M" is a metabolite that appears orange/brown and streaked under long-wave UV light after separation using the intracellular TLC solvent system. It had previously been noted to appear in the secondary metabolite profiles of *Metarhizium* strains isolated from acrids and is a complex of 15 or more metabolic components (Bridge pers.comm.). The biological function of metabolite M is not known, but its loss from the metabolite profile may be more significant. Metabolite M was not detected in many of the replicates of isolate M1 and M2 (except those that had been cryopreserved) immediately after resuscitation from preservation. Following the recovery period, it was detected in all of the replicates of isolate M2. Metabolite M was not detected in a replicate of isolate M1 that had been stored as a mycelial plug in water for 1 year and in 2 replicates that had been lyophilised after 2 years of storage. Many *Fusarium* and *Metarhizium* replicates regained the secondary metabolite profile characteristic of the original profile after the recovery period. However, not all replicates recovered and the number of replicates that failed to recover, increased as the investigation progressed. For example, after 2 years of storage only 1 out of 5 replicates of *Fusarium* isolate F2

that had been stored as a mycelial plug in water recovered the extracellular secondary metabolite profile characteristic of the original isolate. Some replicates that had exhibited typical secondary metabolite profiles immediately after storage, exhibited profiles that differed from the original after the recovery period. For example, 3 replicates of isolate M1 stored as mycelial plugs in water exhibited a metabolite profile that differed from the original after the recovery period, having initially exhibited profiles characteristic of the original isolate. The result indicates that although metabolites not detected following storage can be regained following a recovery period, instability can be induced by further sub-culture. For important isolates, where secondary metabolic stability is essential, it may be advisable for scientists to leave their isolates for 28 days following preservation and storage, but maintain several cultures to reduce the chance of any deterioration that may result from sub-culture.

Occasionally, additional metabolites were detected in metabolite profiles. For example in replicates of *Fusarium oxysporum* isolate F1 that had been cryopreserved and stored for 2 years. This could be because new synthetic pathways have become activated or alternatively the new compounds could be maybe breakdown products of existing metabolites. Where secondary profiles were changed from the original, usually only 1 or 2 metabolites were absent from the profile, but occasionally more metabolites were lost. For example, 6 out of 8 metabolites were not detected after one week of storage of a replicate of isolate M4 that had been lyophilised. This suggests greater disruption in metabolic activity, and may indicate interference in more than one synthetic pathway. During the investigation, some secondary metabolites were more susceptible to loss than others. This could be because some synthetic pathways are more susceptible to disruption. If growth is poor following resuscitation, the precursors that are required for the production of secondary metabolites from a specific synthetic pathway may not be readily available from the products of primary metabolism. Alternatively, intermediary compounds or alternative secondary metabolites may be produced, in response to adaptive mechanisms that the fungus may produce in response to any stresses encountered during preservation and storage. In *Metarhizium* isolates, where specific secondary metabolites characteristic of the original profile were not detected, additional secondary metabolites, not representative of the typical profile were detected. This

could indicate breakdown of a metabolite or that the end product of a pathway has not been synthesised. The formation of a secondary metabolite appears to be dependent on the physiological stability of the fungus. A secondary metabolite may have many precursors and is often a product of a complex synthetic pathway. If the pathway is disrupted, metabolites may not be produced. Disruption of the synthetic pathway could be due to phenotypic factors or genetic repression of the genes that encode for synthesis of essential precursors. Changes in genetic conformation may result in damage to the genes that transcribe the precursors. Thus, if a gene is not expressed the physiology of a fungus may be affected. If a fungus is stressed it may produce "stress factors" that discourage the synthesis of metabolic precursors, similar to the theory of catabolite repression of enzyme production. Ultimately the energy demand would be less, especially if recovery from storage is poor.

If growth is poor the onset of secondary metabolism may be delayed, this would explain why many cultures regained the secondary metabolite profile typical of the original isolate following a recovery period. On the other extreme, Bu'lock (1961) suggests that secondary metabolism is a mechanism of removing excess intermediary compounds during times of environmental stress. However, if such a strategy is employed it may delay the synthesis of metabolites that are produced under non-stressed conditions. In many fungi, production of secondary metabolites may be correlated with morphological differentiation (Garraway and Evans 1991). Bartmen *et al.* (1981) found that mycophenolic acid production in *Penicillium breviocompactum* is associated with the emergence of aerial hyphae. Potential correlations between cultural degeneration and secondary metabolite production are discussed in Chapter 8.

The data obtained from the HPLC data corresponded to trends that were evident from the data obtained using TLC profiles. Replicates from 4 preservation treatments of *Metarhizium* isolate M4, grouped together on the dendrogram. Two distinct groups were identifiable on the dendrogram from the data obtained from replicates of *Fusarium oxysporum* isolate F3. One group contained all the replicates that had been maintained by mycelial plugs in water. The other contained all of the replicates that had been cryopreserved and 2 of the 3 replicates that had been lyophilised. All these later replicates having undergone a cooling process during

preservation. This is further evidence to suggest that the preservation method used can have a specific effect on the metabolic stability of an isolate.

The methods selected allowed an assessment on the effect of preservation method on the stability of secondary metabolite profiles. However, there are limitations with the TLC system. The solvent systems used only allowed the detection of a small number of secondary metabolites. Metabolites that were only produced in small quantities may have been beyond the limits of detection. In this investigation only the metabolites that were strongly identified and exhibited throughout the investigation were used to compile the characteristic metabolite profiles. However, the method was highly reproducible. It should be noted that similar spots appearing at the same Rf position on a TLC plate might not necessarily be the same compound. This could lead to an over-estimate of similarity. The use of HPLC allowed a wider range of metabolites to be detected and used in analysis. However, the preparation of extracts is a time-consuming process. The use of TLC allowed many replicates to be assessed in a relatively short time, which was essential during this investigation where the metabolite profiles of many replicates needed to be established on one day. Improvement in the analysis of TLC plates could be achieved using computerised image analysis with appropriate software. Alternative, post-solvent sprays could be applied, but may result in other metabolites becoming undetectable. The identity of the secondary metabolites was not established during the investigation. Although this information would have been useful, the initial aim of the project was to assess the overall stability of metabolite profiles. To identify individual metabolites would have been time-consuming and limited the number of replicates that were assessed at each testing time and would have compromised the objectives of the project.

Secondary metabolites are compounds that are used for taxonomic and teaching purposes, research and in industrial processes. Changes in secondary metabolism following preservation, as demonstrated in this investigation may have serious consequences. For example, the loss of activity of some secondary metabolites (i.e. destruxins) may affect the ability of the fungus to maintain pathogenesis. Chemotaxonomic studies should not be carried out with fungi that have been preserved, as any changes in metabolite profile induced by preservation

protocol could cause the fungus to be incorrectly classified. The results suggest that storage in water and storage at –20°C may provide the best short-term (<16 weeks) preservation of secondary metabolite activity. These protocols are not at all suitable for longer-term (>16 weeks) preservation, as the stability of the secondary metabolite profiles rapidly deteriorates. Although both lyophilisation and cryopreservation can induce changes in secondary metabolite production, they are the most suitable long-term protocol for the preservation of the test fungi. Numerous replicates of the isolate should be preserved as back-up in case of physiological instability in test cultures. The use of strain-specific protocols should be developed to ensure the stability of secondary metabolite production. As a quality control measure, the secondary metabolite profile of an isolate should be determined on defined media under a set of controlled parameters, before preservation and at least 28-days following preservation.

CHAPTER 5: THE EFFECT OF PRESERVATION AND STORAGE ON EXTRACELLULAR ENZYME PRODUCTION

5.1 Introduction

Fungi produce extracellular enzymes (exoenzymes) which are essential for saprophytic or parasitic modes of nutrition. These enzymes allow the fungus to exploit a wide variety of extracellular carbon sources. Commonly utilised, simple carbon sources include glucose, maltose, mannose and fructose (Barnett and Hunter 1998). Other enzymes allow the utilisation of more complex carbon sources. These include proteases (e.g. trypsin), amylases (e.g. glucoamylase), cellulases, xylanases (e.g. xylosidase, glucuronidase), phosphatases (e.g. alkaline phosphatase), lipases (e.g. esterase) and ligninases (Archer and Wood 1995). Enzyme production is subservient to regulatory control mechanisms (Priest 1984). These include induction by compounds in the substratum (Ericksson 1990) or repression by the end products of metabolism (Priest 1984). Fungal enzyme production may be subject to catabolite repression i.e. enzymes are not synthesised in the presence of the preferred substrate such as glucose (Singleton and Sainsbury 1993). Fungal enzymes are widely utilised in industry and academia. Some enzymes are synthesised for use as biochemical reagents e.g. Proteinase K from *Tritirachium album* or in the production of fermented foods e.g. miso or soy sauce using *Aspergillus oryzae* and Indonesian tempe from *Rhizopus* spp. (Archer and Wood 1995). Other enzymes are produced on a huge scale for use in industry e.g. glucoamylase from *Aspergillus awamori*, cellulase from *Trichoderma reesei*, (Lowe 1992). Many industrial strains are "genetically improved" by mutagenesis or parasexual recombination to enhance enzyme production (Ball 1984). Fungal enzymes are also essential in the bioremediation of organic compounds in waste products such as trichlorophan by *Trichoderma reesei* (Nandan and Raisuddin 1992). The commercial importance of industrial strains means that it is vital that replicates are successfully preserved and stored in culture collections in case the strain in current use loses activity or no longer functions effectively. Maintenance of enzyme activity is vitally important for isolates used in chemotaxonomy and bioassays. It is also important for biological control fungi to remain enzymically stable during maintenance, preservation and storage. Successful activity against target organisms in the field relies upon the production and activity of enzymes involved in pathogenesis e.g. dispepsidases St.Leger (1992).

Fungal enzyme systems have been widely studied. Many workers have used allozyme and isozyme analysis to assess enzyme production in a variety of fungi. Tisserant *et al.* (1998) used isozyme techniques to detect strains of *Glomus* spp. in planta. Many commercial test-kits are available that allow assays of extracellular enzymes from active liquid culture filtrates or from analysis of mycelial growth on specific substrates. The APIZYM system (St.Leger *et al.* 1986, Bridge *et al.* 1993) assays 19 enzymes (Table 1, section 5.3) able to hydrolyse carbohydrates, lipids and proteins. Reactions are chemically developed and enzyme activity chromatically recorded. The API5OCH system is similar to APIZYM but assays for 49 carbohydrate-specific enzymes (Rath *et al.* 1995). Other chromatographic and fluorimetric assaying systems can be tailored to individual needs. 4-methylumbelliferyl bound substrates can be selected to assay for a wide range of extracellular enzymes (Barth and Bridge 1989). Substrates are incubated with active culture filtrate for 4 hours at 37°C and then alkali (sodium bicarbonate) is added. When viewed on a UV transilluminator, utilised substrates then fluoresce due to the release of free 4-methylumbelliferone molecules.

Fusarium oxysporum can utilise a wide variety of substrates and thus can synthesise a wide array of extracellular enzymes. Simple mono-saccharides such as glucose and more complex sugars such as galactose and starch can be utilised (Almeida 1978). Unlike other hyphomycetes, which may show catabolite repression, glucose may not inhibit the synthesis of more complex enzymes in *Fusarium* species (Smith 1994). Enzyme stability is important in *Fusarium* spp. used in food production e.g. the mycoprotein fermentation.

The enzyme system of *Metarhizium* has been well studied because of its activity during insect pathogenesis. St. Leger (1995) found that protein synthesis was stimulated in cultures of *Metarhizium* when cockroach cuticle was added to the nutrient source. This resulted in induction of enzymes (lipases, chitinases and proteases) that were capable of breaking down the complex macromolecular structure of insect cuticle. However, it was discovered that while the cuticle-degrading enzymes where synthesised, enzymes normally produced were repressed.

Bridge *et al.* (1993) established that during the active phase of growth, polysaccharide-degrading enzymes and other complex enzymes were not secreted by the fungus into the culture medium. However, once the glucose supply had been utilised, more complex enzymes were synthesised. Thus *Metarhizium* was subject to catabolite repression, glucose being preferentially utilised when available.

A number of methods have been used to analyse the enzyme profiles of *Metarhizium* spp. These include APIZYM (St.Leger *et al.* 1986, Bridge *et al.* 1993), API50CH (Rath *et al.* 1995), isozyme analysis (Riba *et al.* 1996), allozyme analysis (St.Leger *et al.*1992). and 4MU™ (Sigma) plate tests (Bridge pers.comm.). The production of an extracellular enzyme profile database of *Metarhizium* species has been suggested as an aid to taxonomic characterisation of isolates (Rath *et al.* 1995). Using API5OCH, Rath *et al.* (1995) examined the ability of 134 *Metarhizium* spp. isolates to utilise carbohydrates. Out of 49 carbohydrates assayed, 13 carbohydrates were not utilised by any of the strains and only one was utilised by all isolates (aesculin). The authors were able to distinguish 4 species of *Metarhizium* using the enzyme profiles produced from the API5OCH system. Using APIZYM, Bridge *et al.* (1993) showed that two proteases, trypsin and chymotrypsin are synthesised by *Metarhizium* in order to degrade host insect cuticles. Bridge *et al* (1993) also managed to separate isolates of *M.anisopliae* from *M. flavoviride* (now *M.flavoviride* var. *acridum*) using APIZYM.

The aim of this chapter is to assess the effects of preservation and storage on extracellular enzyme production in *Metarhizium anisopliae* and *Fusarium oxysporum*

5.2 Materials and methods

The materials and methods are listed in Chapter 2 Section 2,

5.3 Results

The enzyme profiles obtained for each isolate using APIZYM strips are listed in Table 1. The enzyme profiles obtained for each isolate using 4MU assays are listed in Table 2.

Table 1: APIZYM enzyme profiles characteristic of the original isolates before preservation.

	ISOLATE			
ENZYME	F1	F2	M1	M2
Alkaline phosphatase	●	●	●	●
Esterase	●	●	●	●
Esterase lipase	●	●	●	●
Lipase	–	O*	–	–
Leucine arylamidase	●	●	O*	O*
Valine arylamidase	●	●	O*	O*
Cystine arylamidase	–	–	O*	–
Trypsin	–	–	O*	O*
Chymotrypsin	–	–	–	●
Acid phosphatase	●	●	●	●
Phosphoamidase	●	●	●	●
α-galactosidase	–	●	●	●
β-galactosidase	–	–	●	●
β-glucuronidase	–	–	–	●
α-glucosidase	–	–	●	–
β-glucosidase	●	●	●	●
β-glucosamidase	●	●	●	●
α-mannosidase	●	●	–	–
α-fucosidase	–	–	●	●

Key: ● , enzyme activity detected; – enzyme activity not detected, O*Not produced by original isolate, but was detected in some replicates after preservation and storage

Table 2: 4MU enzyme profiles characteristic of the original isolates before preservation

	ISOLATE					
ENZYME	F1	F2	F3	M1	M2	M4
β-d-glucosamidase	●	●	●	●	●	●
α-ι-arabinofuranosidase	●	●	–	–	–	–
β-d-chitobiosidase	●	●	●	●	●	●
α-ι-fucosidase	–	–	–	●	●	●
β-d-galactosidase	–	–	–	●	●	●
β-d-glucosidase	●	●	●	●	●	●
β-d-glucuronidase	–	–	–	–	●	●
α-d-mannosidase	●	●	●	–	–	–
β-d-xylosidase	●	●	●	●	–	–
Esterase	●	●	●	●	●	●

Key: ●, enzyme activity detected; – enzyme activity not detected

5.3.1 *Fusarium oxysporum*

Fusarium oxysporum isolate F1

The enzyme profile typical of the original isolate is listed in Tables 1 and 2. Over the period of the investigation, some enzyme activities characteristic of the original profile changed. After 1 day of storage, leucine arylamidase and alkaline phosphatase activity were not detected in a replicate that had been cryopreserved. Alkaline phosphatase and β-glucosamidase activity was not detected in 1 replicate and leucine arylamidase was not detected in another replicate stored as mycelial plugs in water. After 1 year of storage, α-mannosidase activity was not detected in any of the replicates maintained by continual sub-culture, 3 replicates that had been stored as mycelial plugs in water, 2 replicates stored at –20°C and 1 replicate that had been lyophilised. Xylosidase activity was not detected in any of the replicates that had been maintained by continual sub-culture, 2 replicates stored at –20°C, a replicate stored lyophilised and a replicate stored cryopreserved. After 2 years of storage (Table 3/Fig. 1), α-mannosidase, β-xylosidase and β-glucosamidase activities were not detected in some replicates. α-Mannosidase activity was only detected in replicates that had been cryopreserved and lyophilised and in 3 replicates that had been stored at –20°C. β-Glucosamidase activity was not detected in a replicate stored

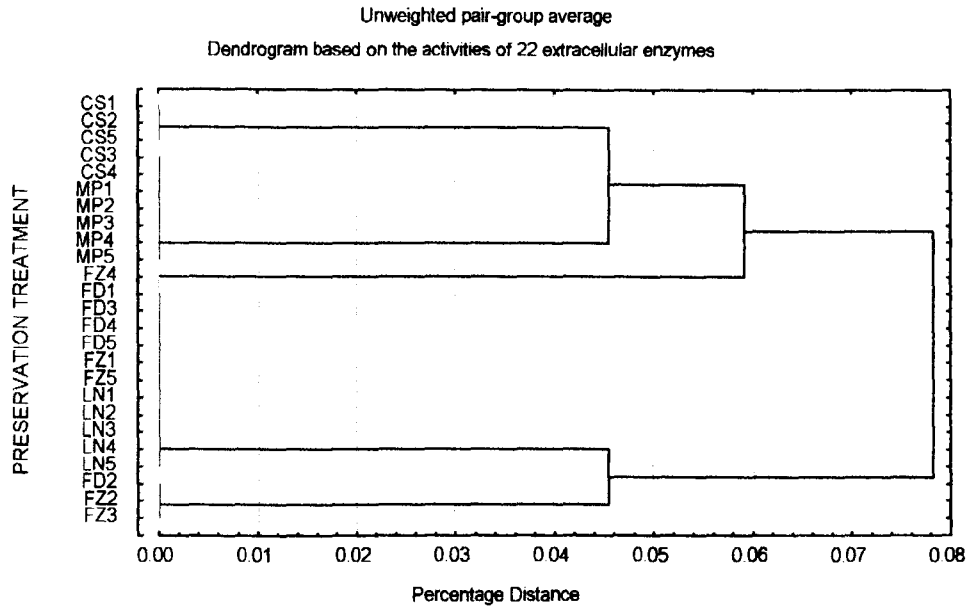
at –20°C. β-Xylosidase activity was not detected in any replicates that had been stored as mycelial plugs in water, 3 out of 5 replicates that had been stored at –20°C, 2 out of 5 replicates that had been maintained by continual sub-culture and a single replicate that had been stored lyophilised.

Table 3: Enzyme activities of replicates of *Fusarium oxysporum* isolate F1 after 2 years of preservation. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at –20°C, LN= Cryopreservation)

Rep	Alkaline phosphatase	Esterase	Esterase Lipase	Lipase	Leucine arylamidase	Valine arylamidase	Cystine arylamidase	Trypsin	Chymotrypsin	Acid phosphatase	Phosphoamidase	α-galactosidase	β-galactosidase	β-glucuronidase	α-glucosidase	β-glucosidase	β-glucosamidase	α-mannosidase	α-fucosidase	α-arabinofuranosidase	β-chitobiosidase	β-xylosidase
Original	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	●	X	●	●	●
CS1	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	X	X	●	●	●
CS2	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	X	X	●	●	●
CS3	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	X	X	●	●	X
CS4	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	X	X	●	●	X
CS5	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	X	X	●	●	●
FD1	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	●	X	●	●	●
FD2	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	●	X	●	●	X
FD3	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	●	X	●	●	●
FD4	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	●	X	●	●	●
FD5	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	●	X	●	●	●
MP1	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	X	X	●	●	X
MP2	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	X	X	●	●	X
MP3	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	X	X	●	●	X
MP4	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	X	X	●	●	X
MP5	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	X	X	●	●	X
FZ1	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	●	X	●	●	●
FZ2	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	●	X	●	●	X
FZ3	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	●	X	●	●	X
FZ4	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	X	X	X	●	●	X
FZ5	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	●	X	●	●	●
LN1	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	●	X	●	●	●
LN2	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	●	X	●	●	●
LN3	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	●	X	●	●	●
LN4	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	●	X	●	●	●
LN5	●	●	●	X	●	●	X	X	X	●	●	X	X	X	X	●	●	●	X	●	●	●

Key: ● enzyme activity detected; X enzyme activity not detected; original refers to enzyme activity of original isolate; Rep, replicate.

Fig. 1: Dendrogram compiled from APIZYM and 4MU enzyme profiles of *Fusarium oxysporum* isolate F1 after 2 years of storage.



Key: CS, continual sub-culture; FD, lyophilised; MP, mycelial plugs in water; FZ, freezing at –20°C, LN, cryopreserved.

Fusarium oxysporum isolate F2

The enzyme profile typical of the original isolate is listed in Tables 1 and 2. Over the testing period of the investigation, some enzyme activities characteristic of the original profile of isolate F2 changed. After 1 day of storage, alkaline phosphatase and leucine arylamidase activity were not detected in a single replicate that had been stored as a mycelial plug in water and a single replicate that had been stored at –20°C. After 1 year of storage, α-mannosidase activity was not detected in a replicate that had been stored at –20°C. Alkaline phosphatase was not detected in a single replicate that had been stored as a mycelial plug in water and in a single replicate that had been cryopreserved. After 2 years of storage (Fig 2), valine arylamidase activity was not detected in 2 replicates and α-mannosidase activity was not detected in another replicate that had been lyophilised. β-Chitobiosidase and α-arabinofuranosidase activity was not detected in 3 replicates that had been stored at –20°C. β-Xylosidase, β-chitobiosidase and α-arabinofuranosidase activity were not detected in a replicate that had been stored at –20°C. β-Xylosidase and α-arabinofuranosidase activities were not detected in a replicate that had been lyophilised. Lipase activity was not detected either before or after 1-day of

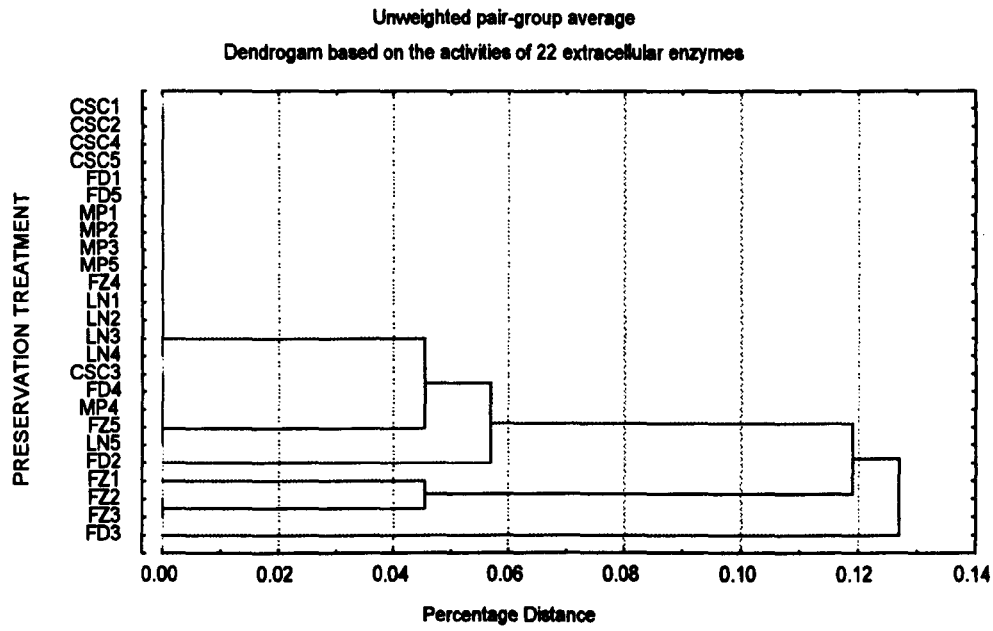
preservation. However, weak lipase activity was detected in replicates that had been stored as mycelial plugs in water and that had been cryopreserved after 1 year of storage. After 2 years of storage (Table 4) lipase activity was detected in all replicates, except 2 replicates that had been stored lyophilised and a replicate that had been maintained by continual sub-culture.

Table 4: Enzyme activities of replicates of *Fusarium oxysporum* isolate F2 after 2 years of preservation. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation)

Rep	Alkaline phosphatase	Esterase	Esterase Lipase	Lipase	Leucine arylamidase	Valine arylamidase	Cystine arylamidase	Trypsin	Chymotrypsin	Acid phosphatase	Phosphoamidase	α-galactosidase	β-galactosidase	β-glucuronidase	α-glucosidase	β-glucosidase	β-glucosamidase	α-mannosidase	α-fucosidase	α-arabinofuranosidase	β-chitobiosidase	β-xylosidase
Original	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
CS1	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
CS2	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
CS3	●	●	●	X	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
CS4	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
CS5	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
FD1	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
FD2	●	●	●	●	●	X	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
FD3	●	●	●	X	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	X	●	X
FD4	●	●	●	X	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
FD5	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
MP1	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
MP2	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
MP3	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
MP4	●	●	●	X	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
MP5	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
FZ1	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	X	X	X
FZ2	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	X	X	●
FZ3	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	X	X	●
FZ4	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
FZ5	●	●	●	X	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
LN1	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
LN2	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
LN3	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
LN4	●	●	●	●	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●
LN5	●	●	●	X	●	●	X	X	X	●	●	●	X	X	X	●	●	●	X	●	●	●

Key: ● enzyme activity detected; X enzyme activity not detected; original refers to enzyme activity of original isolate; Rep, replicate.

Fig. 2: Dendrogram compiled from APIZYM and 4MU enzyme profiles of *Fusarium oxysporum* isolate F2 after 2 years of storage.



Key: CS, continual sub-culture; FD, lyophilised; MP, mycelial plugs in water; FZ, freezing at –20°C, LN, cryopreserved.

Fusarium oxysporum isolate F3

The enzyme profile typical of the original isolate is listed in Tables 1 and 2. Over the period of the investigation, some enzyme activities characteristic of the original profile of isolate F3 changed. After 1 week of storage, xylosidase activity was not detected in a replicate that had been cryopreserved. α-Mannosidase activity was not ~~detected in a~~ replicate ~~that~~ had been cryopreserved. All other replicates exhibited extracellular enzyme profiles that were characteristic of the original isolate before preservation. After 16 weeks of storage, α-mannosidase activity was not detected in a single replicate that had been cryopreserved and a single replicate that had been stored as a mycelial plug in water. After 1 year of storage (Table 5), esterase activity was not detected in a single replicate that had been stored lyophilised. β-Glucosamidase and β-chitobiosidase activities were not detected in a

single replicate that had been maintained by continual sub-culture. After a recovery period this replicate regained β-chitobiosidase activity but not β-glucosamidase activity

Table 5: Enzyme activities of replicates of *Fusarium oxysporum* isolate F3 after 1 year of preservation. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at –20°C, LN= Cryopreservation)

Replicate	β-glucosamidase	α-arabinofuranosidase	β-chitobiosidase	α-fucosidase	β-galactosidase	β-glucosidase	β-glucuronidase	α-mannosidase	β-xylosidase	Esterase
Original	●	X	●	X	X	●	X	●	●	●
CS1	●	X	●	X	X	●	X	●	●	●
CS2	●	X	●	X	X	●	X	●	●	●
CS3	●	X	●	X	X	●	X	●	●	●
CS4	X	X	X	X	X	●	X	●	●	●
CS5	●	X	●	X	X	●	X	●	●	●
FD1	●	X	●	X	X	●	X	●	●	●
FD2	●	X	●	X	X	●	X	●	●	●
FD3	●	X	●	X	X	●	X	●	●	●
FD4	●	X	●	X	X	●	X	●	●	●
FD5	●	X	●	X	X	●	X	●	●	X
MP1	●	X	●	X	X	●	X	●	●	●
MP2	●	X	●	X	X	●	X	●	●	●
MP3	●	X	●	X	X	●	X	●	●	●
MP4	●	X	●	X	X	●	X	●	●	●
MP5	●	X	●	X	X	●	X	●	●	●
FZ1	●	X	●	X	X	●	X	●	●	●
FZ2	●	X	●	X	X	●	X	●	●	●
FZ3	●	X	●	X	X	●	X	●	●	●
FZ4	●	X	●	X	X	●	X	●	●	●
FZ5	●	X	●	X	X	●	X	●	●	●
LN1	●	X	●	X	X	●	X	●	●	●
LN2	●	X	●	X	X	●	X	●	●	●
LN3	●	X	●	X	X	●	X	●	●	●
LN4	●	X	●	X	X	●	X	●	●	●
LN5	●	X	●	X	X	●	0	●	●	●

Key: ● enzyme activity detected; X enzyme activity not detected; original refers to enzyme activity of original isolate

5.3.2 *Metarhizium anisopliae*

Metarhizium anisopliae isolate M1

The enzyme profile typical of the original isolate is listed in Tables 1 and 2. Over the period of the investigation, some enzyme activities characteristic of the original profile of isolate M1 changed.

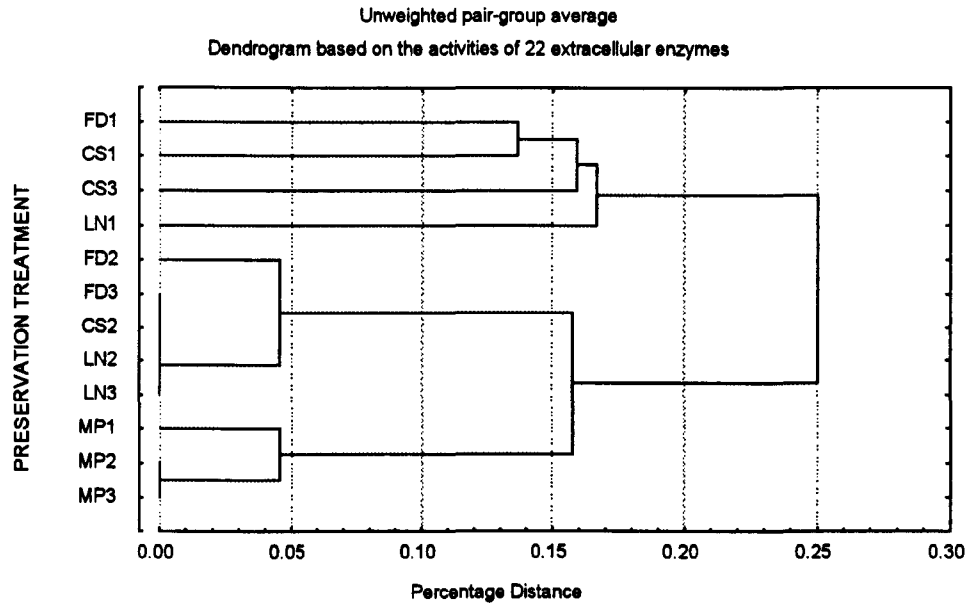
Table 6: Enzyme activities of replicates of *Metarhizium anisopliae* isolate M1 after 2 years of preservation. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at -20°C, LN= Cryopreservation.)

Rep	Alkaline phosphatase	Esterase	Esterase Lipase	Lipase	Leucine arylamidase	Valine arylamidase	Cystine arylamidase	Trypsin	Chymotrypsin	Acid phosphatase	Phosphoamidase	α-galactosidase	β-galactosidase	β-glucuronidase	α-glucosidase	β-glucosidase	β-glucosamidase	α-mannosidase	α-fucosidase	α-arabinofuranosidase	β-chitobiosidase	β-xylosidase
Original	●	●	●	X	X	X	X	X	X	●	●	●	●	X	●	●	●	X	●	X	●	X
CS1	X	●	●	X	●	●	●	X	X	●	●	●	X	X	X	●	●	X	●	X	●	X
CS2	X	●	●	X	X	X	X	X	X	●	●	X	●	X	X	●	●	X	●	X	●	●
CS3	●	●	●	X	●	●	●	X	X	●	●	X	X	X	●	●	●	X	●	X	●	X
FD1	●	●	●	X	●	●	X	X	X	●	●	●	●	X	X	●	●	X	●	X	●	X
FD2	X	●	●	X	X	X	X	X	X	●	●	●	●	X	X	●	●	X	●	X	●	●
FD3	X	●	●	X	X	X	X	X	X	●	●	X	●	X	X	●	●	X	●	X	●	●
MP1	X	●	X	X	X	X	X	X	X	●	●	●	●	X	X	●	●	X	●	X	●	X
MP2	●	●	X	X	X	X	X	X	X	●	●	●	●	X	X	●	●	X	●	X	●	X
MP3	●	●	X	X	X	X	X	X	X	●	●	●	●	X	X	●	●	X	●	X	●	X
LN1	X	●	●	X	●	X	●	X	X	●	●	●	●	X	●	●	●	X	●	X	●	X
LN2	X	●	●	X	X	X	X	X	X	●	●	X	●	X	X	●	●	X	●	X	●	●
LN3	X	●	●	X	X	X	X	X	X	●	●	X	●	X	X	●	●	X	●	X	●	●

Key: ● enzyme activity detected; X enzyme activity not detected; original refers to enzyme activity of original isolate; Rep, replicate.

After 1 year of storage (Table 6 / Fig 3), alkaline phosphatase activity was not detected in 8 replicates, only 2 replicates that had been stored as mycelial plugs in water, 1 replicate that had been stored lyophilised and 1 replicate that had been maintained by continual sub-culture retained alkaline phosphatase activity. Leucine, valine and cystine arylamidase activities, not characteristic in the profile of the original isolate were detected in 2 replicates that had been maintained by continual sub-culture, 1 replicate that had been stored lyophilised and 1 replicate that had been stored cryopreserved. Esteraselipase activity was not detected in 3 replicates that had been stored as mycelial plugs in water. β-Galactosidase activity was not detected in 2 replicates that had been maintained by continual sub-culture. α-Galactosidase activity was not detected in a replicate that had been lyophilised, 2 replicates that had been maintained by continual sub-culture and 2 replicates that had been cryopreserved. β-Xylosidase activity was not detected in any replicates that had been stored as mycelial plugs in water, 2 replicates that had been maintained by continual sub-culture, a single replicate that had been lyophilised and a single replicate that had been cryopreserved. After 2 years of storage, 12 replicates did not exhibited alkaline phosphatase activity. Only a single replicate that had been lyophilised, a single replicate stored as a mycelial plug in water and a single replicate stored cryopreserved retained alkaline phosphatase activity. Esterase lipase activity was not detected in 2 replicates that had been stored as mycelial plugs in water, a single replicate that had been stored lyophilised and a single replicate stored cryopreserved. Leucine, cystine and valine arylamidase activity was not detected in 3 replicates that had been stored cryopreserved and 2 replicates that had been stored lyophilised. Trypsin activity was detected in 3 replicates that had been stored as mycelial plugs in water and 2 replicates that had been stored lyophilised. α-Fucosidase activity was not detected in 2 replicates and α-glucosidase activity not detected in 4 replicates that had been stored cryopreserved. β-Xylosidase activity was detected in 2 replicates that had been cryopreserved and a replicate had been lyophilised.

Fig. 3: Dendrogram compiled from APIZYM and 4MU enzyme profiles of *Metarhizium anisopliae* isolate M1 after 1 year of storage.



Key: CS, continual sub-culture; FD, lyophilised; MP, mycelial plugs in water; FZ, freezing at –20°C, LN, cryopreserved.

Metarhizium anisopliae isolate M2

The enzyme profile typical of the original isolate is listed in Tables 1 and 2. Over the period of the investigation, some enzyme activities characteristic of the original profile of isolate M2 changed. After 1 day of storage, esteraselipase activity was not detected in a replicate that had been lyophilised. α-Fucosidase and β-galactosidase activities were not detected in a replicate that had been stored at –20°C. After 1 year of storage (Fig 4), alkaline phosphatase, esterase, β-galactosidase, β-glucuronidase, β-chitobiosidase and β-glucosidase activities were not detected in a replicate maintained by continual sub-culture. β-Galactosidase, α fucosidase, β-chitobiosidase and β-glucuronidase activity was not detected in a replicate that had been cryopreserved. After 2 years of storage (Table 7 / Fig 5) alkaline phosphatase activity was not detected in a single replicate that had been lyophilised. β-Galactosidase activity was not detected in a single replicate that had been cryopreserved. β-Glucuronidase activity, alkaline phosphatase and phosphoamidase activities were not detected in a replicate that had been cryopreserved. Trypsin activity was not detected in any replicates after 1 day of storage and after 1 year of

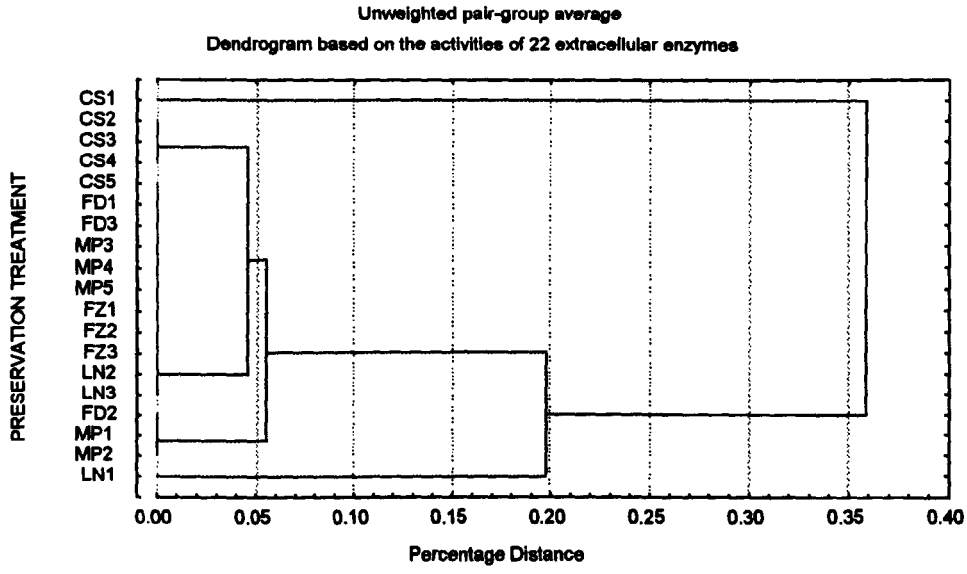
storage was only detected in 3 replicates (2 replicates stored as mycelial plugs in water and a single replicate that had been stored lyophilised). However, after 2 years of storage, trypsin activity was detected in all of the replicates that had been stored at –20°C, all of the replicates that had been stored as mycelial plugs in water, a single replicate that had been stored lyophilised and a single replicates that had been stored cryopreserved.

Table 7: Enzyme activities of replicates of *Metarhiziun anisopliae* isolate M2 after 2 years of preservation. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at –20°C, LN= Cryopreservation)

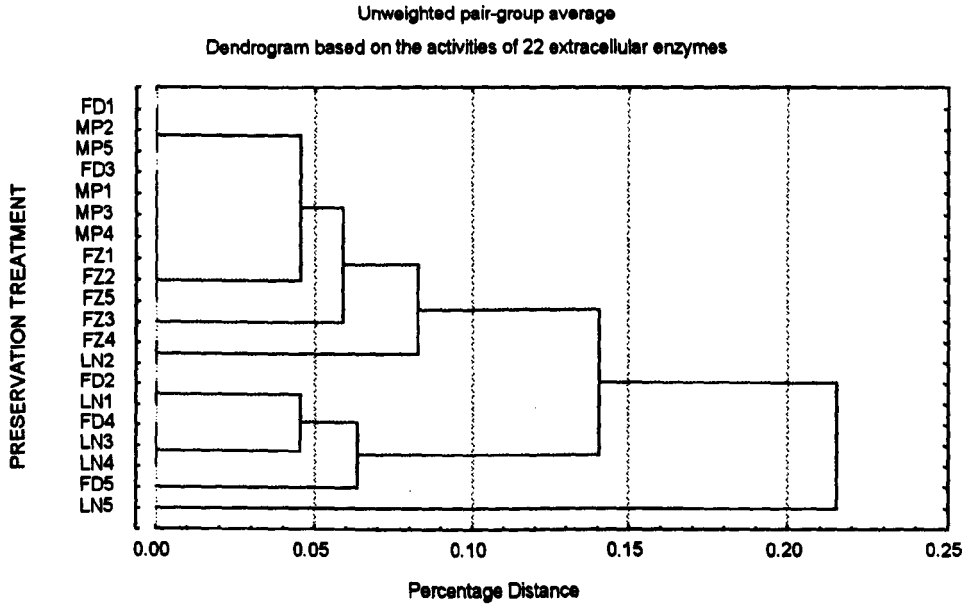
Rep	Alkaline phosphatase	Esterase	Esterase Lipase	Lipase	Leucine arylamidase	Valine arylamidase	Cystine arylamidase	Trypsin	Chymotrypsin	Acid phosphatase	Phosphoamidase	α-galactosidase	β-galactosidase	β-glucuronidase	α-glucosidase	β-glucosidase	β-glucosamidase	α-mannosidase	α-fucosidase	α-arabinofuranosidase	β-chitobiosidase	β-xylosidase
Original	●	●	●	X	X	X	X	X	●	●	●	X	●	●	X	●	●	X	●	X	●	X
FD1	●	●	●	X	●	●	X	●	X	●	●	●	●	●	X	●	●	X	●	X	●	X
FD2	●	●	●	X	X	X	X	X	X	●	●	●	●	●	X	●	●	X	●	X	●	X
FD3	●	●	●	X	●	X	X	●	X	●	●	●	●	●	X	●	●	X	●	X	●	X
FD4	●	●	●	X	X	X	X	X	X	●	●	●	●	●	X	●	●	X	●	X	●	X
FD5	X	●	●	X	x	X	X	X	X	●	●	●	●	●	X	●	●	X	●	X	●	X
MP1	●	●	●	X	●	X	X	●	X	●	●	●	●	●	X	●	●	X	●	X	●	X
MP2	●	●	●	X	●	●	X	●	X	●	●	●	●	●	X	●	●	X	●	X	●	X
MP3	●	●	●	X	●	X	X	●	X	●	●	●	●	●	X	●	●	X	●	X	●	X
MP4	●	●	●	X	●	X	X	●	X	●	●	●	●	●	X	●	●	X	●	X	●	X
MP5	●	●	●	X	●	●	X	●	X	●	●	●	●	●	X	●	●	X	●	X	●	X
FZ1	●	●	●	X	●	X	X	●	X	●	●	●	●	●	X	●	●	X	●	X	●	X
FZ2	●	●	●	X	●	X	X	●	X	●	●	●	●	●	X	●	●	X	●	X	●	X
FZ3	X	●	●	X	●	X	X	●	X	●	●	●	●	●	X	●	●	X	●	X	●	X
FZ4	●	●	●	X	●	●	X	●	X	●	●	●	X	●	X	●	●	X	●	X	●	X
FZ5	●	●	●	X	●	x	X	●	X	●	●	●	●	●	X	●	●	X	●	X	●	X
LN1	●	●	●	X	X	X	X	X	X	●	●	●	●	●	X	●	●	X	●	X	●	X
LN2	●	●	●	X	●	●	X	●	X	●	●	●	X	●	X	●	●	X	●	X	●	X
LN3	●	●	●	X	X	X	X	X	X	●	●	●	●	●	X	●	●	X	●	X	●	X
LN4	●	●	●	X	X	X	X	X	X	●	●	●	●	●	X	●	●	X	●	X	●	X
LN5	X	●	●	X	X	X	X	X	X	●	●	X	●	X	X	●	●	X	●	X	●	X

Key: ● enzyme activity detected; X enzyme activity not detected; original refers to enzyme activity of original isolate; Rep, replicate.

Fig. 4: Dendrogram compiled from APIZYM and 4MU enzyme profiles of *Metarhizium anisopliae* isolate M2 after 1 year of storage and B. after 2 years of storage.



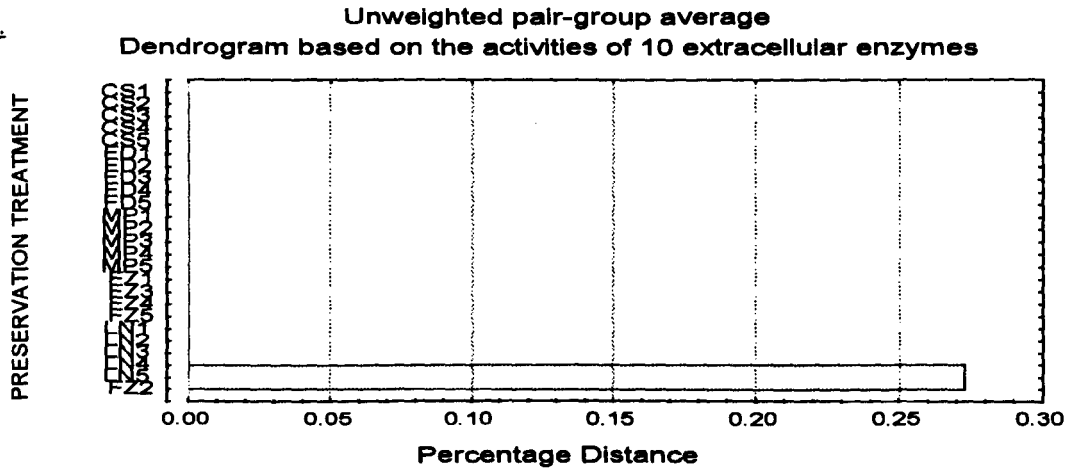
B.



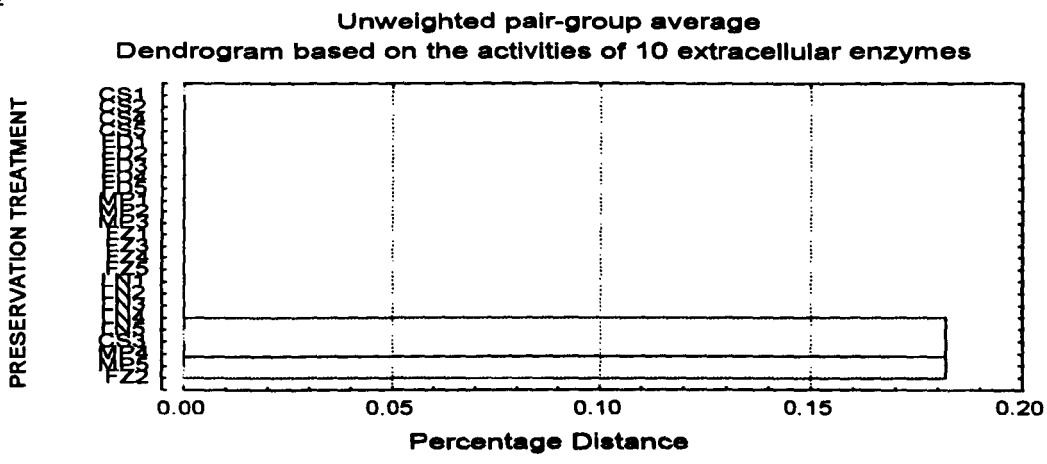
Key: CS, continual sub-culture; FD, lyophilised; MP, mycelial plugs in water; FZ, freezing at –20°C, LN, cryopreserved.

Fig. 6: A. Dendrogram compiled from 4MU enzyme profiles of *Metarhizium* spp. isolate M4 after 1 week of storage. B. after 16 weeks of storage and C. after 1 year of storage.

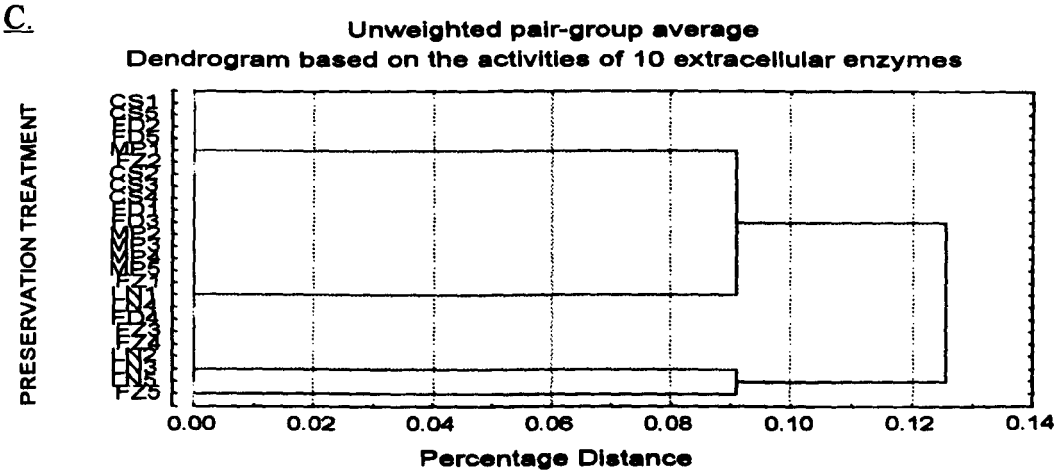
A.



B.



C.



Key: CS, continual sub-culture; FD, lyophilised; MP, mycelial plugs in water; FZ, freezing at –20°C, LN, cryopreserved.

Metarhizium spp. isolate M4

The enzyme profile typical of the original isolate is listed in Tables 1 and 2. Over the period of the investigation, some enzyme activities characteristic of the original profile of isolate M4 changed. Enzyme profiles became increasingly changed as the investigation proceeded. After 1 week of storage (Fig 6A), α-fucosidase, β-galactosidase and β-glucuronidase activities were not detected in a replicate that had been stored at –20°C. After 16 weeks of storage (Fig 6B) α-fucosidase and β-glucuronidase activity was not detected in 2 replicates that had been stored as mycelial plugs in water and a single replicate that had been maintained by continual sub-culture. After 1 year of storage, chitobiosidase activity was not detected in 3 replicates that had been stored at –20°C, a single replicate that had been lyophilised and 3 that had been cryopreserved. β-Galactosidase activity was not detected in 2 replicates that had been maintained by continual sub-culture, 2 replicates that had been stored as mycelial plugs in water and 2 replicates that had been stored at –20°C. After a recovery period, β-galactosidase activity was detected in all of the replicates that had initially lost activity. β-Chitobiosidase activity was recovered in a replicate that had been stored cryopreserved, but was not recovered in any of the other replicates.

Table 8: Enzyme activities of replicates of *Metarhizium anisopliae* isolate M4 after 1 year of preservation. (CS=Continual Sub-culture, FD=lyophilisation, MP=Mycelial Plugs in water, FZ=Freezing at –20°C, LN= Cryopreservation)

Replicate	β-glucosamidase	α-arabinofuranosidase	β-chitobiosidase	α-fucosidase	β-galactosidase	β-glucosidase	β-glucuronidase	α-mannosidase	β-xylosidase	Esterase
Original	●	X	●	●	●	●	●	X	X	●
CS1	●	X	●	●	X	●	●	X	X	●
CS2	●	X	●	●	●	●	●	X	X	●
CS3	●	X	●	●	●	●	●	X	X	●
CS4	●	X	●	●	●	●	●	X	X	●

CS5	●	X	●	●	X	●	●	X	X	●
FD1	●	X	●	●	●	●	●	X	X	●
FD2	●	X	●	●	X	●	●	X	X	●
FD3	●	X	●	●	●	●	●	X	X	●
FD4	●	X	X	●	●	●	●	X	X	●
FD5	●	X	●	●	X	●	●	X	X	●
MP1	●	X	●	●	X	●	●	X	X	●
MP2	●	X	●	●	●	●	●	X	X	●
MP3	●	X	●	●	●	●	●	X	X	●
MP4	●	X	●	●	●	●	●	X	X	●
MP5	●	X	●	●	●	●	●	X	X	●
FZ1	●	X	●	●	●	●	●	X	X	●
FZ2	●	X	●	●	X	●	●	X	X	●
FZ3	●	X	X	●	●	●	●	X	X	●
FZ4	●	X	X	●	●	●	●	X	X	●
FZ5	●	X	X	●	X	●	●	X	X	●
LN1	●	X	●	●	●	●	●	X	X	●
LN2	●	X	X	●	●	●	●	X	X	●
LN3	●	X	X	●	●	●	●	X	X	●
LN4	●	X	●	●	●	●	●	X	X	●
LN5	●	X	X	●	●	●	●	X	X	●

Key: ● enzyme activity detected; X enzyme activity not detected; original refers to enzyme activity of original isolate.

5.4 Discussion

Extracellular enzyme production in *Fusarium oxysporum* and *Metarhizium anisopliae* can be changed by preservation regime and is indicative of the physiological stability of strains following resuscitation from storage. Analyses of the dendrograms compiled from the enzyme profiles of the replicates for each isolate at each testing time showed increased instability as the investigation progressed. Clusters often contained all of the replicates preserved by a specific method in isolates of both *Fusarium* and *Metarhizium*. Stability of enzyme profiles was strain-specific. For example, in *Fusarium oxysporum* isolate F2 after two years of preservation, the enzyme profiles typical of the original isolate were produced in 60% of the replicates, but in isolate F1 only 44% of the replicates produced the enzyme profile typical of the original isolate. In both cases up to 3 enzymes from the profile of the original isolate were not detected. In *Metarhizium anisopliae* isolate M1 27% of replicates maintained the enzyme profile typical of the original isolate after two years of preservation, but only 12% of replicates of isolate M2 retained enzyme profile typical of the original isolate. Preservation regime had little effect on the enzyme profiles produced by replicates of *Fusarium oxysporum* isolate F3. After one year of storage, only three replicates produced profiles that differed from the original profile exhibited before preservation. However, profiles were altered in two replicates that had been cryopreserved and one that had been lyophilised. This would suggest that the physical preservation processes have affected the physiological stability of these replicates. This is discussed further in Chapter 8. Over the investigation period, the similarity between the enzyme profiles produced by the replicates from the different preservation methods decreased. This was seen over 1 year for replicates of *F. oxysporum* isolate F3 and between the one and two year testing times in replicates of *F.oxysporum* isolates F1 and F2.

After two years of storage, none of the replicates of *Fusarium oxysporum* isolate F1 maintained by continual sub-culture and stored as mycelial plugs in water, along with two replicates stored at –20°C and one replicate stored lyophilised, retained the enzyme profile typical of the original isolate that was exhibited before preservation. Most of the replicates that had different enzyme profiles had either lost α-mannosidase or β-xylosidase activity or both. Xylose ('wood sugar') occurs in

xylans, which forms the major hemicellulose component of plant cell walls (Singleton and Sainsbury 1993). It may be important for phytopathogenic fungi to be able to synthesise xylosidase, an enzyme that may be used during pathogenesis. Loss of production of this enzyme may affect the ability of the fungus to induce pathogenesis and maintain a parasitic mode of nutrition. Mannosidase is also found in plant cells (Singleton and Sainsbury 1993) and may also be required for the maintenance of parasitism in plant hosts. Although not assayed for in this investigation, the metabolite swainsonine, produced by some isolates of *Metarhizium* (Sim and Perry 1995) is reported to inhibit the synthesis of mannosidase (Bridge pers.comm). In some replicates of *Fusarium oxysporum* isolate F2 that had been lyophilised and stored at –20°C, arabinofuranosidase activity was not detected. This enzyme may also be involved in the initiation of pathogenesis and the maintenance of parasitism. Arabinofuranosyl residues form part of the hemicellulose component of cell walls in grasses (Singleton and Sainsbury 1993). As with xylosidase and mannosidase, the non-synthesis of this enzyme may affect the phytopathogenic fitness of the fungus, especially in replicates where the loss of activity of this enzyme is associated with the loss of other enzymes.

Other enzymes that were occasionally lost in replicates of *Fusarium oxysporum* included chitobiosidase, glucosamidase and leucine arylamidase. These enzymes, like those that are involved in the initiation and maintenance of pathogenesis, breakdown substrates that yield more complex carbon sources than those that breakdown substrates that yield "simple" sugars. Simple carbon sources may be preferentially utilised when growth is poor or the fungus has been stressed as a result of preservation and storage. Chitobiose is a complex sugar (a disaccharide consisting of (1-4)-β-linked *N*-acetyl-D-glucosamine) and so chitobiosidase utilisation may require more metabolic energy, making chitobiosidase synthesis wasteful. Similarly, glucosamidase is an enzyme that may be involved in pathogenesis and also yield complex carbon sources. Alkaline phosphatase activity was also lost in some replicates, it is an enzyme that is produced by all eukaryotic organisms (Singleton and Sainsbury 1993). It is not known whether enzymes are not being expressed as a result of the regulation of phenotypic factors i.e. whether the genes coding for the synthesis of an enzyme are simply "switched off" or whether

the genes have been physically disrupted as a result of the stresses encountered during preservation and storage. Poor suppression of dormancy may promote irreversible adaptation of the fungus to a specific environmental condition and loss of ability to synthesise specific enzymes. A potential method to determine the mechanism of the failure of a replicate to produce an enzyme could be established using molecular methods. Primers could be designed from known segments of the sequence of a gene that encodes for the synthesis of an enzyme. This primer could then be used in PCR. If the enzyme is not being synthesised, but a band is produced on separation of PCR product on a gel, we can deduce that the enzyme is not being expressed i.e. that its transcription has been "switched off". Alternatively if the enzyme is not produced and there is not a band on a gel following PCR we can assume that the enzyme is not being produced because of a conformational change at the genetic level. The use of Reverse transcription-PCR (RT-PCR) can also be applied to measure gene expression (Reidy *et al.* (1995). RT-PCR a very sensitive method that requires only limited amounts of specific RNA to be effective (Edel 1998). The theories for genetic change are discussed in Chapter 6. If a fungus is stressed during preservation and storage it is possible that it may produce stress factors that discourage the synthesis of more complex enzymes (such as β-chitobiosidase). Similar to the theory of catabolite repression, enzymes that allow the utilisation of simple substrates would be synthesised in preference to more complex substrates. Ultimately the energy demand would be less, especially if recovery from storage is poor. It is possible that the products of secondary metabolism could also inhibit the synthesis of some enzymes (see final discussion).

Increasing storage time led to further changes of the enzyme profiles in all isolates of *Metarhizium*. In isolate M4, (irrespective of the method of preservation protocol used), 4% of replicates showed alterations in enzyme profiles after one week of storage, 16 % after sixteen weeks of storage and 52% after one year of storage. Further degeneration was also observed in the enzyme profiles between the one and two year testing times in isolates M1 and M2. A number of the cryopreserved and lyophilised replicates also showed differences from the enzyme profile characteristic of that produced by the original isolate and the possible reasons for this are discussed in Chapter 8. Some replicates, for example a replicate of isolate

M2 that had been stored cryopreserved for two years, failed to produce β-galactosidase, α-fucosidase, β-chitobiosidase or β-glucuronidase activity; this may suggest more serious physiological disruption. Generally, where profiles were changed after preservation and storage only one or two enzymes were not detected. The most common enzymes that were not detected in replicates that had disrupted enzyme profiles included galactosidase, glucuronidase, fucosidase, and chitobiosidase. It is likely that other enzymes (i.e. those that breakdown substrates that yields simple sugars that are easier for the fungus to utilise) may be synthesised in preference to those that were not detected. The sugar fucose (polydeoxygalactose) is rare in nature and is not easily utilisable (Bridge pers.comm). Synthesis of the enzyme fucosidase will ultimately require more metabolic energy to produce. If growth is poor after resuscitation the fungus may employ a more direct strategy to ensure a quicker return to physiological stability. Fucosidase may also be inhibited by some products of secondary metabolism (Bridge pers.comm).

In replicates of *Metarhizium* isolates, enzyme activity that was not detected before preservation was detected as the investigation proceeded. In replicates of *Metarhizium* isolate M1, trypsin, leucine arylamidase, cystine arylamidase and valine arylamidase enzyme activities were detected as the investigation progressed. For example, after two years of storage leucine arylamidase activity was detected in all replicates that had been stored as mycelial plugs in water but was not detected in some replicates that had been lyophilised and cryopreserved. In replicates of isolate M2 trypsin, leucine arylamidase and valine arylamidase activities were not detected in any replicates before or after one day of preservation. After one year of storage, trypsin activity was detected in two replicates that had been stored as mycelial plugs in water and one that had been lyophilised. After two years of storage, trypsin activity was detected in all replicates stored at –20°C and as mycelial plugs in water, two replicates that had been stored lyophilised and one replicate that had been stored cryopreserved. Protease production in *Metarhizium* is generally subject to catabolite repression (Clarkson pers.comm). Why should the fungus produce enzymes that require expenditure of metabolic energy when the substrate already contains high levels of simple sugars? St.Leger *et al.* (1995) suggest that because *Metarhizium* exists in diverse microcosms in the host or natural environment, it is appropriate for

it to possess considerable physiological adaptability. To achieve this, subtle regulatory systems are required to modulate virulence factor expression (i.e. enzymes involved in pathogenesis) appropriate to the local environment of the pathogens. Trypsin has been implicated in cuticle breakdown during pathogenesis, its synthesis generally promoted by the presence of the insect cuticle (St.Leger 1995). However, if *Metarhizium* can easily express more complex enzymes in the presence of glucose what is the mechanism that is causing the change of metabolic strategy, especially as there are no protease induction factors in the medium? It is possible that the physical preservation process and length of storage time are causing stress to the fungus, which on resuscitation will induce an "emergency strategy"; this may include the production of enzymes not normally produced because of disruption of the subtle regulatory pathways controlling enzyme synthesis in *Metarhizium*. This is demonstrated in the detection of trypsin activity in all of the replicates stored at –20°C and as mycelial plugs in water with increasing storage times. If the stability of the fungus is not affected by the preservation protocol, proteases are subject to the more typical catabolite repression in the presence of simple sugars.

If replicates were subject to a recovery period (28 days on a maintenance medium prior to inoculation into liquid culture), enzyme activities that were initially not detected after resuscitation from storage may be regained. A replicate of *Fusarium oxysporum* isolate F3 regained β-chitobiosidase activity but β-glucosamidase activity was not recovered. In replicates of *Metarhizium* isolate M4, β-galactosidase activity was recovered in six replicates where it was previously undetected. However, out of seven replicates that had lost β-chitobiosidase activity, only a replicate that had been cryopreserved regained activity of this enzyme after the recovery period. These results indicate that although enzymes lost as a result of preservation and storage can be recovered, some enzyme activities may be permanently lost. However, as long as the genes that express undetectable enzymes have not been damaged, it may be possible to induce enzyme synthesis by growing the fungus on a different nutrient source or by adding specific growth factors to the media. For example, cockroach cuticle extract could be added to induce trypsin synthesis (St.Leger 1995).

The methods employed were adequate in assessing the stability of enzyme profiles produced by replicates after preservation. However, the range of 4MU substrates investigated could be increased to encompass a more diverse range of substrates, tailored to the individual genera of fungi assessed. The use of a U.V micro-titre plate reader would allow quantification of the 4MU system and may reduce the chances of reader error. The data obtained from APIZYM strips was reproducible. However, the analysis of APIZYM strips should not be attempted by more than one researcher, as consistent interpretation is dependent on the individual. The use of computerised image analysis could allow more accurate determination of the colour. Alternative methods such as Biolog would provide a wider range of 95 available substrates. However, its use for fungi is not widely recorded and plates are notoriously difficult to interpret (Lane pers.comm., Buddie pers.comm.).

Enzymes are used for taxonomic identifications and in industrial processes. As any preservation regime can induce changes in the enzyme profiles of an isolate, mycologists should preserve type strains, and isolates for which enzyme stability is essential by a variety of preservation regimes. Although any preservation regime can induce changes in enzyme profile, the results from this investigation suggest that cryopreservation was the most suitable protocol for the test fungi. However, the use of strain-specific protocols should be examined to maintain maximal stability of enzyme production. As a quality control measure, the enzyme profile of an isolate should be determined on defined media under a set of controlled parameters, before preservation and at least 28 days following preservation.

CHAPTER 6 – THE EFFECT OF PRESERVATION AND STORAGE ON GENETIC STABILITY

6.1 Introduction

Molecular microbiology has developed significantly over the last 12 years. The use of the polymerase chain reaction (PCR) has been applied to many disciplines within the field of mycology (Edel 1998). The study of genes and their expression has been undertaken and aided the comprehension of the molecular basis of pathogenesis in a number of fungi. For example, Talbot *et al.* (1996) studied the *MPG1* gene of *Magnaporthe grisea*, and showed it to encode a hydrophobin associated in surface interactions during pathogenesis. Characterisation of the gene encoding for PR1a (a cuticle-degrading, subtilisin-like endoprotease) in *Metarhizium anisopliae* has allowed the gene to be used in the genetic manipulation of a potential mycoinsecticide (St.Leger *et al.* 1996). PCR-based techniques have been used to study populations, aid taxonomy, to characterise type strains and allow the identification of specific strains. Lodwig *et al.* (1999) used PCR fingerprint patterns, obtained with micro-satellite primers to distinguish clonal lineages within East African populations of *Fusarium oxysporum* f.sp. *cubense*. PCR techniques have allowed the direct identification of fungi in the environment. For example, Clapp *et al.* (1995) used genus-specific primers derived from 18S rDNA to detect mycorrhizal fungi direct from root samples. New more powerful techniques, for example, Single Strand Conformation Polymorphism (SSCP) can detect sequence variation in fungi in field samples (Clapp pers.comm). Couteaudier *et al.* (1998) summarised the range of methods available to establish the genetic structure of populations of the entomopathogen *Beauveria*. Characterisation of sub-populations was achieved using RFLP-PCR analysis of the internal transcribed spacer (ITS) regions and a combination of PCR fingerprints and vegetative compatibility group (VCG) testing.

Genetic stability of strains following preservation and storage is essential. Any changes in PCR fingerprinting patterns, which are induced as a result of preservation could be disastrous. Damage at the genetic level could affect the physiological stability of an isolate. If a gene is disrupted, a valuable metabolite or enzyme may not be synthesised resulting in considerable losses in industry. Damage to an isolate used in molecular research could result in significant losses in time and money if changes go undetected. If polymorphisms are detected in the fingerprint of

an environmental strain or registered patent there could be legal complications. Alternatively, changes in the fingerprints of type and reference strains could affect taxonomic and population studies. There are few examples of changes in genetic stability following preservation. Kuhls *et al.* (1995) noticed that presumably identical strains of *Trichoderma* obtained from different culture collections had deviating PCR fingerprints. Kelly *et al.* (1994) found that an isolate of *Fusarium oxysporum* f.sp. *ciceris* maintained for 12 years, did not conform to the typical PCR fingerprints of other strains of the same species, and concluded that the strain had deteriorated after 12 years of maintenance. Horgen *et al.* (1996) found that chromosomal abnormalities and polymorphisms in RFLP fingerprints were associated with strain degeneration in *Agaricus bisporus*.

Random Amplified Polymorphic DNA (RAPD) analysis (a PCR fingerprinting technique) has been widely used to provide fingerprints of fungal genomic DNA (e.g., Bentley *et al.* 1995, Ozino *et al.* 1998). RAPDs have been used to provide species-specific patterns and their use has been concentrated on population studies (e.g. Kelley *et al.* 1994) and to clarify the identity of type strains (Kuhls *et al.* 1995). RAPD-PCR has many advantages over other techniques that provide species-specific banding patterns such as Restriction Fragment Length Polymorphism's (RFLP's). RAPD's do not require specific nucleotide sequence details for primer design (Edel 1998), DNA fragments of undefined length can be amplified (Edel 1998), only nanogram quantities are required (Bridge & Arora 1998) and samples can be processed quickly (Bridge *et al.* 1997). However, the number and nature of bands produced are often difficult to interpret (Bridge *et al.* 1997) and results may not be reproducible because of low stringency of the primers, which emanate from the low PCR annealing temperature. A technique was developed that took the advantages of the RAPD method but significantly improved reproducibility. Primers that had previously been used as fingerprinting probes were adapted for RAPD-like PCR. The tandem repeat sequence of the M13 phage or simple sequence repeats, which are complementary to the flanking regions or the core sequence of variable number tandem repeats (VNTRs) of the microsatellite DNA (Bridge *et al.* 1997) were used. Banding patterns were produced that were simple and easier to interpret than RAPD patterns, that could distinguish between strains of the same species and were more reproducible than RAPD fingerprints (Bridge *et al.* 1997).

The technique can be applied to a wide range of organisms and is highly cost effective.

Metarhizium anisopliae, in common with all mitotic fungi, is asexual. However, genetic exchange can occur as a result of mutation, parasexual processes and heterokaryosis. Parasexual cycles occur in some mitotic fungi as a non-sexual process resulting in the formation of diploid recombinant nuclei. It has been demonstrated in *Metarhizium* (Al Aidroos 1980). Genetic recombination created from protoplast fusions (Silviera and Azevedo 1987) and forced heterokaryon formation (Bogo *et al.* 1996) has also been reported. Azevedo *et al.* (1987) suggests that parasexuality is a valuable tool for genetic studies and the breeding of new *Metarhizium* strains. It is not known if genetic recombination occurs naturally in the environment. However, Pipe *et al.* (1995) reported that some *M.anisopliae* var. *majus* isolates could be heterozygous diploids. *Metarhizium anisopliae* is not thought to have a sexual stage (teleomorph), which reduces the prospects of natural intraspecific genetic recombination. However, Liang *et al.* (1991) report that one of the least recognised species of *Metarhizium* (*M.taii*) has a sexual stage (*Cordyceps taii*).

Unlike other species of *Fusarium*, *F. oxysporum* has no known teleomorph. It may have a single pair of allelomorphic mating type factors A/a, but Burnett (1984) suggests that the fungus may be strictly anamorphic. Genetic exchange may occur as a result of heterokaryosis or parasexuality of vegetatively compatible isolates (Woo *et al.* 1998). Buxton (1954) mixed morphological and colour variants of *F.oxysporum* f.sp. *gladioli* together in culture to produce heterokaryons via anastomosis of germ tubes. The resulting cultures consisted of a mosaic of homokaryotic and heterokaryotic growth. However, the long-term stability of heterokaryons is doubted. Production of intraspecific hybrids of *F.oxysporum* f.sp. *radicis-lycopersici* and *F.oxysporum* f.sp. *lycopersici* have been achieved by protoplast fusion (Madhosingh 1994). The hybrids showed significant differences in behaviour from their parents, indicating a changed genetic basis for the expression of the altered characteristics. The author suggests that new hybrids could be produced to help control *Fusarium* diseases. Many methods to establish the degree of molecular differentiation of *Fusarium oxysporum* have been developed. Electrophoretic

Karyotyping, using PFGE (Pulsed Field Gel Electrophoresis) to determine chromosome size and number has been applied to assess the relatedness of several isolates of *F.oxysporum* f.sp. *dianthi* (Migheli *et al.* 1995). Bridge *et al.* (1995) developed simple mitochondrial DNA probes to characterise mitochondrial DNA and showed that mtDNA polymorphisms were present in and between different special forms. The use of PCR techniques to characterise *F.oxysporum* has become widely used to estimate the genetic divergence and relationships among isolates (e.g. Bentley *et al.* 1994, Crowhurst *et al.* 1995, Lodwig *et al.*1999). Dipietro *et al.* (1994) discovered a retrotransposon-like sequence in *F.oxysporum* and postulated that it might be widespread throughout the species. Anaya & Roncero (1996) found that the *skippy* retrotransposon could be rearranged as a result of genomic stress. The work described in this chapter aimed to monitor the effects of preservation and storage time on the genetic stability of the test fungi.

6.2 Materials and methods

Materials and methods are described in Chapter 2, section 2.4.5

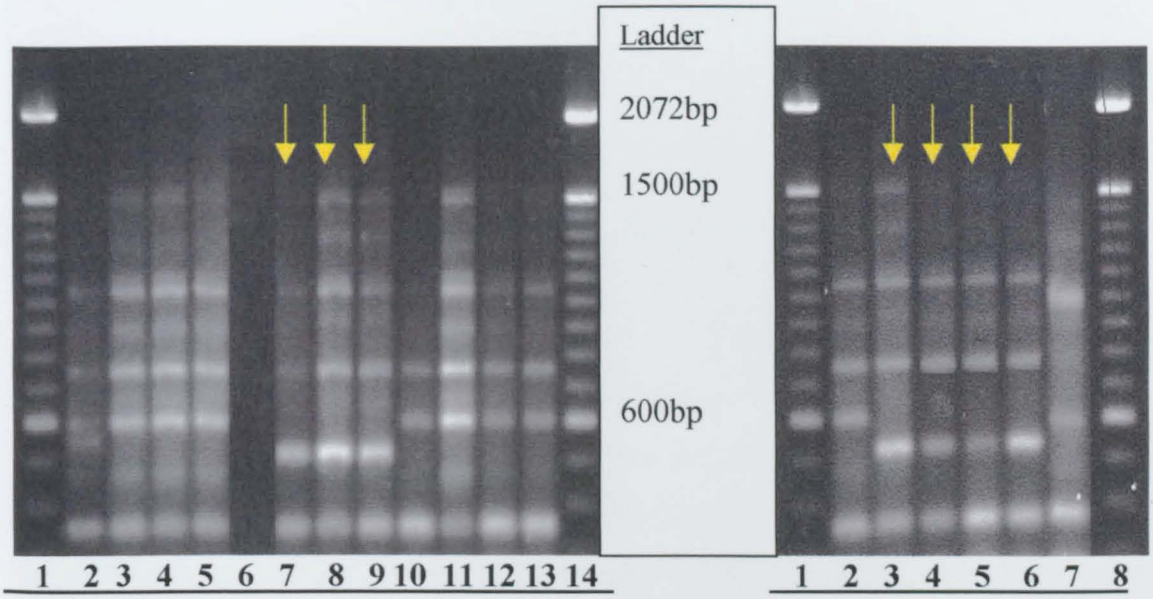
6.3 Results

6.3.1 *Metarhizium anisopliae*

Metarhizium anisopliae isolate M1

Nine bands were obtained with MR primer, 3 bands were obtained with RY primer and 3 bands were obtained with GACA primer. Polymorphisms were detected with MR primer in some replicates that had been lyophilised and cryopreserved, after 1 (Fig 1) and 2 (Fig 2) years of storage. A band of approx. 600bp, common to the representative fingerprint, was lost and an additional band of approx. 525bp was visible. After 1 year, 3 out of five replicates that had been lyophilised and 4 out of 5 replicates that had been cryopreserved exhibited the polymorphism. After 2 years, 3 out of 5 replicates that had been lyophilised and cryopreserved exhibited the polymorphism. All other replicates exhibited the characteristic fingerprint.

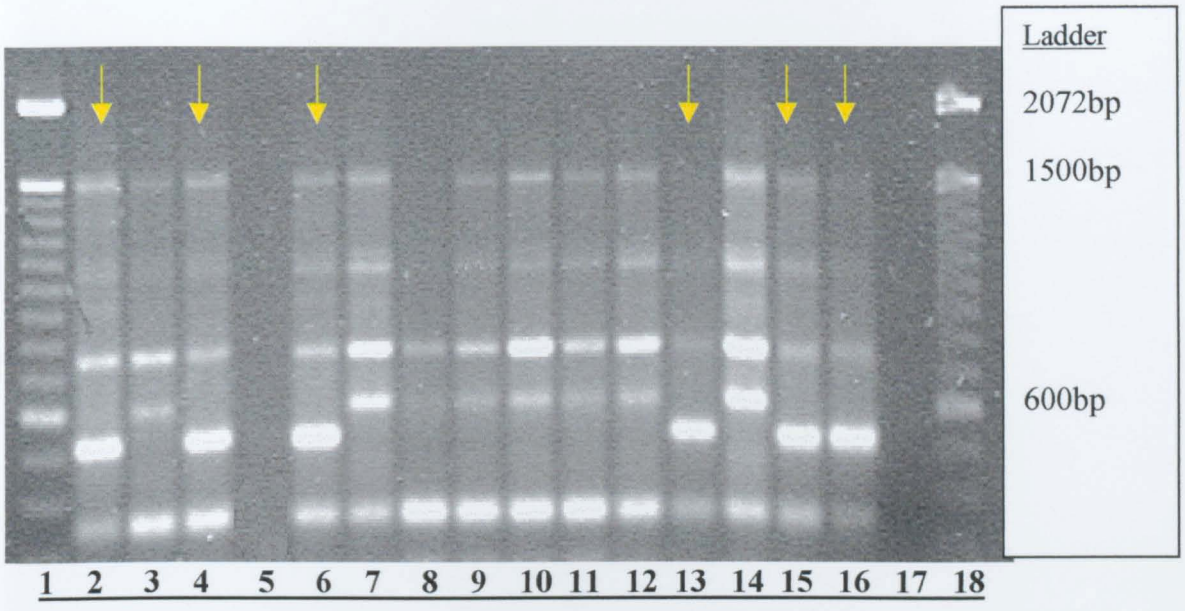
Fig.1: PCR fingerprints obtained with MR primer of replicates of *Metarhizium anisopliae* isolate M1 after 1 year of storage



Key, Left frame, lanes left to right, 1, 100bp ladder; 2,3,4,5,6, continual-sub culture; 7,8,9,10, lyophilised; 11,12,13, mycelial plugs in water; 14, 100 bp ladder. Right frame: 1, 100bp ladder, 2, mycelial plugs in water; 3,4,5,6,7 cryopreservation; 8, 100 bp ladder. bp= base pairs.

Replicates exhibiting a polymorphism are indicated with a yellow arrow.

Fig.2: PCR fingerprints obtained with MR primer of replicates of *Metarhizium anisopliae* isolate M1 after 2 years of storage

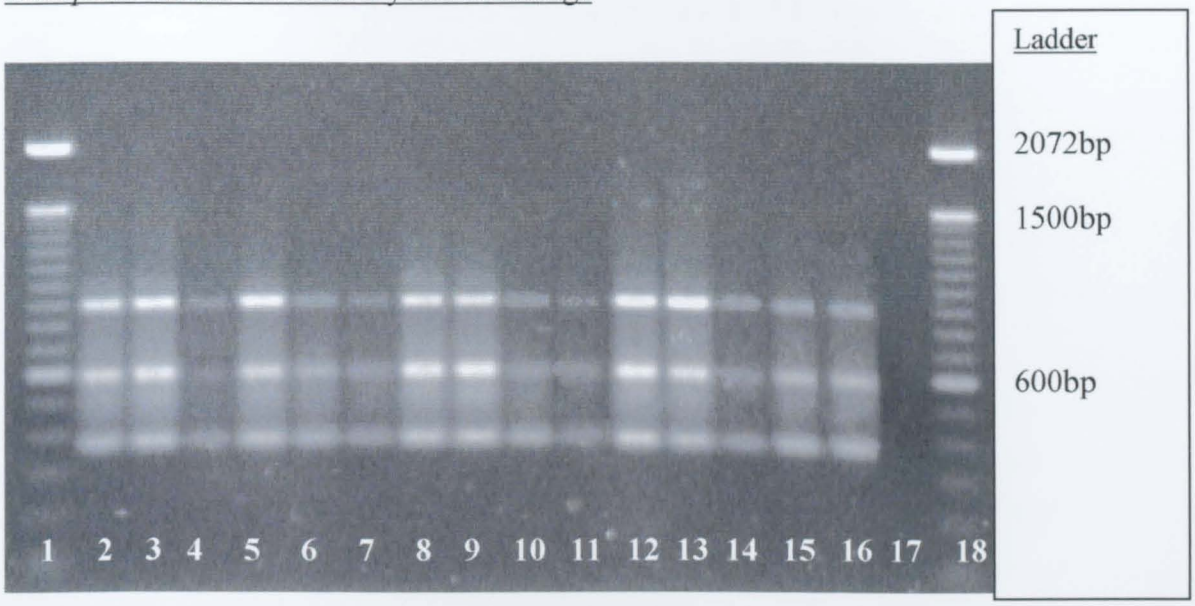


Key, lanes left to right: 1, 100bp ladder; 2,3,4,5,6, lyophilised; 7,8,9,10,11, mycelial plugs in water; 12,13,14,15,16, cryopreservation; 17, control; 18, 100 bp ladder. bp= base pairs.

Replicates exhibiting a polymorphism are indicated with a yellow arrow.

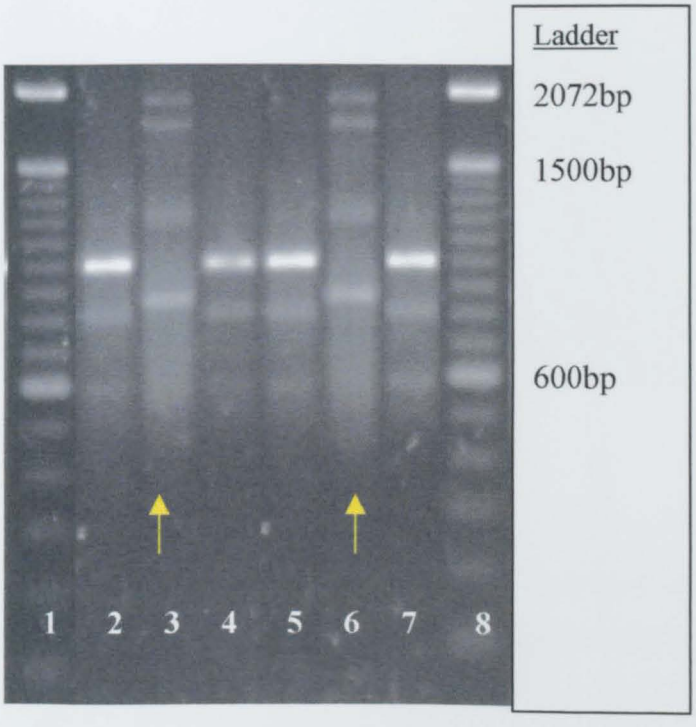
The PCR fingerprints of all the replicates obtained with GACA primer (Fig 3) were identical throughout the investigation. The fingerprints obtained with RY primer also exhibited similar banding patterns from most replicates during the investigation. However, 2 replicates that had been cryopreserved (and had also exhibited a polymorphism with MR primer) exhibited polymorphisms with RY primer after 1 year of storage (Fig 4). Bands present at approx.700bp and 100bp in the representative profile were absent and 4 additional bands were detected. Bands were obtained at approx.775bp and 1300bp and 2 bands between approx.1500bp and 2072bp.

Fig.3: PCR fingerprints obtained with GACA primer of replicates of *Metarhizium anisopliae* isolate M1 after 2 years of storage



Key, lanes left to right: 1, 100bp ladder; 2,3,4,5,6, lyophilised; 7,8,9,10,11, mycelial plugs in water; 12,13,14,15,16, cryopreservation; 17, control; 18, 100 bp ladder. bp= base pairs.

Fig.4: Example of PCR fingerprints obtained with RY primer of replicates of *Metarhizium anisopliae* isolate M1 after 1 year of storage



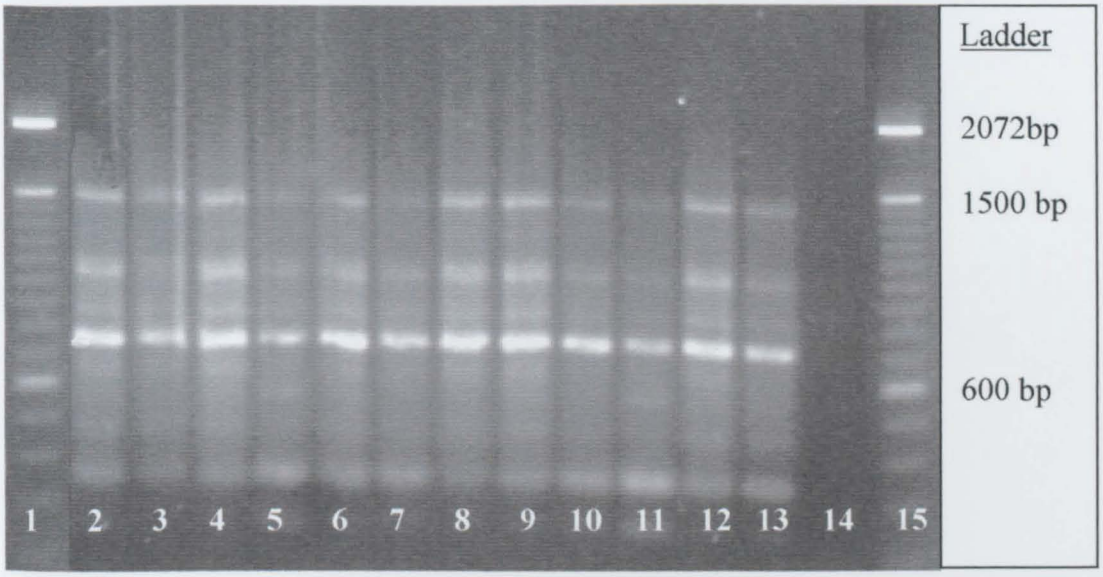
Key, lanes left to right: 1, 100bp ladder; 2, mycelial plugs in water; 3,4,5,6,7, cryopreservation; 8, 100 bp ladder. bp= base pairs.

Replicates exhibiting a polymorphism are indicated with a yellow arrow.

Metarhizium anisopliae isolate M2

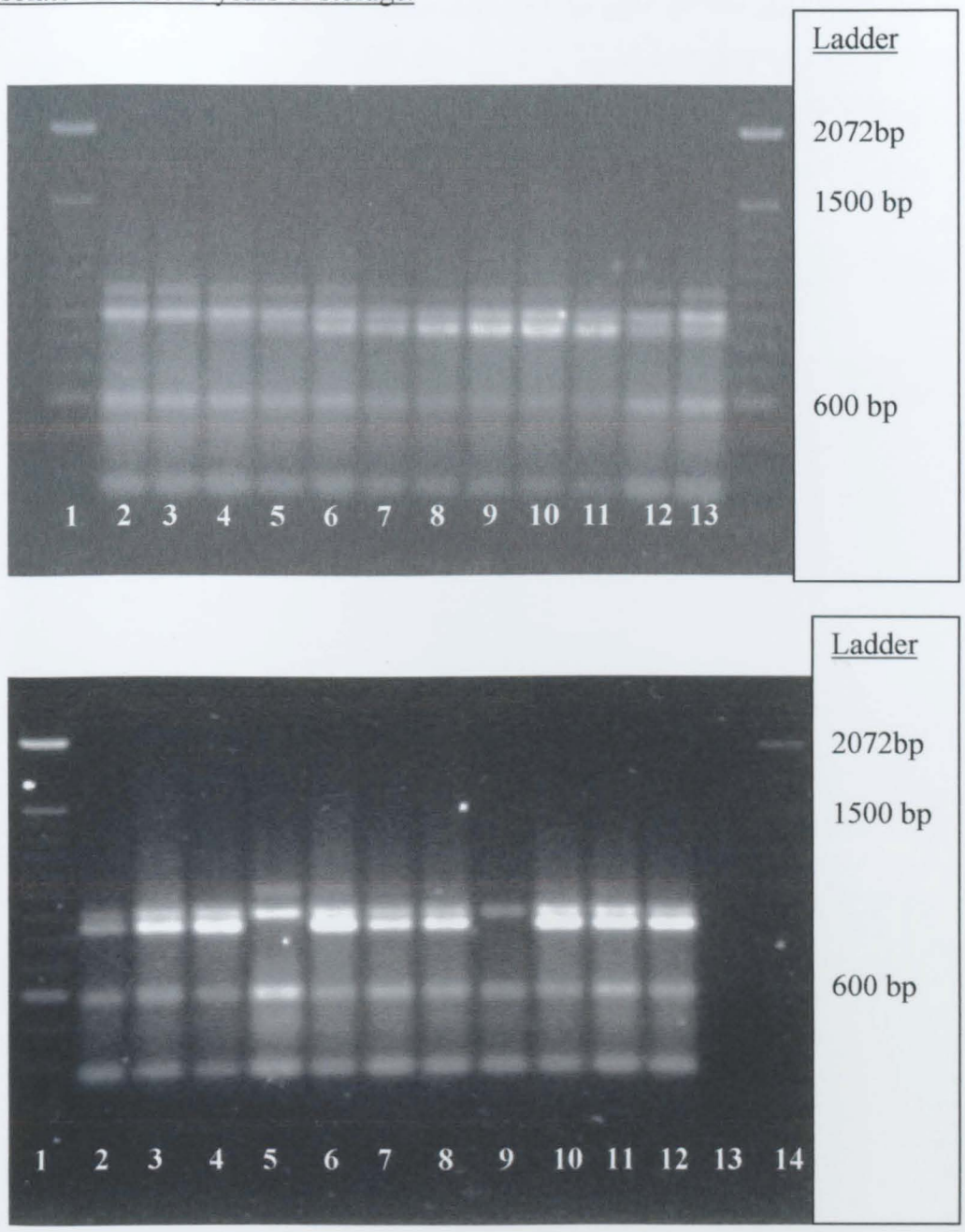
The PCR fingerprint patterns obtained with MR, RY and GACA primers of replicates of *Metarhizium anisopliae* isolate M2 were all identical after 1 year of storage. Five bands were produced with GACA primer, 9 bands were produced with MR primer and only 1 band was obtained with RY primer. After 2 years of storage, the PCR fingerprints with MR primer (Fig 5) and RY primer from all of the replicates resembled the banding patterns obtained after 1 year of storage. However, a polymorphism was detected with GACA primer in the fingerprints of 3 replicates (Fig 6). A band of approx. 850bp in size was not detected and the fingerprint consisted of only 4 bands. The polymorphism was detected in 2 replicates that had been lyophilised and a replicate that had been cryopreserved. A repeat of the PCR procedure verified the result.

Fig.5: Example of PCR fingerprints obtained with MR primer of replicates of *Metarhizium anisopliae* isolate M2 after 2 years of storage



Key, lanes L to R: 1, 100bp ladder; 2,3,4,continual sub-culture; 5,6 lyophilisation; 7,8, mycelial plugs in water; 9,10,11 storage at –20°C; 12, 13, cryopreservation; 14, control; 15; 100 bp ladder. bp= base pairs.

Fig.6: PCR fingerprints with GACA primer of replicates of *Metarhizium anisopliae* isolate M2 after 2 years of storage.

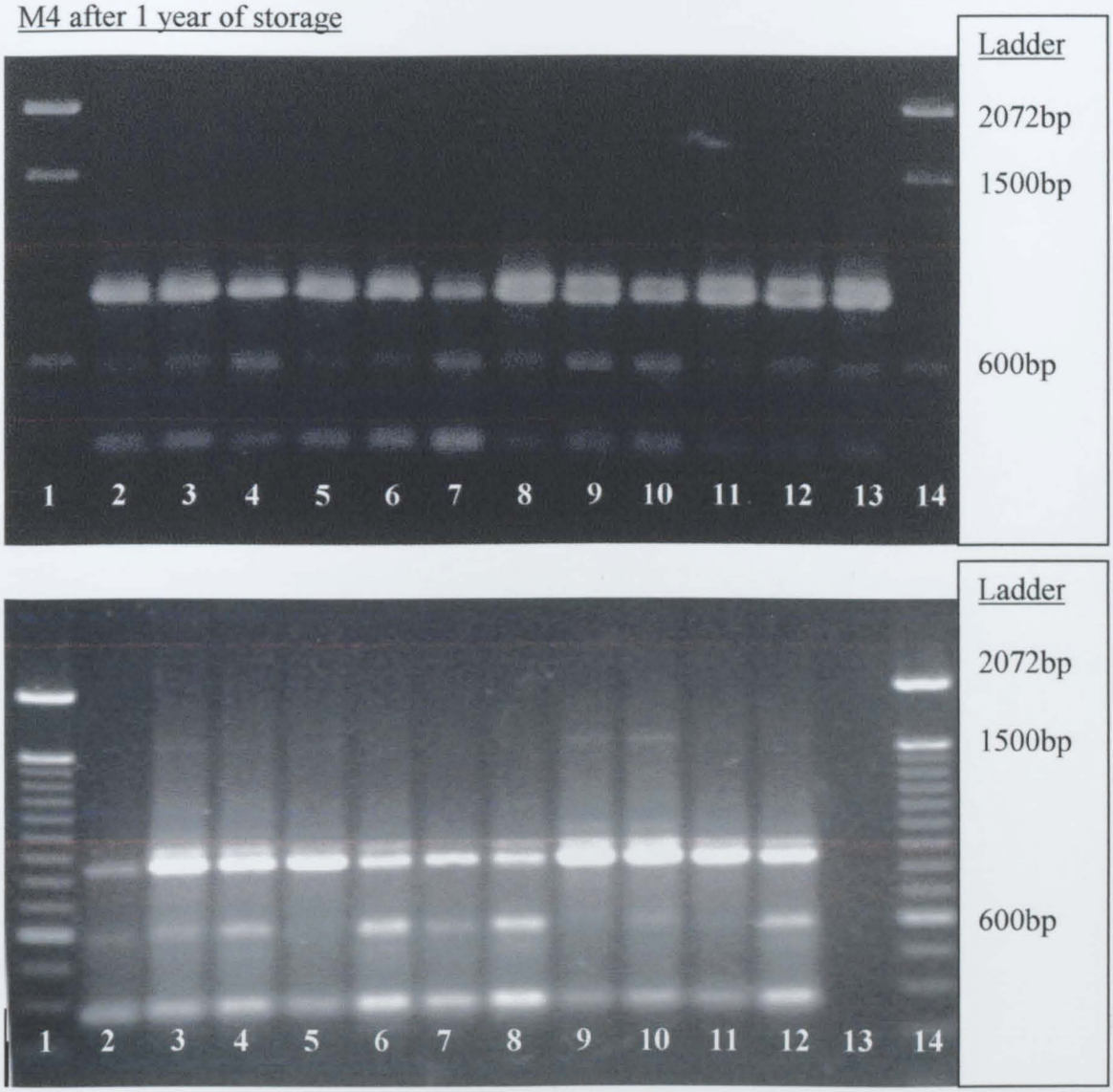


Key, top, lanes L to R: 1, 100bp ladder; 2,3,4,5,6 lyophilisation; 7,8,9,10,11, mycelial plugs in water; 12, 13 storage at –20°C; 14, 100 bp ladder.
Bottom: 1, 100bp ladder; 2,3,4 storage at –20°C; 5,6,7,8, cryopreservation; 9,10,11,12 cryopreservation (repeat) 13, control; 14; 100 bp ladder. bp= base pairs.
Polymorphisms (band deletion at 850 bp) are in lanes 1, 3 top and lane 5 bottom.

Metarhizium isolate M4

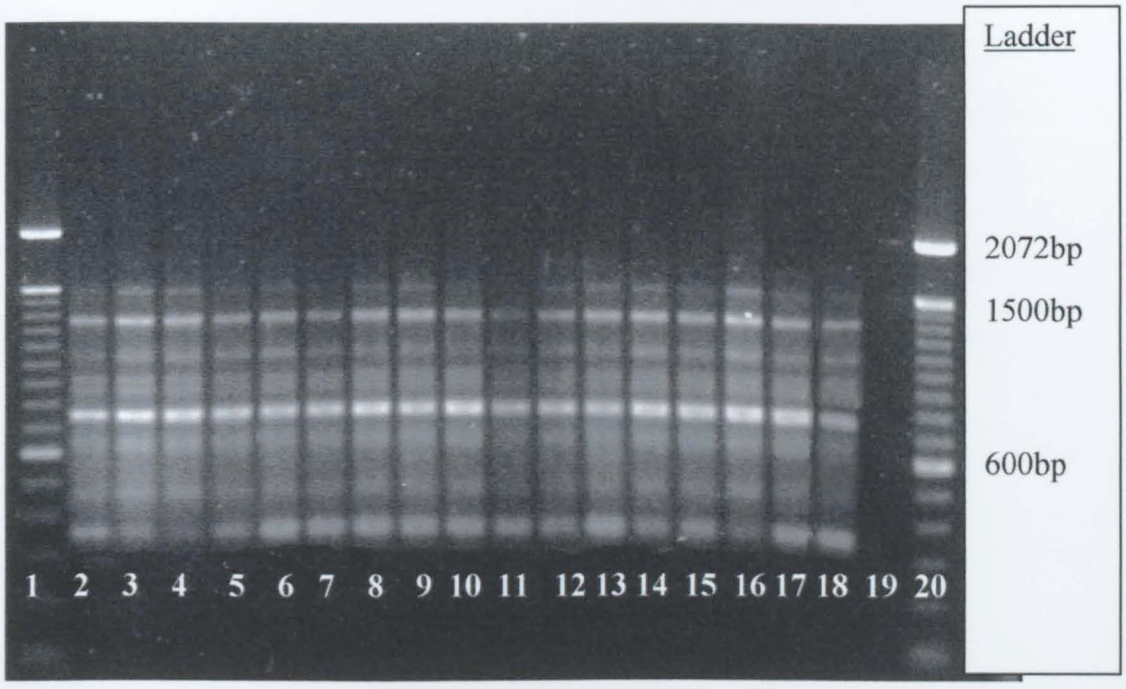
No polymorphisms were detected in any of the replicates of isolate M4 during the investigation. Four strong bands were obtained with GACA primer (Fig 7). 10 bands were obtained with MR Primer (Fig 8) and 3 bands were obtained with RY primer (Fig 9).

Fig.7: PCR fingerprints with GACA primer of replicates of *Metarhizium* spp. isolate M4 after 1 year of storage



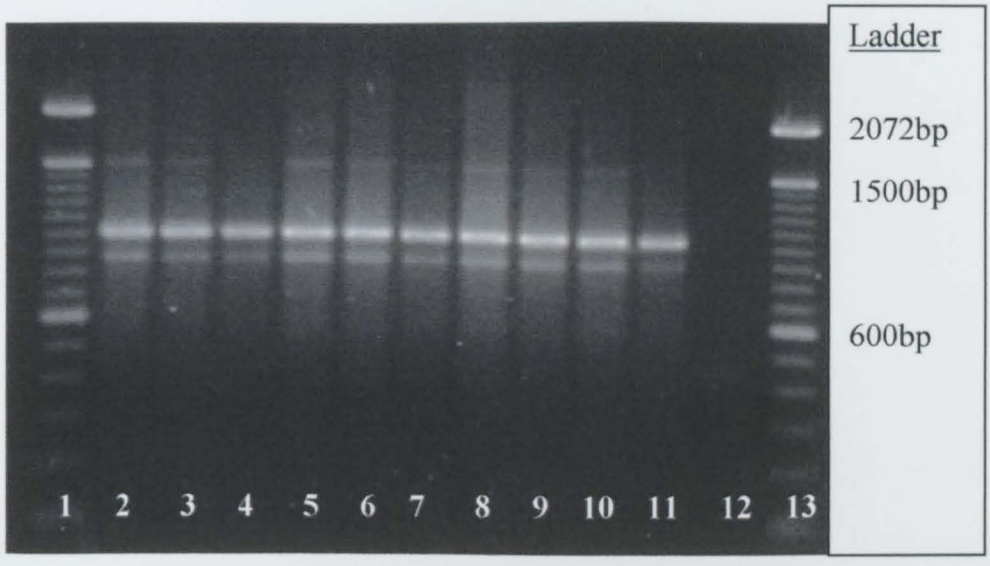
Key, top, lanes L to R: 1, 100bp ladder; 2,3,4,5, continual sub-culture; 6,7,8,9,10, lyohilisation; 11,12,13, mycelial plugs in water; 14, 100 bp ladder;
Bottom: 1, 100bp ladder; 2,3, mycelial plugs in water; 4,5,6,7,8, storage at –20°C; 9,10,11,12, cryopreservation; 13, control; 14; 100 bp ladder. bp= base pairs.

Fig.8: PCR fingerprints with MR primer of replicates of *Metarhizium* spp. isolate M4 after 1 year of storage



Key, lanes L to R: 1, 100bp ladder; 2,3,4,5, continual sub-culture; 6,7,8, lyophilisation; 9,10,11, mycelial plugs in water; 12,13,14,15 storage at –20°C; 16,17,18, cryopreservation; 19, control; 20, 100 bp ladder. bp= base pairs.

Fig.9: Example of PCR fingerprints with RY primer of *Metarhizium* spp. isolate M4 after 1 year of storage



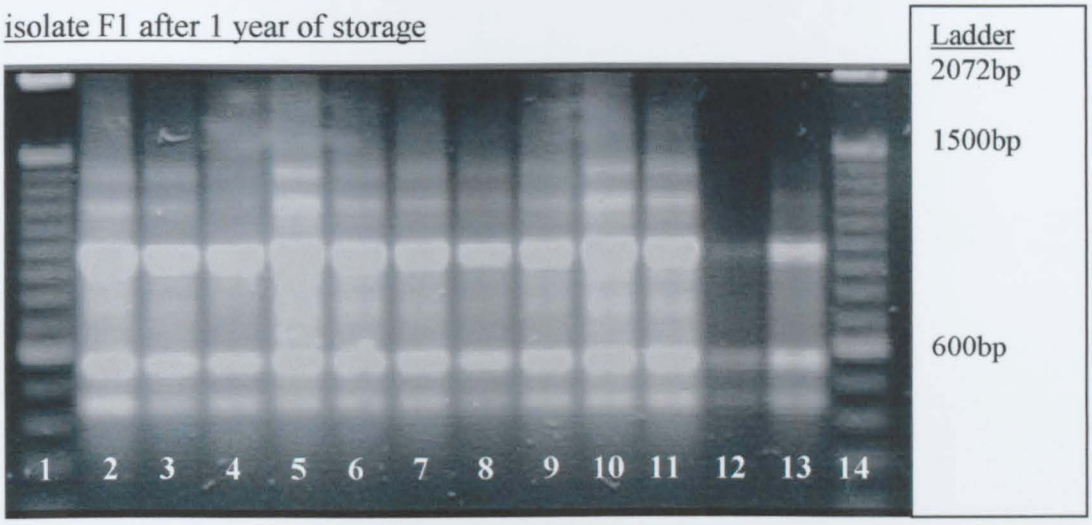
Key, lanes L to R: 1, 100bp ladder; 2,3,mycelial plugs in water; 4,5,6,7, storage at –20°C; 8, 9,10,11 cryopreservation; 12, control; 13, 100 bp ladder. bp= base pairs.

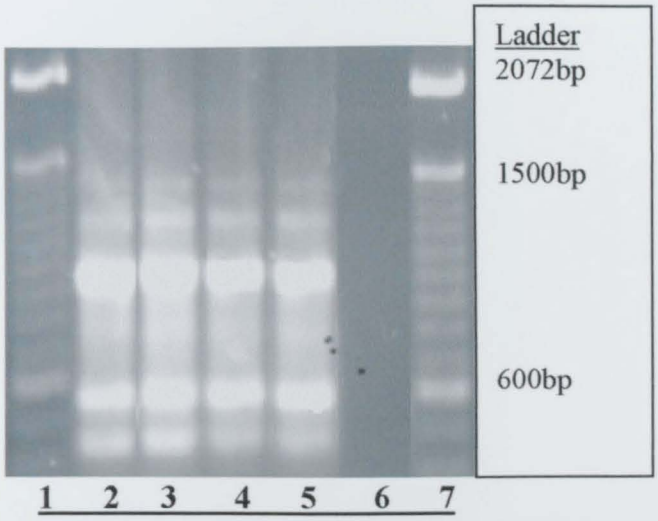
6.3.2 *Fusarium oxysporum*

Fusarium oxysporum isolate F1

Six bands were obtained with MR primer (Fig 10), 6 bands were obtained with GACA primer (Fig 11) and 5 bands were obtained with RY primer (Fig 12). The PCR fingerprints of all replicates (irrespective of preservation treatment) with all three primers remained stable throughout the investigation.

Fig.10: PCR fingerprints with MR primer of replicates of *Fusarium oxysporum* isolate F1 after 1 year of storage

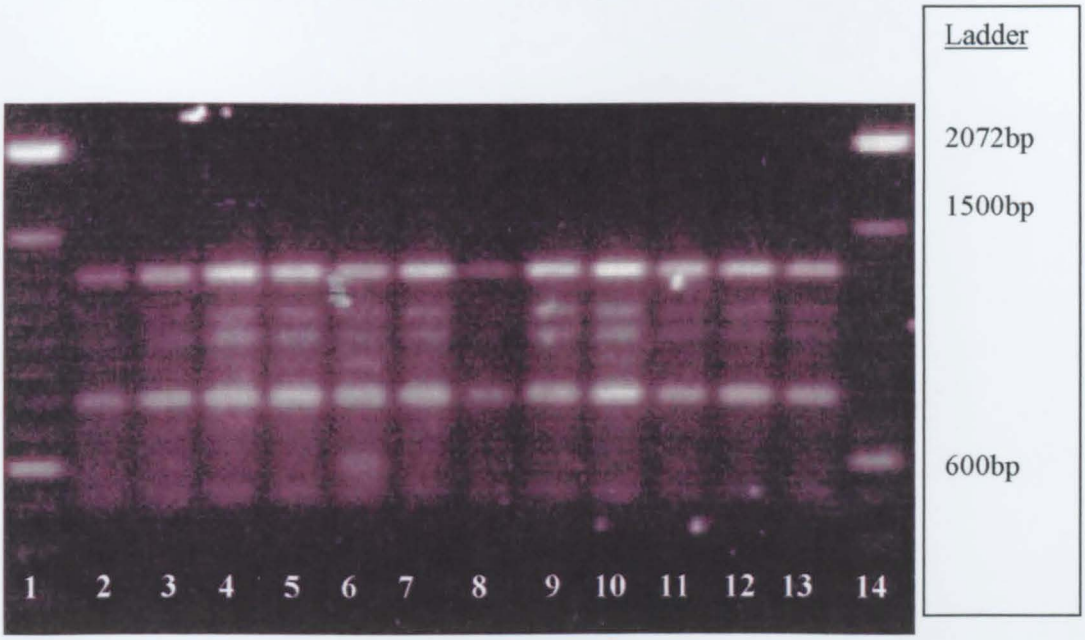


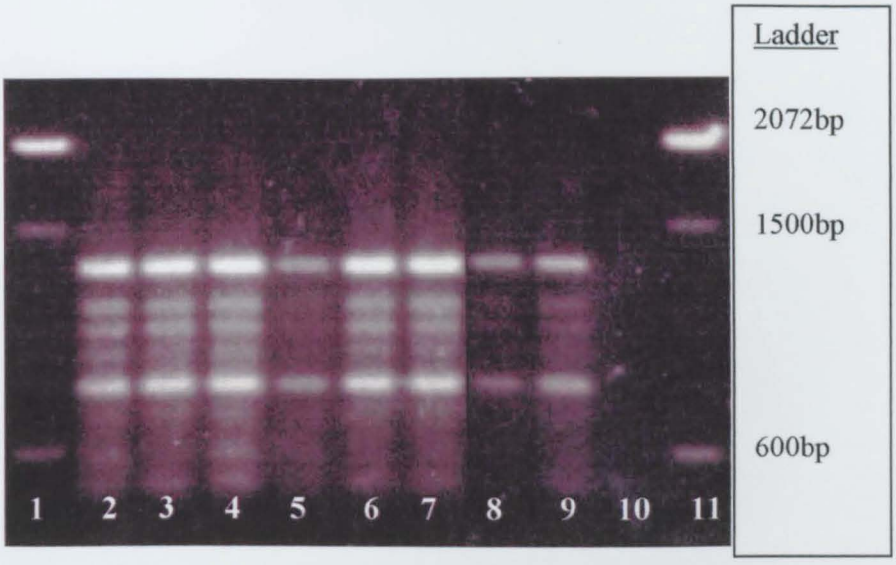


Key, top, lanes L to R: 1, 100bp ladder; 2,3,4,5, continual sub-culture, 6,7,8, lyophilised, 9,10,11, mycelial plugs in water; 12,13, frozen at –20°C; 14, 100bp ladder.

Bottom: 1, 100bp ladder; 2, frozen at –20°C; 3,4,5, cryopreserved; 6, control; 7, 100bp ladder. bp= base pairs.

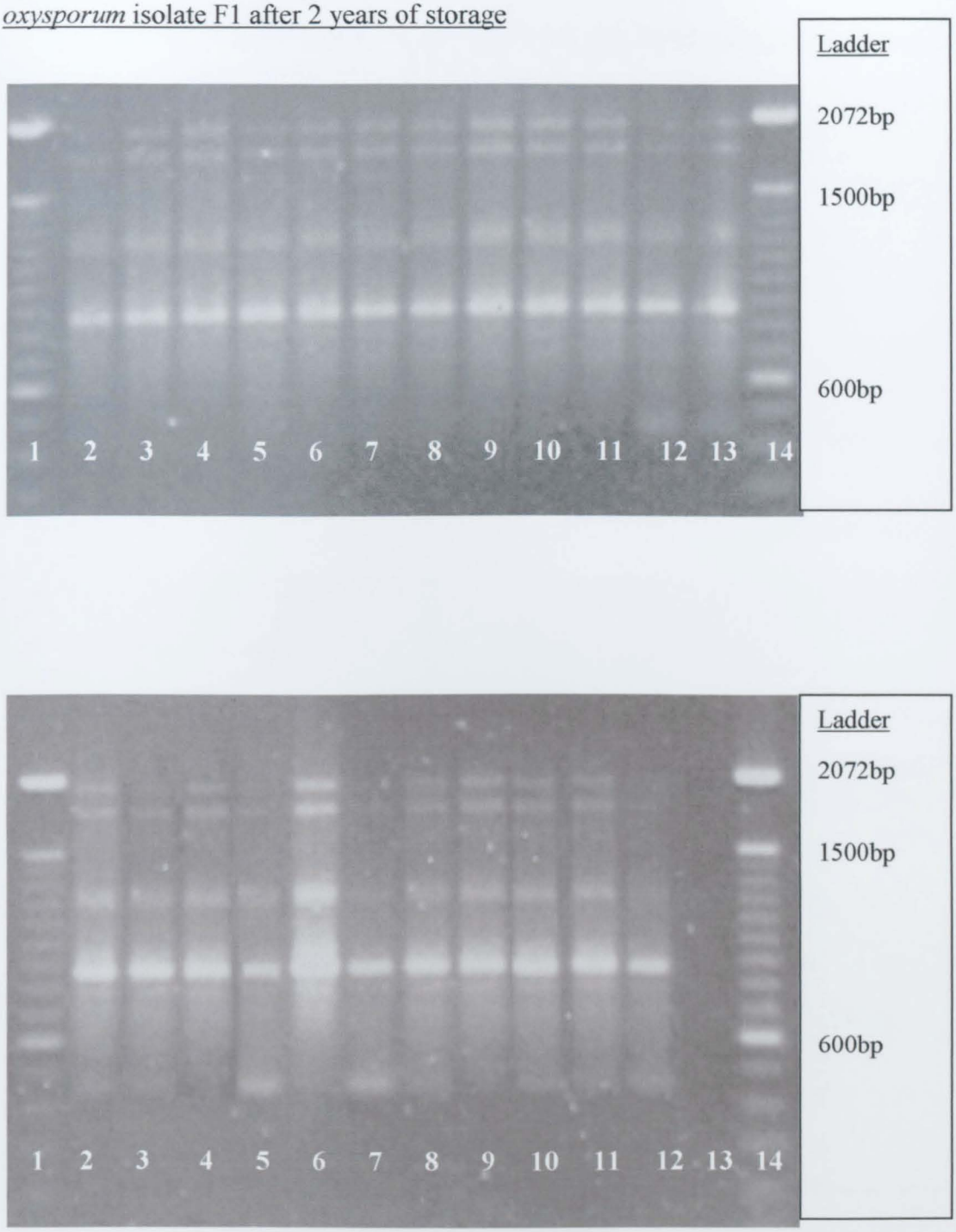
Fig.11: Example PCR fingerprints with GACA primer of replicates of *Fusarium oxysporum* isolate F1 after 2 years of storage





Key, top, lanes L to R: 1, 100bp ladder; 2,3,4,5, continual sub-culture, 6,7,8,9 lyophilised; 10,11,12, 13 mycelial plugs in water; 14, 100bp ladder.
Bottom: 1, 100bp ladder; 2,3,4,5, frozen at –20°C; 6,7,8,9, cryopreserved; 10, control; 11, 100bp ladder. bp = base pairs.

Fig.12: Example PCR fingerprints with RY primer of replicates of *Fusarium oxysporum* isolate F1 after 2 years of storage

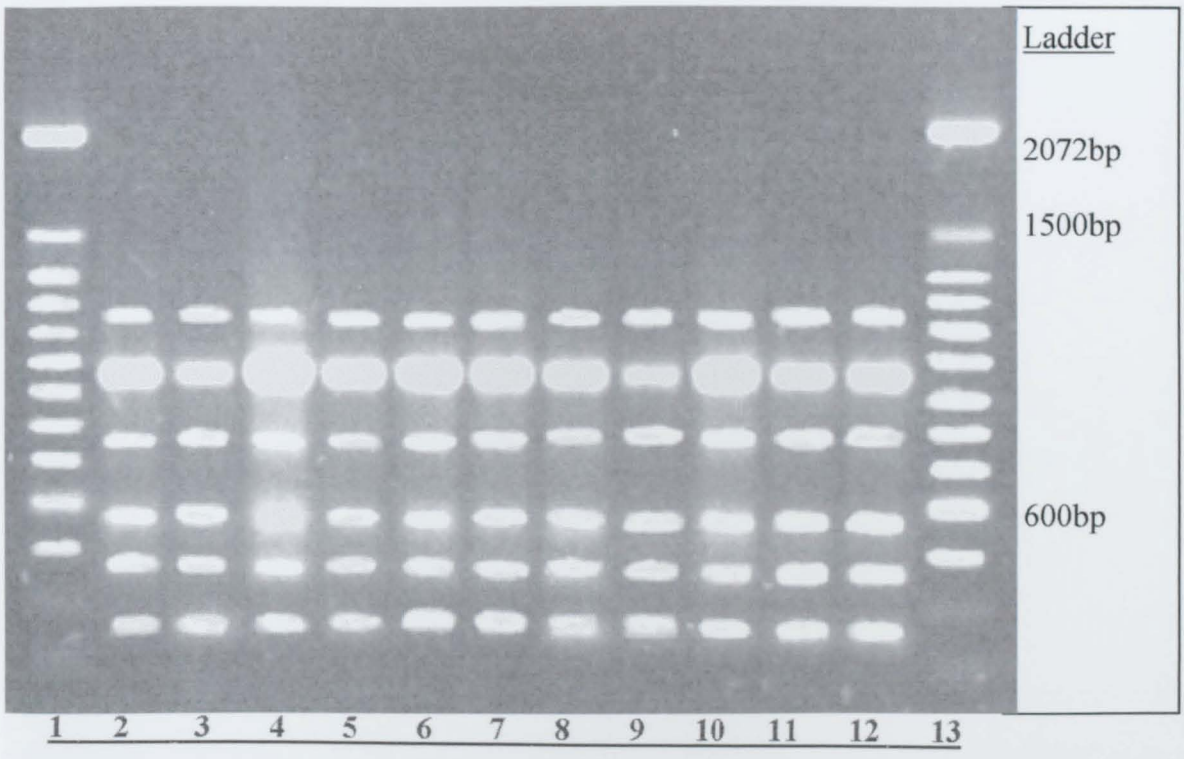


Key, top, lanes L to R: 1, 100bp ladder; 2,3,4,5, continual sub-culture, 6,7,8,9,10, lyophilised;11,12, 13 mycelial plugs in water; 14, 100bp ladder.
Bottom: 1, 100bp ladder; 2,3, mycelial plugs in water; 4,5,6,7,8, frozen at –20°C; 9,10,11,12, cryopreserved; 13, control; 14, 100bp ladder. bp = base pairs

Fusarium oxysporum isolate F2

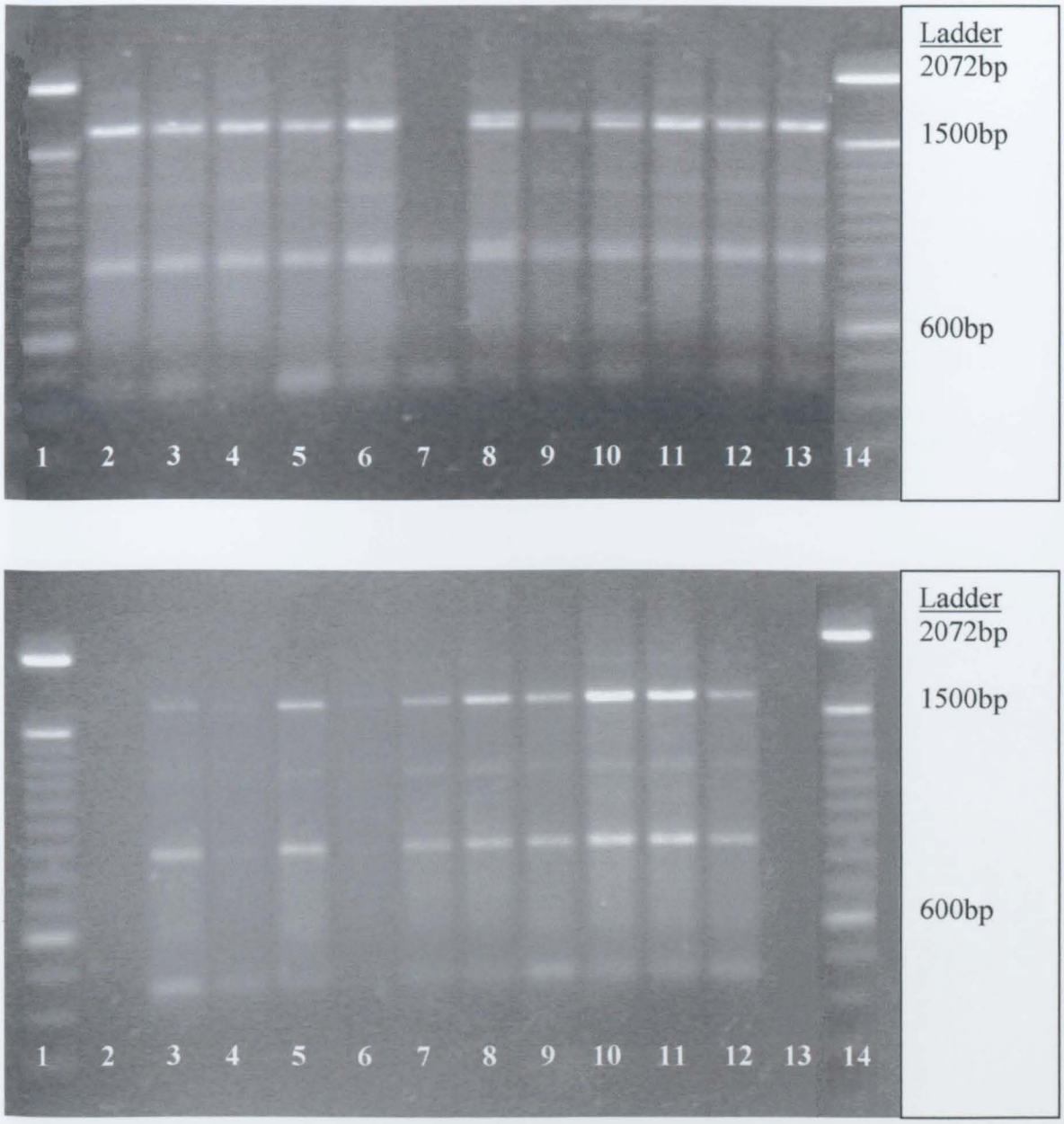
Six bands were obtained with MR primer (Fig 13), 6 bands were obtained with RY primer (Fig 14) and 5 bands were obtained with GACA primer (Fig 15). The PCR fingerprints of all replicates (irrespective of preservation treatment) with all three primers remained stable throughout the 2-year testing period (i.e. no polymorphisms were detected)

Fig.13: Example PCR fingerprints with MR primer of replicates of *Fusarium oxysporum* isolate F2 after 2 years of storage. Bands on this gel have been digitally enhanced.



Key, lanes, L to R: 1, 100bp ladder; 2,3,4,5,6, continual sub-culture, 7,8,9,10, lyophilised, 11,12, mycelial plugs in water; 13, 100bp ladder.

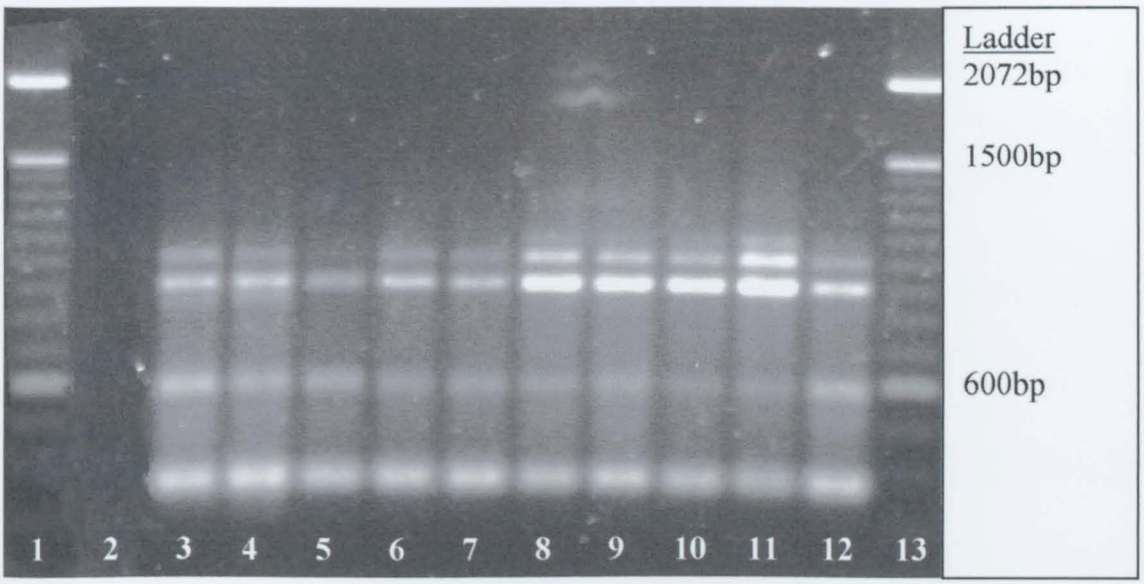
Fig.14: PCR fingerprints with RY primer of replicates of *Fusarium oxysporum* isolate F2 after 2 years of storage.

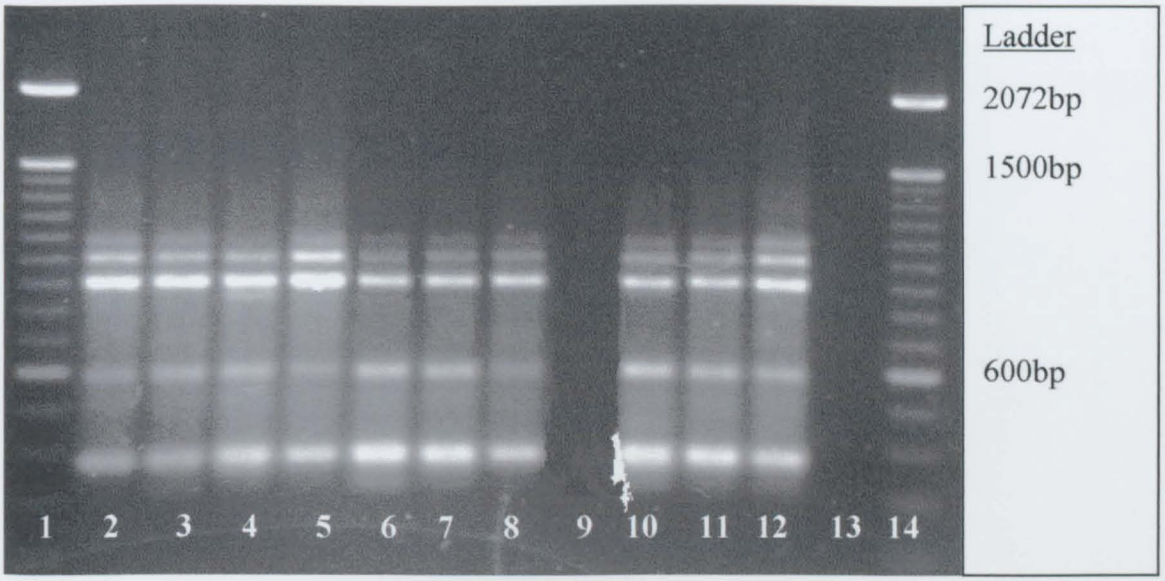


Key, top, lanes L to R: 1, 100bp ladder; 2,3,4,5,6, continual sub-culture, 7,8,9,10, lyophilised, 11,12,13, mycelial plugs in water; 14, 100bp ladder.

Bottom: 1, 100bp ladder; 2,3, mycelial plugs in water; 4,5,6,7 frozen at –20°C; 8,9,10,12, cryopreserved; 13, control; 14, 100bp ladder.

Fig.15: PCR fingerprints with GACA primer of replicates of *Fusarium oxysporum* isolate F2 after 2 years of storage.





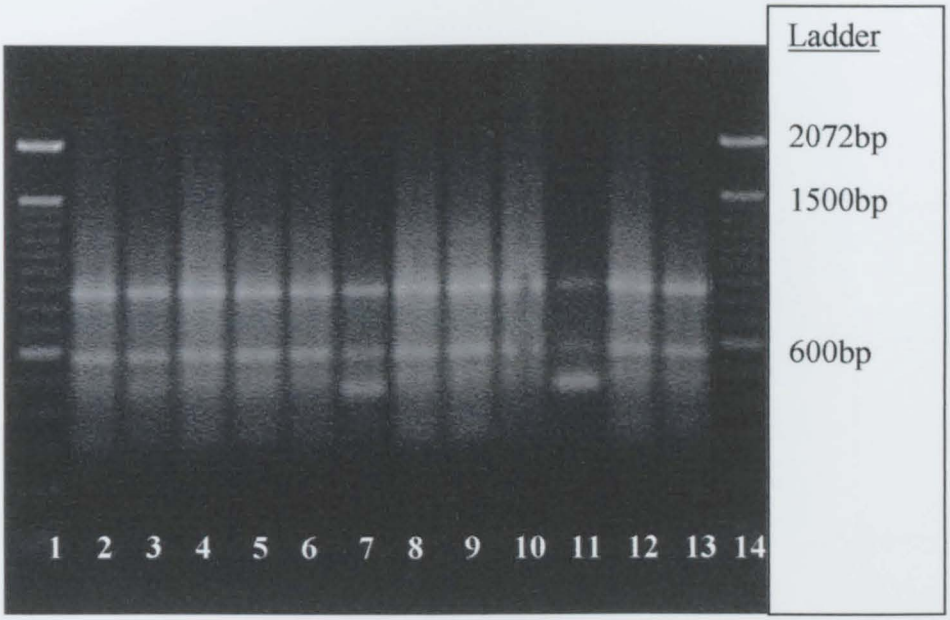
Key, top, lanes L to R: 1, 100bp ladder; 2,3,4,5,6, continual sub-culture, 7,8,9 lyophilised, 10,11,12, mycelial plugs in water; 13, 100bp ladder.

Bottom: 1, 100bp ladder; 2,3, mycelial plugs in water; 4,5,6,7 frozen at –20°C; 8,9,10,12, cryopreserved; 13, control; 14, 100bp ladder

Fusarium oxysporum isolate F3

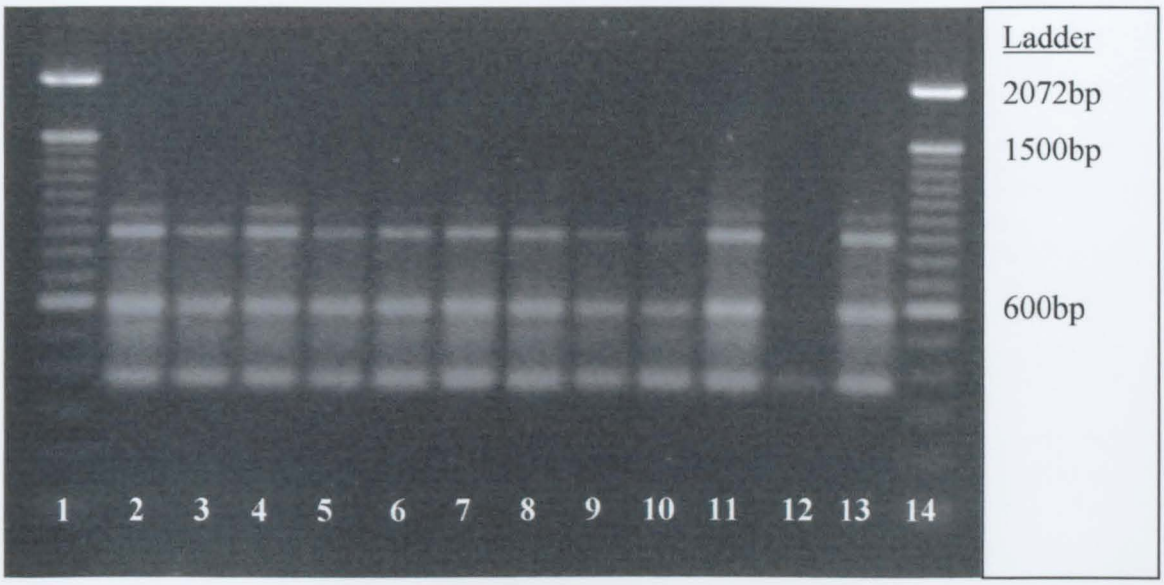
Four bands were obtained with GACA primer, 5 bands were obtained with MR primer and 3 strong bands were obtained with RY primer (Fig 16). After 1 day of storage, the characteristic banding patterns were exhibited by all replicates with all 3 primers. After 16 weeks of storage, the characteristic banding patterns were exhibited by all of the replicates with GACA (Fig 17) and RY primers. However, 2 polymorphisms were detected with MR primer (Fig 18) in 2 replicates that had been maintained by continual sub culture. A band of approx. 750bp, common to the characteristic fingerprint profile, was not detected in the replicates that exhibited a polymorphism. A band of approx. 650bp, only weakly visible in replicates exhibiting the characteristic fingerprint, was more intense in the replicates that exhibited a polymorphism. After 1 year of storage, the representative banding patterns were exhibited by all replicates with all 3 primers.

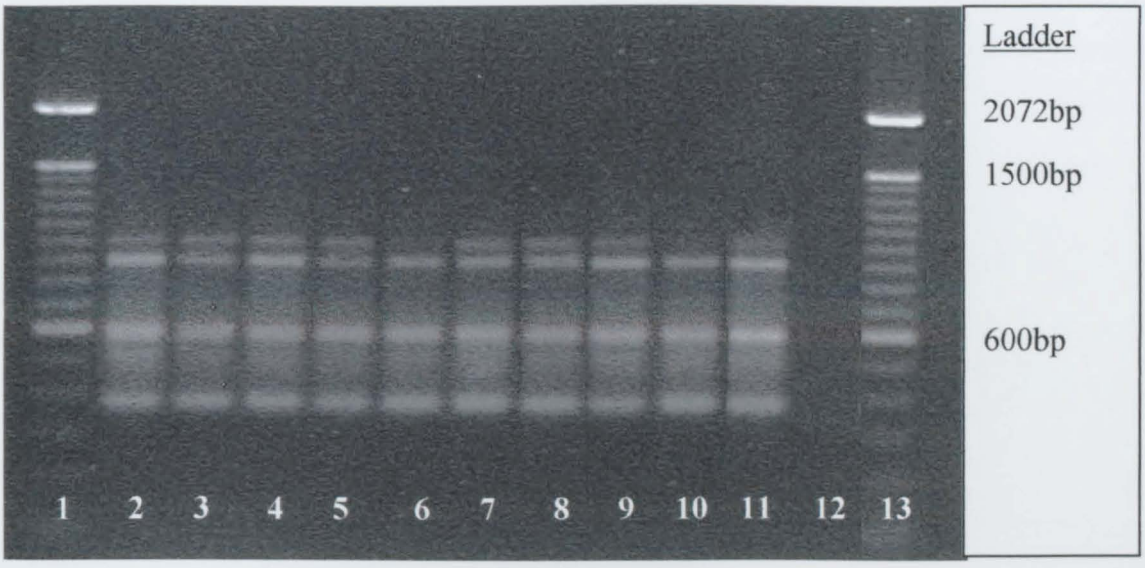
Fig.16: Example PCR fingerprints with RY primer of replicates of *Fusarium oxysporum* isolate F3 after 1 year of storage.



Key, lanes, L to R: lane 1, 100bp ladder; 2,3,4, continual sub-culture; 5,6,7,8, lyophilised; 9,10,11,12,13, mycelial plugs in water; 14, 100bp ladder.

Fig.17: PCR fingerprints with GACA primer of replicates of *Fusarium oxysporum* isolate F3 after 16 weeks of storage.

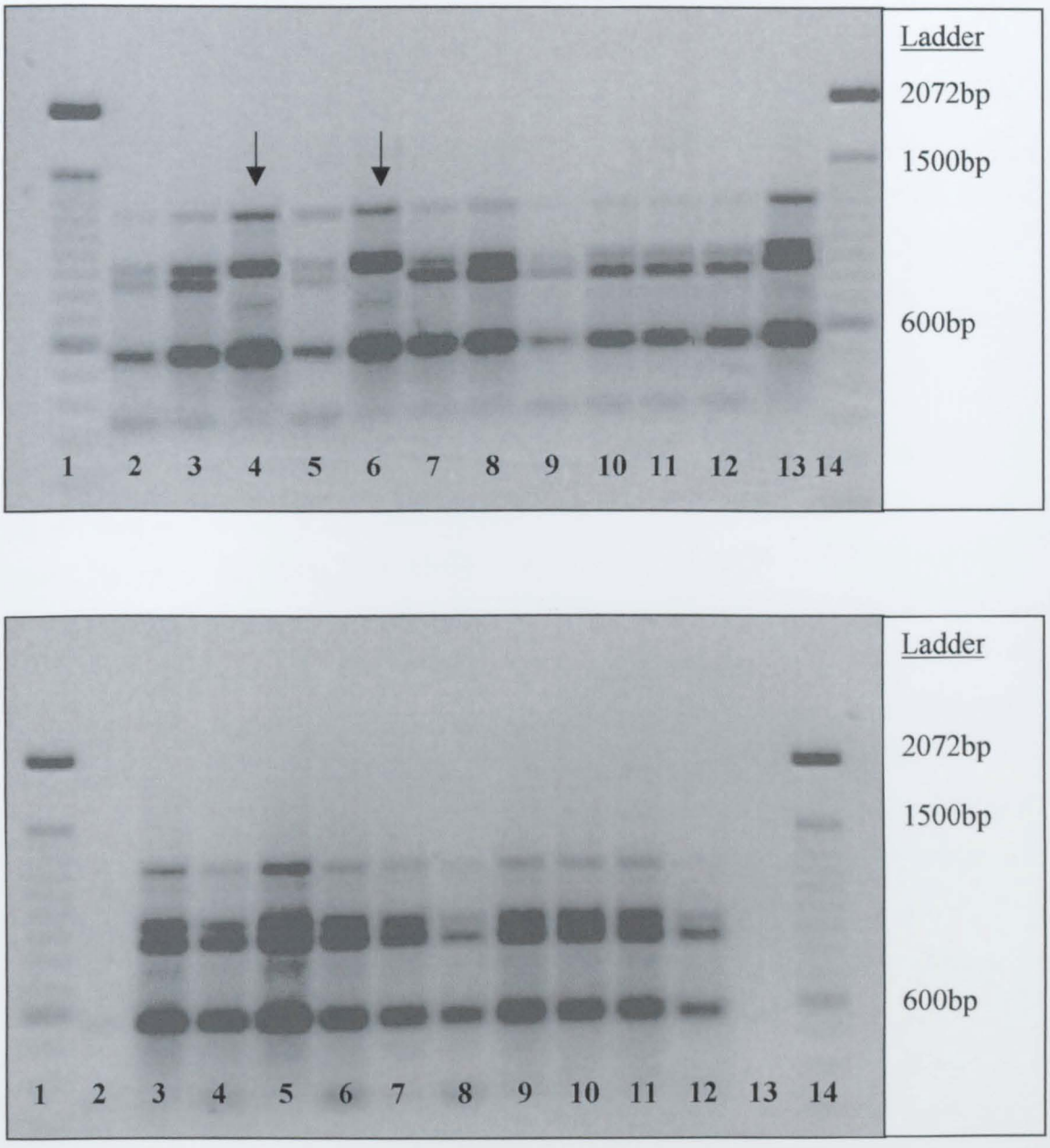




Key, top, lanes L to R: 1, 100bp ladder; 2,3,4,5,6, continual sub-culture; 7,8,9,10, lyophilised; 11,12,13, mycelial plugs in water; 14, 100bp ladder.

Bottom: 1, 100bp ladder; 2,3, mycelial plugs in water; 4,5,6,7 frozen at –20°C; 8,9,10,11, cryopreserved; 12, control; 13, 100bp ladder.

Fig.18: PCR fingerprints with MR primer of replicates of *Fusarium oxysporum* isolate F3 after 16 weeks of storage. Image colour reversed.



Key, top: lanes L to R: 1, 100bp ladder; 2,3,4,5,6, continual sub-culture; 7,8,9,10, lyophilised; 11,12,13, mycelial plugs in water; 14, 100bp ladder.

Bottom: 1, 100bp ladder; 2,3, mycelial plugs in water; 4,5,6,7 frozen at –20°C; 8,9,10,11,12, cryopreserved; 13, control; 14, 100bp ladder.

Replicates exhibiting polymorphisms indicated are by black arrows.

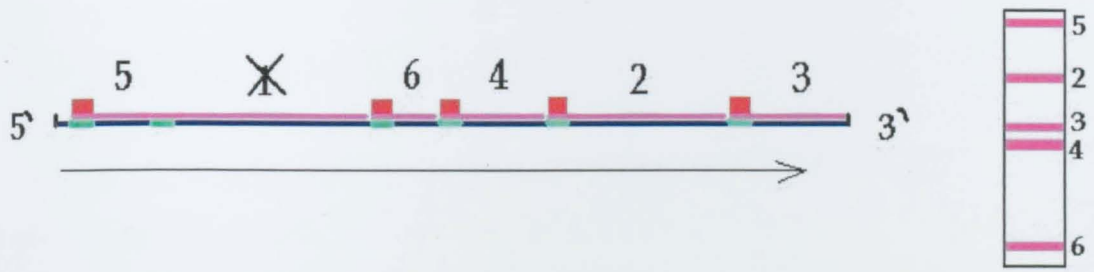
6.4 Discussion

Preservation regime can affect the genetic stability of the test fungi. VNTR and SSR-derived PCR fingerprints are indicative of the genetic stability of strains and may be changed following preservation and storage and are useful because they are generated from regions throughout the genome. Polymorphisms were detected in replicates of *Metarhizium anisopliae* isolates M1 and M2 and in *Fusarium oxysporum* isolate F3. The PCR fingerprints of *Fusarium oxysporum* isolates F1 and F2 and *Metarhizium* spp. isolate M4 remained stable throughout the investigation, which suggests that preservation regime does not induce genetic instability in these isolates. However, alternative primers may have detected changes in genetic stability that were not detected with the primer set used in this investigation. The apparent genetic stability of *Fusarium oxysporum* isolates F1 and F2 is surprising as *Fusarium* spp. are reported to be extremely labile in culture, especially when cultures are maintained by continual sub-culture where deterioration is widely reported (Booth 1971, Wing *et al.* 1995, Kim 1997). Even in replicates that exhibited metabolic instability and cultural deterioration, the PCR fingerprints produced with each primer were stable. This suggests that the physiological and cultural changes may be phenotypic or pleomorphic in nature rather than genetic. The polymorphisms in replicates of isolate F3 were detected after 16 weeks of storage in 2 replicates that had been maintained by continual sub-culture. A band of approx. 850bp in the typical fingerprint was absent. The band appearing at approx. 925bp was more intense and slightly larger (approx. 950bp) and the band appearing at 750bp was more intense than the equivalent band in other replicates. Banding pattern may have changed for several reasons. Firstly, the primer did not attach to the binding site that promotes amplification of a particular band. Consequently, the band was not synthesised and other bands were larger. Secondly, the primer-binding site (transposed) moved within the genome, causing a different sized band to be expressed. A third possibility could be that a sequence (e.g. an insertion sequence, transposon or foreign DNA) was inserted between the binding sites, thus altering the size of the PCR product. Any of these changes could result after an alteration in the conformation or sequence of the template DNA (Fig 19). However, microsatellites often occur in flanking genes that may be located on different chromosomes. Therefore, a single banding pattern may be composed of bands derived from several chromosomes.

Fig 19.Simplified diagrammatic illustration of the potential mechanisms that may induce changes in banding pattern.

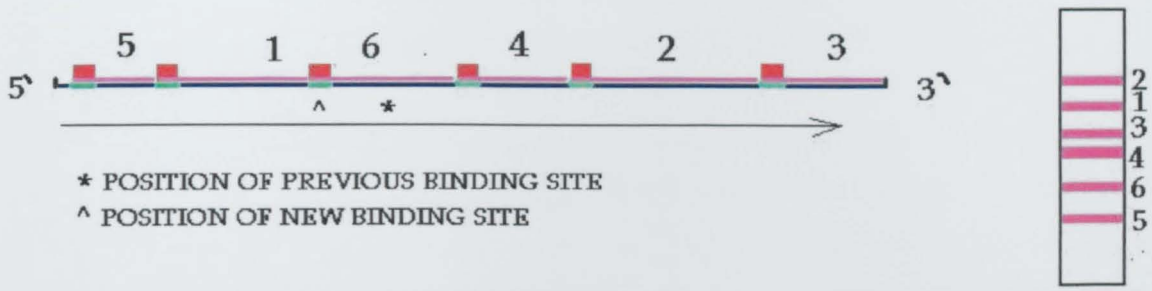
A. Typical banding strategy

B. Polymorphism induced by primer not attaching to binding site. (The primer does not bind to the site that produces band 1, consequently band 5 is larger and band 1 is deleted)



C. Polymorphism induced by sequence insertion. (A sequence (red line) is inserted between the primer binding sites that produce bands 2 and 4, consequently band 4 becomes larger.

D. Polymorphism resulting from the shifting of primer binding site. (The primer binding site that produces band 6 shifts to the left as a result of a conformational change, consequently band 1 becomes smaller and band 6 becomes larger



* POSITION OF PREVIOUS BINDING SITE
^ POSITION OF NEW BINDING SITE

template DNA strand, new DNA product, oligonucleotide primer, primer binding site, DNA insertion sequence, arrows indicate direction of new DNA synthesis.

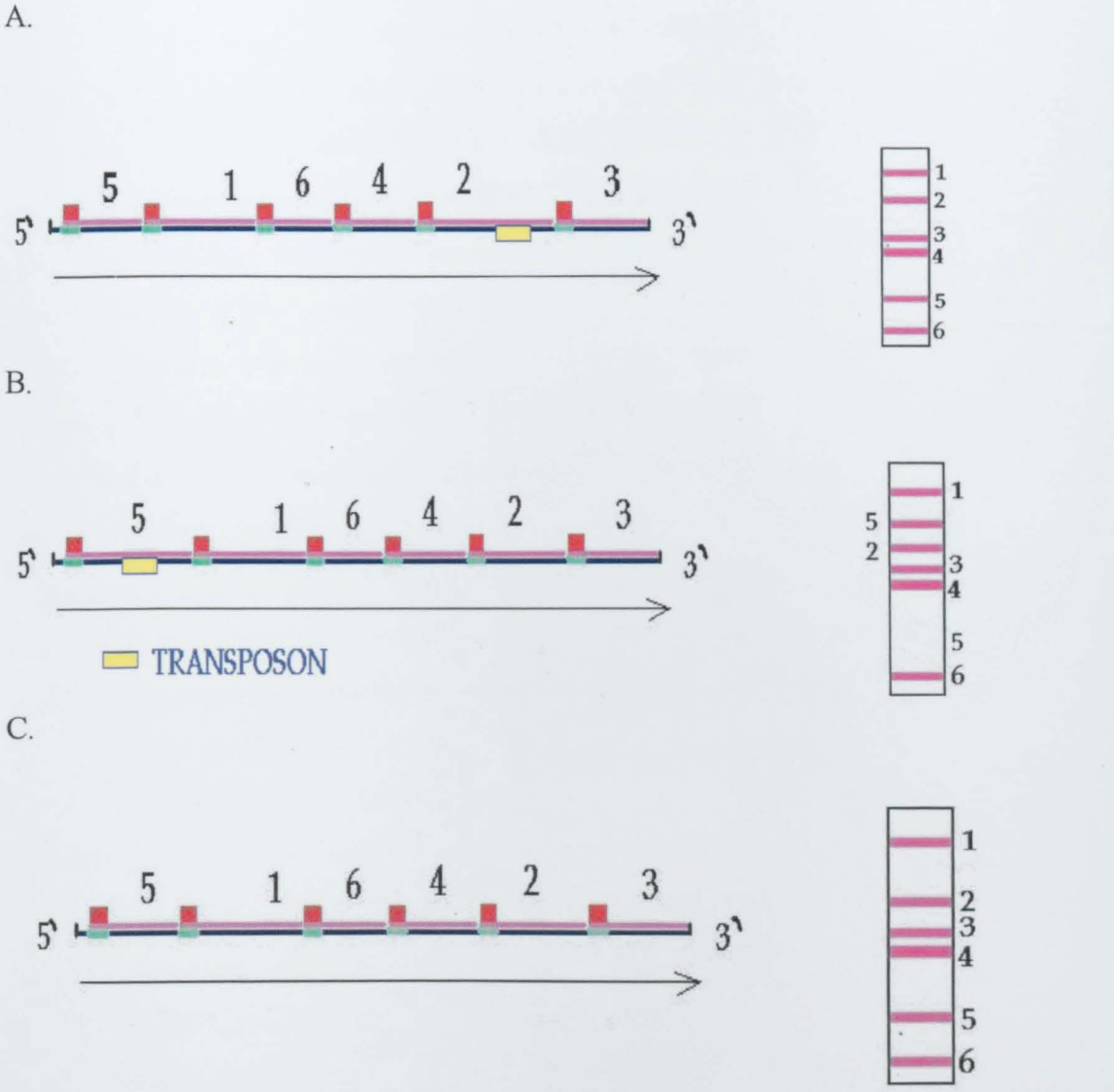
The polymorphism in the replicates that had been maintained by continual sub-culture may have resulted from cultural deterioration or environmental influences. The polymorphisms could result from any of the mechanisms that induce mutation, which are discussed in the next section. However, this does not address the fact that an identical polymorphism occurred in 2 replicates. This could be because an area of the genome of isolate F3, is susceptible to a specific event, which would have resulted in the same genomic re-organisation and the same change in PCR fingerprint banding pattern in the 2 replicates. However, the same polymorphism was not seen at other testing-times when further genetic deterioration might have been expected. Either, the event causing the polymorphism had not happened again, allowing the organism to recover or the culture may have been genetically heterogeneous (because of selection resulting from strain drift) and when sub-cultured the polymorphic form may not have been transferred. Therefore, the polymophic form would be lost and further detection of the polymorphism would be entirely due to chance. Alternatively, because PCR is a competitive process, and if two or more forms are present in the population, only the banding pattern from the dominant population would be exhibited.

Although fingerprints with GACA primer were homologous, polymorphisms were detected with MR primer in replicates of *Metarhizium anisopliae* isolates M1 that had been cryopreserved and lyophilised. A band of approx. 600bp was not detected and a band of approx. 500bp appeared in the replicates exhibiting polymorphism. The "new" band, approx. 100bp smaller than the band that was lost, indicates that a section of the template DNA was not replicated during the PCR extension phase. This could be because of either a conformational change or a transposable element being cleaved from a section of the template DNA. Because no new band was detected it could be assumed that the element was not re-inserted into the DNA between the MR primer binding sites. Polymorphisms were also detected with RY primer in 2 replicates that exhibited polymorphisms with MR primer, indicating more severe genetic disruption as a result of preservation. Polymorphisms were also detected in replicates of isolate M2, but with GACA primer. A replicate had been cryopreserved and 2 that had been lyophilised lost a band of approx. 750bp that was present in the original fingerprint. No extra bands were detected. The fingerprints with MR and RY primers were the same throughout the investigation. Because the polymorphisms were detected throughout the investigation in

cryopreserved and lyophilised replicates of *Metarhizium* isolate M1, it is probably the physical preservation processes that affect the genetic stability of an isolate rather than the length of storage *per se*. Because replicates of isolate M4 that had been cryopreserved and lyophilised did not exhibit polymorphisms after PCR fingerprinting it would appear that the genetic response to preservation is strain-specific.

Spontaneous or random mutations may be induced by natural or synthetic mutagens. Physical mutagens include heat, ionising radiation and ultraviolet radiation (Singleton and Sainsbury 1993). Chemical mutagens include alkylating agents and base analogues (Singleton and Sainsbury 1993). However, mutations may be induced by the stresses that occur during preservation and some possible mechanisms are discussed below. Transposons (or transposable elements of transposons) are segments of DNA that are able to insert themselves at random sites throughout the genome (Clutterbuck 1995). Transposable elements have been shown to alter genes and genomes by promoting changes in gene sequence, expression and possible chromosomal organisation (Daboussi 1997). Transposable elements (TE's) have also been shown to cause spontaneous mutations (Daboussi 1997). The possible action of transposition and how banding pattern may be affected is illustrated in Fig 20. Six families of transposons have been detected within *Fusarium oxysporum* (Daboussi and Langin 1994). Transposons may also be present in the nitrate reductase gene of *Metarhizium anisopliae* (Clarkson pers.com). Fungal transposable elements have been shown to be factors in genetic instability and may influence adaptation to environmental conditions (Daboussi 1997). As conditions are imposed on the fungus as a result of continual sub-culture, the fungus may adapt to the conditions via the rearrangement of transposable elements. Stress has been implicated in the movement of transposons. Anaya and Roncero (1996) found that chlorate can activate rearrangement of the retrotransposon *skippy*. Stress may be induced during the cooling or thawing/drying stages of the cryopreservation and lyophilisation protocols. In the *Metarhizium* replicates where polymorphisms were detected, an area of the genome may be more susceptible to transposition during preservation (as a consequence of biochemical imbalance or ice damage), explaining why the same change occurred in a number of the replicates. However, the nature and mechanism of how stress induces rearrangement remains unclear (Anaya and Roncero 1996).

Fig 20, Simplified diagrammatic illustration of the potential mechanism of transposition, that may induce changes in banding pattern. The typical fingerprint is illustrated in diagram A, with the transposon a segment of the sequence that produces band 2. Movement of the transposon (diagram B), results in the changing of banding pattern. Band 5 is larger following insertion of the transposon and band 2 is smaller following the deletion of the transposon sequence. Alternatively, the transposon may move out of the areas between primer binding sites, resulting in band 2 becoming smaller (diagram C.)



There are other mechanisms that could induce genetic instability including mycoviruses, DNA methylation, plasmids, chromosomal aberrations, heterokaryosis, parasexuality and small intron insertion sequences. Chromosomal aberrations may induce genetic instability. Horgen *et al.* (1996) examined the karyotype in lines of *Agaricus bisporus* and found that entire chromosomes had been lost in degenerate strains. Zolan (1995) suggested that fungi appear to have chromosomes that may be partially or wholly dispensable. However, it was reported that many deliberate attempts to induce karyotypic changes by repeated sub-culture had been largely unsuccessful (Zolan 1995). Unlike other methods of preservation that slow down the cell cycle during preservation, lyophilisation and cryopreservation are protocols that may stop the cell cycle during mitosis. It is not known if the cell cycle returns to normal after resuscitation from preservation or whether there is permanent damage to the chromosomes, which may lead to the formation of genetically different sub-populations. Extrachromosomal inheritance of plasmids or mtDNA may also result in genetic instability. Inessential mitochondrial chromosomes may become inherited into the main chromosomal body (Clutterbuck 1995). Plasmids are extragenomic DNA molecules that can integrate covalently into genomic DNA (Griffiths 1995). Increased insertion of plasmids into genomic DNA has been correlated with the onset of senescence in *Neurospora.* Akins *et al.* (1990) found that plasmids might equally be deleted from the genome of *Neurospora.* Either chromosomal changes or plasmid insertion/deletion could induce changes in PCR fingerprint banding pattern. However, chromosomal aberrations may be a more random event and not the cause of polymorphisms during this investigation.

Mycoviruses and related elements contribute to fungal diversity (Buck *et al.* 1998). Viruses and virus-like particles have been detected in isolates of *Metarhizium anisopliae* (Bogo *et al.* 1996). Virus-like elements may become inserted into the fungal genome and are transmitted intracellularly (Meinhardt *et al.* 1997). Extracellular transmission has not been proved (Clutterbuck 1995). Buck (1998) suggests that some phenotypic effects may be the result of virus infection. If a virus was present in the isolate before preservation, it is possible that it may replicate itself and become inserted into the fungal genome in younger cultures. However, the chances of transmission into the same site in the genome would be remote and insertion would probably involve a more random event dependent on the

physiological fitness of a particular replicate. Therefore, it is unlikely to have disrupted the banding patterns of the replicates in this investigation, but could affect the banding patterns of individual replicates under alternative culture conditions. Cytosine methylation levels may change during culture. Reynelopez *et al.* (1997) found that DNA methylation occurs during fungal morphogenesis and that changes could be detected with RFLPs. Kim (1997) found that methylation levels changed following successive sub-cultures. There is no information in the literature to suggest that methylation levels would affect banding patterns of RAPD-like PCR. Methylation cannot be ruled out as a causative mechanism of polymorphisms during this investigation.

Neither *Metarhizium anisopliae* or *Fusarium oxysporum* has a teleomorph, thus natural sexual genetic recombination is improbable. However, heterokaryosis and parasexuality are potential mechanisms that could allow genetic exchange. Heterokaryosis, a state where a fungus may have two or more genetically distinct nuclei, formed via anastomosis (Hawksworth *et al.* 1995), is considered to be "not the ideal system of co-operation" (Carlile 1987). Parasexuality is a four-step process that involves heterokaryon formation, diploidisation, somatic crossing over and haploidisation. Clutterbuck (1995) suggests that each step occurs at low frequency and their occurrence may be accidental. Parasexuality is unlikely to have occurred in the replicates of *Metarhizium* that exhibited polymorphisms during this investigation, as it is likely to be a rare and more random event. Polymorphism may also occur as a result of selection if genetically different sub-populations exist within a culture. However, selection is equally likely to occur as a result of changes at the genetic level.

The methods employed were suitable for assessing the genetic stability of the test fungi. The primers used provided reproducible strain-specific fingerprints throughout the investigation. However, these primers can only assess the stability of a small part of the genome, and it is possible that genetic aberrations may have gone unnoticed in replicates that had appeared to be stable with the primer set used in this investigation. A larger suite of primers i.e. $(CAT)_5$ (Desenzo and Harrington 1994) or $(CA)_8$ (Latge *et al.* 1998) could enlarge the areas of the genome that are investigated and increase the chances of detecting polymorphisms. Nevertheless, MR primer,

which is derived from the M13 phage is widely considered to be the universal fingerprint primer (Bridge pers.comm) and may be the primer of choice to conduct stability assays. Other molecular techniques could be adapted to assess the stability of isolates, for example AFLP or RFLP-PCR. The potential of transposon-based PCR system and the use of sequencing to determine the causative mechanism of polymorphism are discussed in Chapter 8.

The results from this investigation would suggest that molecular taxonomic studies carried out with fungi that have been preserved should be treated with caution, as any changes of PCR fingerprint, induced by preservation protocol could cause the fungus to be incorrectly grouped. It would appear that storage at –20°C and by mycelial plugs in water are the best methods for maintaining genetic stability although longer storage periods may induce genetic instability. Although the lyophilisation and cryopreservation regimes induced polymorphisms in some of the test fungi during this investigation, they are probably the best options for the long-term preservation of fungi. The use of strain-specific protocols should be developed to ensure the genetic stability of an isolate. Numerous replicates of the isolate should be preserved in case of difficulties with cultures. As a quality control measure, the PCR fingerprints of an isolate should be determined with a set of primers before preservation and assessed again following storage.

CHAPTER 7: VARIABILITY OF ISOLATE CHARACTERISTICS DUE TO PRESERVATION IN DIFFERENT CULTURE COLLECTIONS OR SECTORING

7.1 Introduction

The Worldwide Federation of Culture Collections (WFCC) issues guidelines for the establishment of culture collections (Hawksworth 1991). However, as independent organisations, culture collections operate their own procedures and regulations. Cultures may be maintained on different nutrient agars, stored under contrasting environmental conditions and dispatched differently. Preservation protocols and equipment will vary and operators will trained and managed under different regulations. Subsequently, isolates will be subject to different conditions in different organisations. In the UK this problem has been partly redressed with the establishment of the United Kingdom National Culture Collection (UKNCC) an umbrella organisation that formulates quality policy and co-ordinates services between member collections (UKNCC 1999). Nevertheless, there is a need to develop new and existing protocols (Smith, pers.comm.), to standardise techniques and procedures and assess the suitability of those already in place. This chapter consists of two experiments that aimed to assess the stability of synonymous strains deposited in different culture collections and establish the effect of sectorisation in plate culture.

Experiment 1: Comparison of synonymous isolates deposited in different culture collections. Synonymous strains of *Metarhizium* deposited in different culture collections were assessed for viability, culture morphology, and physiological and genetic similarity

Experiment 2: Investigation of sectorisation in an isolate of *Metarhizium*. Sub-cultured lines were established from sectors, formed by an isolate of *Metarhizium*. An assessment was made of the physiological and genetic similarity of the different lines over time.

7.2 Comparison of synonymous isolates deposited in different culture collections.

7.2.1 Introduction

When obtaining an isolate from a culture collection, scientists expect the culture to be a true representative of the strain requested, and this is especially important if the isolate is to be used for taxonomic comparisons, in research or as a teaching strain. If the culture does not possess the characteristics that were described when the isolate was submitted to the collection, the economic and scientific consequences could be damaging. Changes may result from misidentification or incorrect labelling, contamination with other species or from changes induced by the effects of preservation regime. For example, different lines of an isolate of *Schizosaccharomyces malidevorans* obtained from the Australian Wine Research Institute (AWRI) and Centraalbureau voor Schimmelcultures (CBS) culture collections, were examined for determination of mating type and the ability of the lines to sporulate and assimilate maltose. It was concluded that the CBS line had lost its homothallic capability compared to the isolate maintained by the AWRI (Bridge and May 1988).

The aim of this investigation was to assess whether synonymous strains of *Metarhizium* deposited in different major international culture collections show similarity in their culture characteristics, physiology and PCR fingerprint profiles.

7.2.2 Material and methods

Lines (replicates of the same isolate deposited in different international public service culture collections) of 6 isolates of *Metarhizium* spp. were selected (Table 1). One freeze-dried replicate of each catalogued isolate was obtained from each culture collection. Replicates were resuscitated onto SDA and a series of investigations carried out to compare the culture characteristics, physiological and genetic profiles of each culture. Cultures of each line were compared by examination of culture characteristics (growth rate, sporulation, culture morphology) thin layer chromatography of extracellular secondary metabolites, fluorogenic enzyme tests

(using 4-methylumbellifryl bound substrates) and PCR fingerprinting of genomic DNA. Details of methods are described in chapter 2.

Table 1 - Details of synonymous isolates and original accession numbers.

Code	Isolate	Centre origin / number
M5	*Metarhizium anisopliae* var. *anisopliae*	CBS 431.64
		IMI 164266
		CECT 2952
		MUCL 9815
M6	*Metarhizium anisopliae* var. *anisopliae*	CBS 464.70
		IMI 147690
M7	*Metarhizium anisopliae* var. *anisopliae*	CBS 130.22
		IMI 170143
M8	*Metarhizium anisopliae* var. *majus*	CBS 248.64
		MUCL 9644
M9	*Metarhizium anisopliae* var. *majus*	CBS 643.67
		NRRL 13970
M10	*Metarhizium flavoviride*	CBS 218.56
		NRRL 13971
		IHEM 3994

CBS-Centraalbureau voor Schimmelcultures- AG Baarn, Netherlands

IMI-International Mycological Institute (CABI Bioscience UK), Egham, UK

CECT-Coleccion Espanola De Cultivos Tipo- Universidad de Valencia, Spain

MUCL-Mycothèque de l'Université Catholique de Louvain, Louvain, Belgium

NRRL-Agricultural Research Service Culture Collection, Peoria, USA

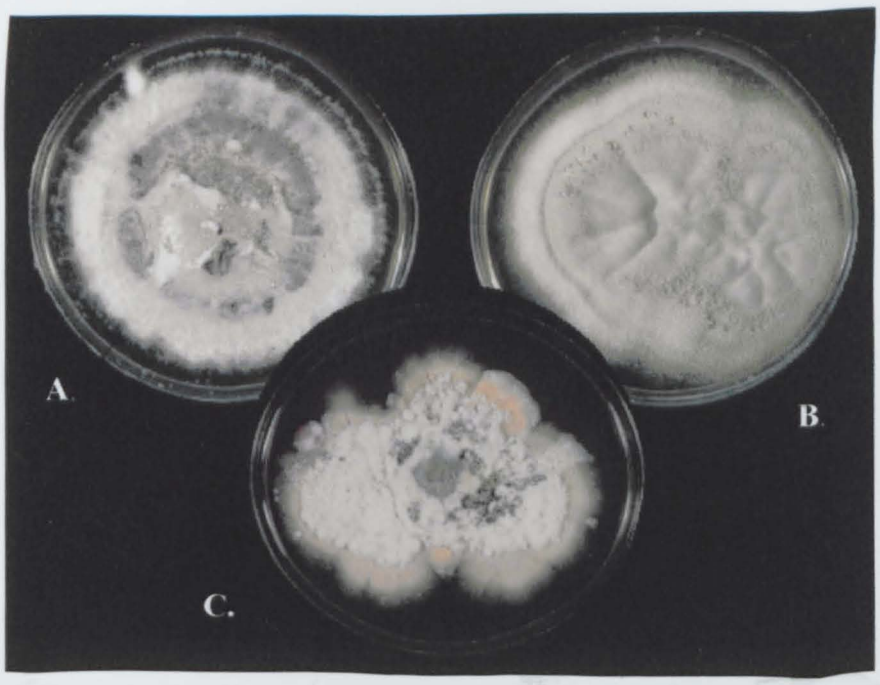
IHEM-Scientific Institute of Public Health - Louis Pasteur, Belgium

7.2.3 Results

7.2.3.1 Viability and culture characteristics

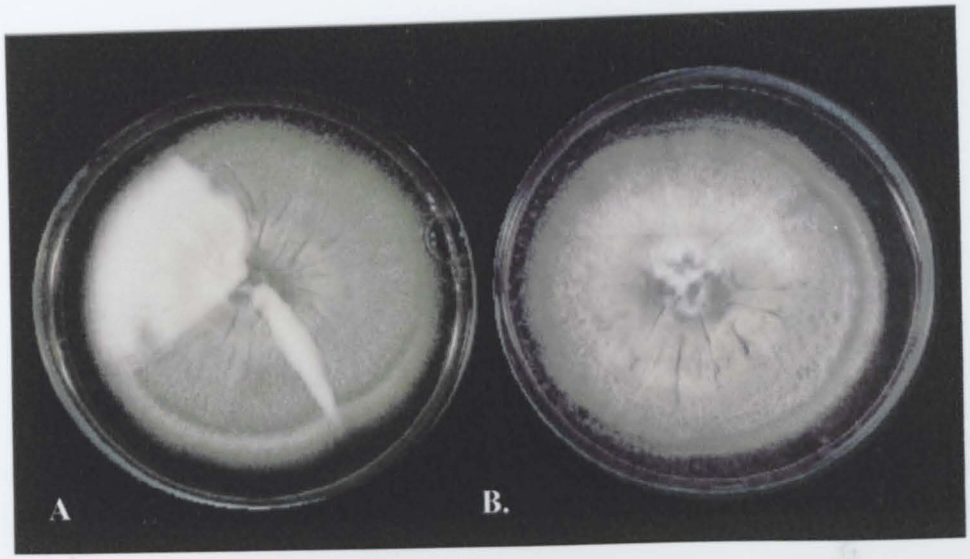
Most cultures obtained were viable. However, conidia of cultures M8 CBS 248.64 and M5 CECT 2592 did not germinate after resuscitation from storage, even after incubation under black light. Replicate lines of each isolate generally showed similar culture morphology. There were no observable differences in culture morphology and sporulation between cultures of M9 CBS 643.67 and M9 NRRL 13970 and between cultures of M6 CBS 130.22 and M6 IMI 147690. Cultures of the different lines of isolate M10 all exhibited similar culture morphology, but none sporulated under the experimental conditions imposed during this investigation. However, the culture morphology of the cultures of the different lines of isolate M5 were different (Fig 1). Cultures of line M5 MUCL 9815 produced aggregations of aerial mycelium, which was preceded by mycelium with an irregular culture margin and a yellow hue. Sporulation was limited and patchy. Cultures of lines M5 CBS 431.64 and M5 IMI 164266 exhibited similar mycelial culture morphology but differed in the degree of sporulation. M5 CBS 431.6 sporulated more heavily towards the centre of the mycelium whereas in line M5 IMI 164266, sporulation occurred throughout the mycelium.

Fig 1: Cultures of different lines of *Metarhizium anisopliae* isolate M5 (A=CBS 431.64;B=IMI 164266; C=MUCL 9815) after 28 days growth on SDA at 25°C



Cultures of the different lines of isolate M7 showed similar cultural morphology and sporulation of the same distribution, abundance and colour. However, two cultures of line M7 CBS 130.22 formed sectors. Sector morphology was the same as exhibited by the mycelium from which it arose. However, the sectors did not sporulate (Fig 2).

Fig 2: Cultures of different lines of *Metarhizium anisopliae* isolate M7 (A=CBS 130.22; B=IMI 170143) after 28 days growth on SDA at 25°C



7.2.3.2 Radial growth

There was no significant difference ($P>0.05$) in radial growth between cultures of the different lines of isolates M6, M7 (Fig 4) and M9. Cultures of M5 CBS 431.64 had a faster growth rate (2.2 mm day^{-1}) ($P<0.05$) than cultures of M5 IMI 164266 (1.6 mm day^{-1}) and M5 MUCL 9815 (1.5 mm day^{-1}) which had similar growth rates (Fig 3). Conidia from the cultures of *Metarhizium flavoviride* isolate M10 NRRL 13971 did not germinate for 6 days after inoculation onto SDA. However, once growth was initiated there was no significant difference ($P>0.05$) in growth rates between the three lines isolates of isolate M10 (Fig 5).

Fig 3: Graph showing radial growth of cultures of the different lines of *Metarhizium anisopliae* isolate M7, maintained on SDA at 25°C.

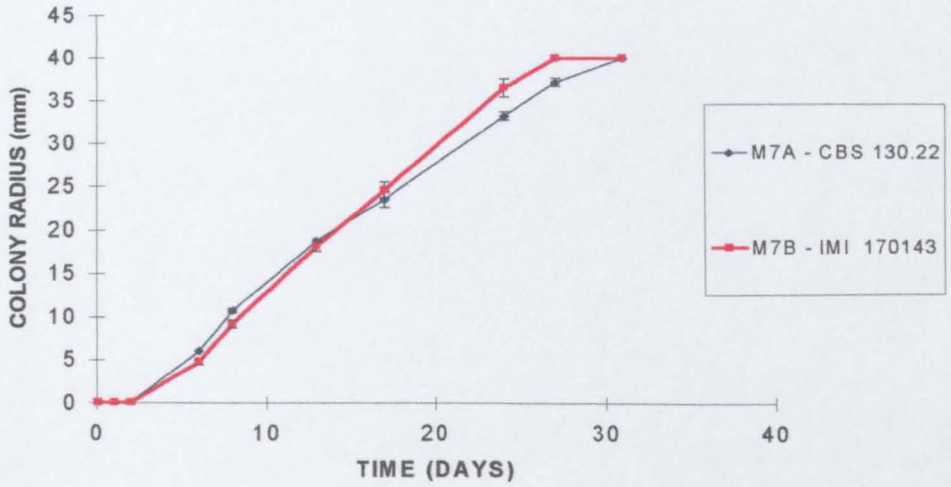


Fig 4: Graph showing radial growth of cultures of the different lines of *Metarhizium anisopliae* isolate M5, maintained on SDA at 25°C.

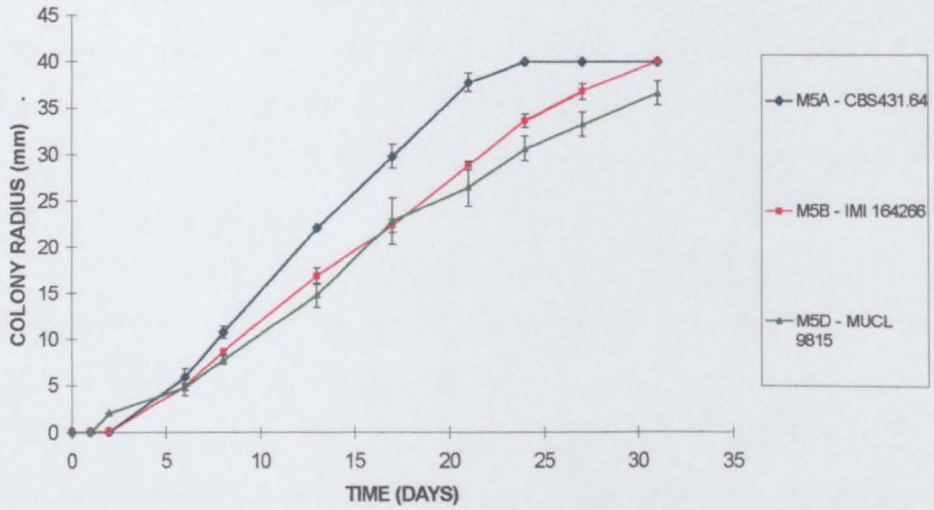
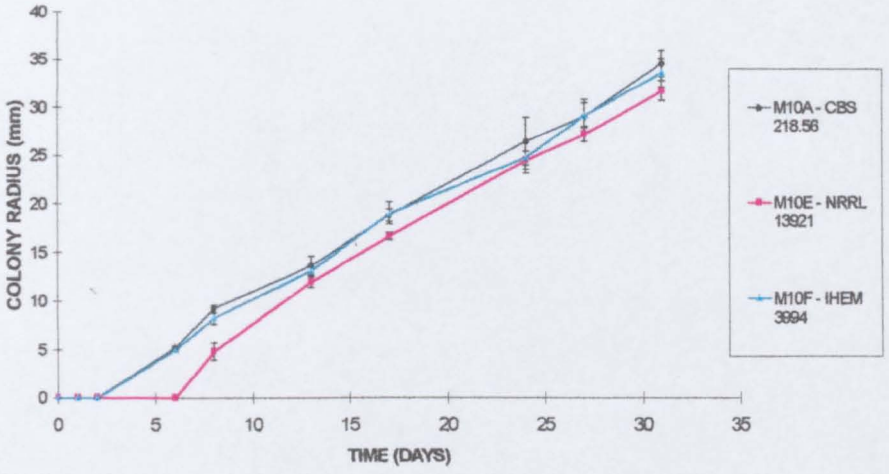


Fig 5: Graph showing radial growth of cultures of the different lines of *Metarhizium flavoviride* isolate M10, maintained on SDA at 25°C.



7.2.3.3 Secondary metabolites profiling

The extracellular secondary metabolite profiles from cultures of the different lines of isolate M5 are listed in Table 2. M5 MUCL 9815 produced 4 detectable extracellular secondary metabolites, only 3 of which were produced by cultures of M5 CBS 431.64 and M5 IMI 164266, which each produced 5 metabolites with identical properties. Cultures of the different lines of isolates M6 and M9 produced different numbers of metabolites. Cultures of line M6 CBS 464.70 produced 6 metabolites ($Rf_{(x100)}$ 7.7, 15.9, 23.5, 24.6, 33.5,96.3) with the same properties of those produced by replicates of cultures of line M6 IMI 147690. However, cultures of line M6 IMI 147690 produced an extra metabolite ($Rf_{(x100)}$ 96.3). Cultures of line M9 CBS 643.67 produced 5 metabolites ($Rf_{(x100)}$ 7.3, 9.0, 11.5, 25.2, 33.4). However, cultures of M9 NRRL 13970 only produced 4 metabolites (The metabolite at $Rf_{(x100)}$ 25.2, bright orange under UV light, was not detected). Cultures of lines M7A and M7B each produced 10 secondary metabolites, all of which had identical properties and Rf values. The 3 lines of isolate M10 all produced different profiles, only 3 metabolites were common to all 3 lines. The extracellular secondary metabolite profiles from cultures of the different lines of isolate M10 are listed in Table 3.

Table 2: Extracellular secondary metabolite profiles of cultures of the different lines of *Metarhizium anisopliae* isolate M5

Spot Rf (x100) & characteristics◆	**M5A** (CBS 431.64)	**M5B** (IMI 164266)	**M5D** (MUCL 9815)
4.8 +/- 0.25 white/yellow UV	●	●	●
8.8 +/- 0.51 white/yellow UV	●	●	●
11.6 +/- 0.43 purple after spray	●	●	X
24.5 +/- 0 UV	X	X	●
34.9 +/- 0.72 yellow UV	●	●	●
41.7 +/- 0.32 orange after spray	●	●	X

Key: ● metabolite detected; x metabolite not detected; ◆ colour under UV light.

Table 3: Extracellular secondary metabolite profiles of cultures of the different lines of *Metarhizium flavoviride* isolate M10

Spot Rf (x100) & characteristics	**M10A** (CBS 218.56)	**M10E** (NRRL 13971)	**M10F** (IHEM 3994)
Rf 5.3 +/- 0.6 White/yellow UV	●	●	●
Rf 7.53 +/- 0.990 White/yellow UV	●	●	X
Rf 9.6 +/- 0.7 White/yellow UV	●	●	●
Rf 26.2 +/- 0.3 Orange after spray	X	X	●
Rf 28.8 +/- 0.5 Blue after spray	X	●	X
Rf 35.2 +/- 0.5 UV	●	●	●

Key: ● metabolite detected; x metabolite not detected; ◆ colour under UV light.

7.2.3.4 Assays of extracellular enzymes

Cultures of lines CBS 431.64 and IMI 164266 of isolate M5 utilised 7, 4-methylumbelliferyl bound substrates, line D utilised 6 substrates but did not utilise the substrate for β-xylosidase (Table 4). Cultures of the different lines of isolates M6 and M7 each produced homologous extracellular enzyme profiles. Cultures of line

M9 NRRL 13971 did not utilise the substrates for α-arabinofuranosidase and β-xylosidase, whereas cultures of line M9 643.67 produced both α-arabinofuranosidase and xylosidase activity.

Table 4: Extracellular enzyme profiles of cultures of the different lines of *Metarhizium* deposited in different culture collections

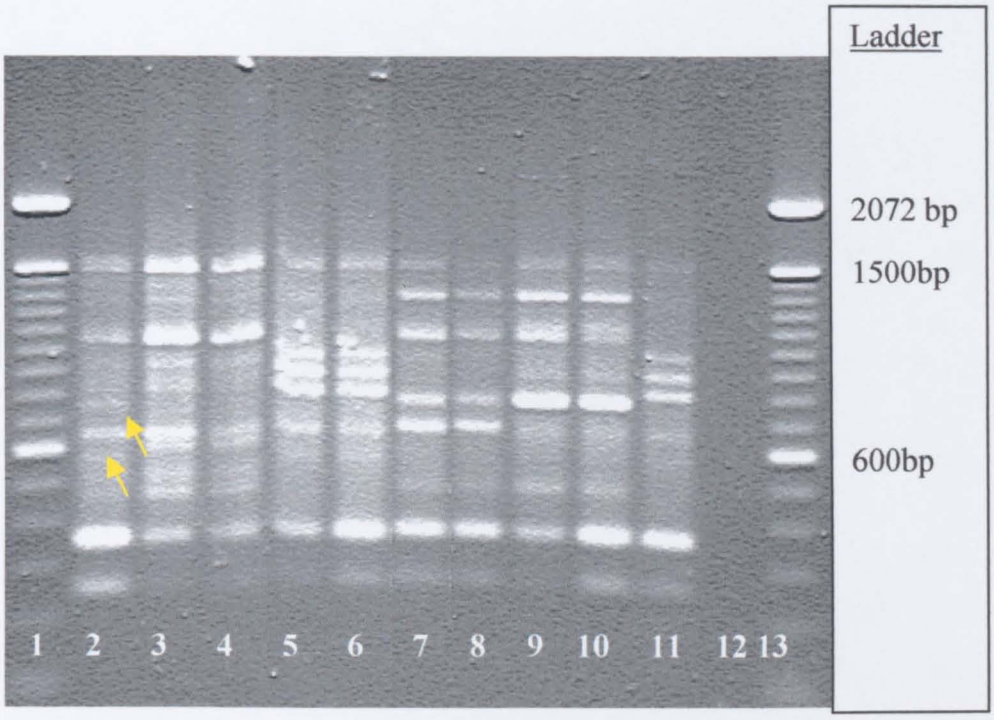
	M5	M5	M5	M6	M6	M7	M7	M9	M9
ENZYME	CBS 431.64	IMI 164266	MUCL 9815	CBS 464.7	IMI 147690	CBS 130.22	IMI 170143	CBS 643.67	NRRL 13970
Glucosamidase	✹	✹	✹	✹	✹	✹	✹	✹	✹
Arabinofuranosidase	-	-	-	-	-	-	-	-	-
Chitobiosidase	✹	✹	✹	✹	✹	✹	✹	✹	✹
Fucosidase	✹	✹	✹	✹	✹	-	-	X	✹
Galactosidase	✹	✹	✹	✹	✹	✹	✹	✹	✹
Glucosidase	✹	✹	✹	✹	✹	✹	✹	✹	✹
Glucuronidase	-	-	-	✹	✹	-	-	-	-
Mannosidase	-	-	-	-	-	-	-	-	-
Xylosidase	✹	✹	X	-	-	-	-	X	✹
Esterase	✹	✹	✹	✹	✹	✹	✹	✹	✹

✹ Enzyme activity detected; **X** Enzyme activity not detected; **-** Enzyme not detected in isolate profile of any line.

7.2.3.5 Assessment of genetic similarity.

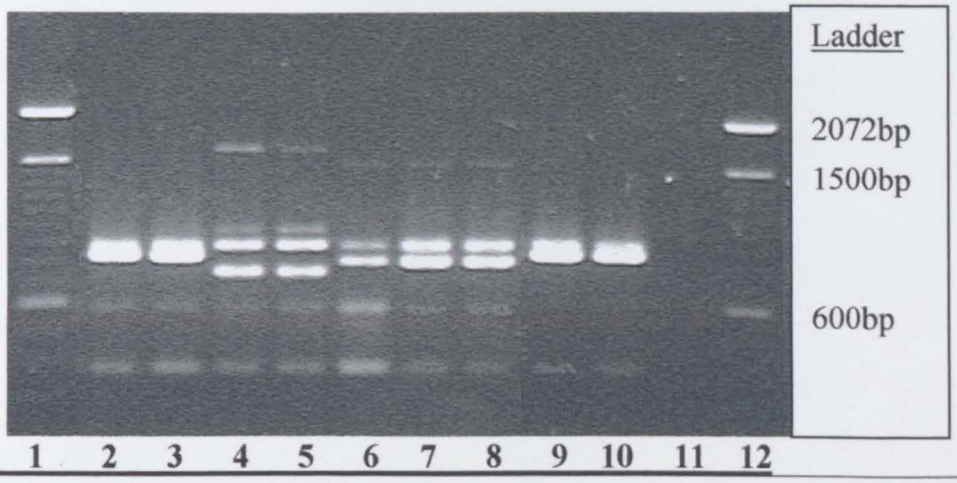
PCR fingerprints were obtained with GACA (Fig 7) and MR primers (Fig 8). No polymorphisms were detected between cultures of lines M6 CBS 464.70 and M6 IMI 147690, M7CBS 130.22 and M7 IMI 170143 and M9 CBS 643.67 and M9 NRRL 13970 with either primer. Cultures of the different lines of isolate M9 produced 6 bands with GACA and 9 bands with MR primer. Cultures of the different lines of isolate M6 produced 3 bands with GACA and 8 bands with MR primer. Cultures of the different lines of isolate M7 produced 4 bands with GACA and 10 bands with MR primer. A polymorphism was detected between cultures of the different lines of isolate M5. Lines M5 IMI 164266 and M5 MUCL 9815 produced 11 bands with MR primer. However, 12 bands were produced by cultures of line M5 CBS 431.64. Two additional "faint" bands were detected at approx. 575bp and 750bp and a band of approx. 600bp visible in the fingerprints of lines M5B and M5D had disappeared. All replicate lines of isolate M5 produced 5 bands with GACA

Fig. 7: PCR fingerprints with MR primer obtained from cultures of the different lines of *Metarhizium* isolates deposited in different culture collections



Key, lanes L to R: 1, 100bp ladder; 2, M5 CBS 431.64; 3, M5 IMI 164266; 4, M5 MUCL 9815; 5, M9 CBS 643.67; 6, M9 NRRL 13970; 7, M6 CBS 464.7; 8, M6 IMI 147690; 9, M7 CBS 130.22, 10, M7 IMI 170143; 12, control; 13, 100bp ladder. bp =base pairs. Polymorphisms in lane 2 are indicated with yellow arrows.

Fig. 8: PCR fingerprints with GACA primer obtained from the cultures of the different lines of isolates deposited in different culture collections



Key, lanes L to R: 1, 100bp ladder; 2, M7 CBS 130.22; 3, M7 IMI 170143; 4, M9 CBS 643.67; 5, M9 NRRL 13970; 6, M5 CBS 431.64; 7, M5 IMI 164266; 8, M5 MUCL 9815; 9, M6A CBS 464.7; 10, M6B IMI 147690; 11, control; 12, 100bp ladder. bp =base pairs.

7.2.4 Discussion

Different lines of isolates of *Metarhizium* deposited in different culture collections vary in physiological, cultural and genetic attributes. For example, in isolate M5, cultures of each of the 3 lines exhibited different culture morphologies. Replicates of line M5 MUCL 9815 had degenerative culture morphology and poor sporulation, which did not recover after further sub-culture. Culture morphology was similar amongst cultures of the different lines of isolates M6, M7 M9 and M10. Although a sector was detected in a culture of line M7. These results illustrate that isolates obtained from different culture collections may exhibit different culture morphology. If this was to happen to a type strain or an isolate used for taxonomic comparisons or for teaching or research, assumptions of the cultural behaviour of an isolate may be falsely attributed as being representative of the isolate. Different culture characteristics may be the result of cultures becoming accustomed to growth on different nutrient agars and the fungus may become irreversibly adapted to the conditions during maintenance imposed in different culture collections in addition to any stresses encountered during the preservation and storage procedures. However, deterioration of cultures could also have occurred during handling before deposit into a culture collection. Growth rates were similar amongst cultures of the different lines of isolates M6, M7, M9 and M10. However, the growth rates of culture of lines M5 CBS 431.64, M5 IMI 164266 and M5 MUCL 9815 were significantly different ($P<0.05$), cultures of line M5 CBS 431.64 growing faster. Although line M5 MUCL 9815 exhibited atypical culture morphology, it had a similar growth rate to line M5 IMI 164266. If growth rates are required for physiological investigations, the source of the culture may affect the validity of the results obtained. Very few lines exhibited similar extracellular secondary metabolite profiles. Only 2 of the 3 lines of isolate M5 and both lines of isolate M7 exhibited similar extracellular secondary metabolite profiles, no other lines of any isolates exhibited similar profiles. These results suggest that secondary metabolite profiles are easily changed by the stresses that are encountered during the preservation and storage procedures of different culture collections. The results correspond with the data obtained in chapter 4 (i.e. that secondary metabolite profiles may be easily disrupted by preservation and storage regime and that the metabolite profiles of some strains may be more resistant to preservation regime). Cultures of line M5 MUCL 9815 and M9 CBS 643.67 failed to

exhibit all of the enzyme activities that were detected in other lines of the same isolates. Notably α-fucosidase and β-xylosidase activity was not detected in line M9 CBS 643.67 but was detected in line M9 NRRL 13970. Without knowing the enzyme profile of the isolate prior to preservation it cannot be known which of the lines (if any) were exhibiting the extracellular enzyme profile that was typical of the original isolate. The physiological differences between lines that were determined during this investigation indicate the risks that may be taken when using preserved cultures of the organisms tested in research.

Kuhls *et al.* (1995) noted that strains obtained from different culture collections often had different PCR fingerprint patterns. However, in this experiment only 1 polymorphism was detected between the lines of isolates obtained from different culture collections. This was a polymorphism detected in line M5 CBS 431.64, where 2 bands were different from the fingerprints exhibited by lines M5 IMI 164266 and M5 MUCL 9815. The potential causes and mechanisms of genetic changes have been discussed in chapter 6. However, a different banding pattern is unlikely to be due to contamination with another species. With 10 bands common to all 3 lines it is highly likely that the line is a replicate of isolate M5. Although no polymorphisms were detected among lines of the other isolates, it does not necessarily infer that they are genetically identical, as changes may have occurred outside of the areas of the genome that are specific to the primers used in this investigation. There appears to be no correlation between genetic and physiological instability on this basis.

The stability of an isolate may be affected by the preservation regime. However, the different regimes operated by different culture collections and the way a culture is maintained, processed and handled may increase the chances of long-term instability of its culture characteristics, physiology stability and genetics. Some collections will sub-culture all of the strains of a particular genus on the same day (Smith pers.comm.). In a genus such as *Metarhizium*, where spores can easily become air-borne, this increases the chances of cross-contamination. Collections may maintain their isolates on different nutrient agars, and this may promote selective pressures. Isolates that are frequently requested may be resuscitated, replicated and

re-preserved many times, substantially increasing the potential for physiological and genetic damage. Collection procedures and preservation protocols will be different, often at the discretion of the curator and within strict financial constraints. Without appropriate levels of financial and staff commitment, cultures may become neglected, further increasing the chances of strain drift. There is a need for all public service culture collections to harmonise procedures, in order to reduce the chances of strain deterioration. The cultural, physiological and genetic properties of an isolate should be determined before preservation, so future comparisons can be made to establish the stability of a resuscitated isolate. Scientists should be cautious when using cultures obtained from culture collections in major research projects. Incorrect supply or atypical behaviour could result in major disruption to research programmes. However, if culture collections use quality control and comply to recommended quality management systems (Smith pers.comm.) such as those advocated by the UKNCC, then there is an improved likelihood that the culture supplied is characteristic of the original isolate.

7.3 Investigation of sectorisation in an isolate of *Metarhizium*

7.3.1 Introduction

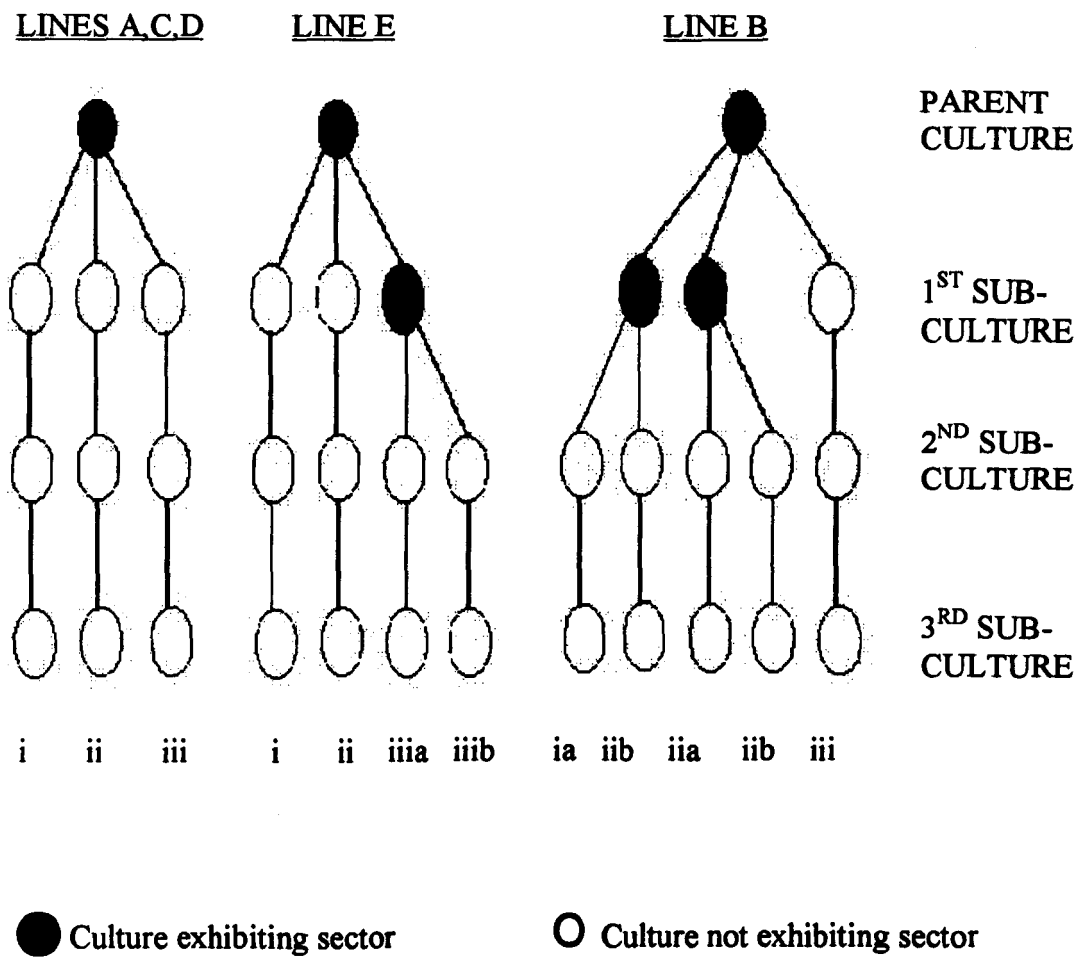
The entomopathogenic fungus *Metarhizium* is used commercially as a biological control agent against insect pests such as the desert locust (*Shistocerca gregaria*) (Prior *et al.* 1992). Successful application in the field requires accurate formulation to ensure viability and stability of the fungal product (Moore *et al.* 1994). For this to happen, maintenance of parent cultures must be tightly controlled and monitored to ensure the fungus retains its pathogenicity. In culture, *Metarhizium* spp. are commonly maintained on Sabouraud's Dextrose Agar (SDA), a nutrient agar that promotes excellent vegetative growth and sporulation (Prior pers.comm.). However, sectors are commonly formed on agar culture that differ in cultural morphology from the parent culture. For example, some sectors do not sporulate. Hawksworth *et al.* (1995) describe the formation of sectors as mutation or selection in plate cultures resulting in one or more sectors of the culture having a changed form of growth. Proser (1993) concluded that sector formation resulted from atypical growth; the reason for which was not well understood. Kim (1997) observed attenuation in cultures of *Fusarium oxysporum* f.sp. *niveum*. After eighteen successive sub-cultures, sectors were detected that exhibited variation in colonial morphology and pigmentation. However, it was concluded that sector characteristics remain stable, even after further sub-culture. Stock cultures of wood-decaying basidiomycetes are also subject to progressive senescence, and show an increased tendency to sector in culture (Gramss 1991b). In this investigation, sub-cultures were taken from sectors, when they were grown on fresh media. The culture morphology, physiological activity and genetic stability of replicates sub-cultured from the sectors, was analysed using thin layer chromatography of secondary metabolites, extracellular enzyme tests and PCR fingerprinting using microsatellite primers.

7.3.2 Materials and methods

Five replicates of *Metarhizium* spp. isolate M4 (I 97-1123) that had been stored for 16 weeks were selected. For each culture of which exhibited sectoring, the culture morphology of each sector was described (Fig 9). Three agar blocks were sub-cultured from each sector onto plates of SDA, maintained at 25°C and then sub-cultured every 21 days (Fig 9). Each replicate was examined for cultural morphology and sporulation. The physiology and genetic stability of each line was assessed after

a 2nd sub-culture. Thin layer chromatography of secondary metabolites, extracellular enzyme tests and an assessment of genetic stability was carried out according to the methods described in Chapter 2.

Fig 9: Culture histories of lines investigated in sectorisation investigation



(a) and (b) refer to lines taken from cultures that formed sectors after the 1st sub-culture; a= subbed from non-sectoring zone; b= subbed from sector exhibiting recovery in the ability to sporulate.

Line A was subbed from a non-sporulating sector of a culture that had been lyophilised; Line B was subbed from a non-sporulating sector that had been stored cryopreserved; Line C was subbed from a non-sporulating sector of a culture that had been cryopreserved; Line D was subbed from a limited sporulating sector of a culture that had been maintained by continual sub-culture, Line E was subbed from a sector with limited sporulation sector of a culture that had been cryopreseved.

7.3.3 Results

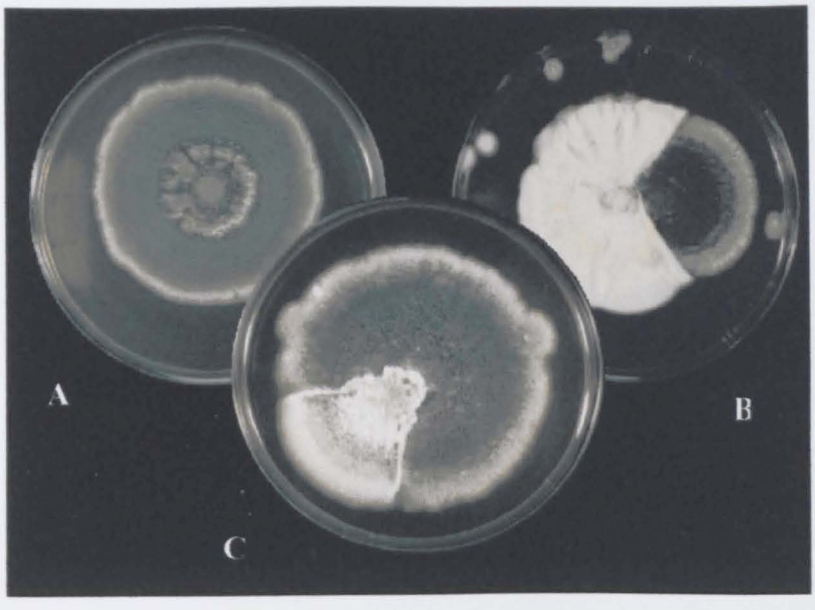
7.3.3.1 Observation of culture characteristics

Sectors were frequently formed in plate cultures of *Metarhizium* isolate M4 (Fig 9). Sectorisation in *Metarhizium* can be classified into 4 types based on criteria derived from analysis of culture morphology and the extent of sporulation (Table 5). The typical culture morphology exhibited by the original isolate consisted of mycelium producing aerial hyphae and conidiophores. However, some sectors exhibited atypical culture morphology, which consisted of mycelium that was resupinate (i.e. was compact and did not produce aerial structures). None of the sectors formed in this investigation produced conidia to the extent exhibited by the mycelium from which they arose and some sectors ceased to sporulate altogether.

Table 5 : Classification of cultural morphology

Type	Culture morphology	Sporulation
1	Typical	Normal
2	Typical	Reduced
3	Typical	Non-sporulating
4	Atypical	Non-sporulating

Fig 10: Sectorisation in *Metarhizium* isolate M4



A = typical culture morphology, B = typical culture morphology yielding sector of atypical non-sporulating culture morphology, C = typical culture morphology yielding typical sector with limited sporulation.

After continual sub-culture of the lines derived from sectors, the morphology exhibited by the cultures rarely recovered to the cultural morphology exhibited by the original isolate (Fig 10a). However, on sub-culture, 2 replicates of line B and 1 replicate of line E, (Fig 9) originally sub-cultured from a non-sporulating sector exhibiting typical culture morphology (type 3), formed further sectors which recovered the ability to sporulate (Fig 11). However, sporulation was limited and no line recovered levels of sporulation typical of the original isolate. Further sectorisation was observed in many of the replicates, but they either retained the cultural morphology that was exhibited by the mycelium from which they arose or they degenerated further. Cultures subbed from atypical sectors of line C (type 4) did not show any evidence of recovery.

Fig11: Culture morphology of replicates of *Metarhizuium* isolate M4 sub-cultured from sectors.

A = Replicate Biii, exhibiting typical non-sporulating culture morphology, B = Replicate Dii, exhibiting typical limited sporulting culture morphology, C= Replicate Eiii, exhibiting typical non-sporulating culture morphology yielding limited sporulating sector.

Table 6: Summary of culture morphology exhibited by lines originally sub-cultured from sectors after 3 sub-cultures

Line / replicate	Mycelium morphology	Sporulation	Type*
Vi, Vii , Viii	Typical	Non-sporulating	3 (3)
W(a) i	Typical	Non-sporulating	3 (3)
W(b) ii	Typical	Limited sporulating	2 (3)
W(a)ii	Typical	Non-sporulating	3 (3)
W(b)ii	Typical	Limited sporulation	2 (3)
Wiii	Typical	Non-sporulating	3 (3)
Xi, Xii, Xiii	Atypical	Non-sporulating	4 (4)
Yi, Yii ,Yiii	Typical	Limited sporulation	2 (2)
Zi, Zii	Typical	V. limited sporulation	3 (2)
Z(a)iii	Typical	V. limited sporulation	3 (2)
Z(b)iii	Typical	Limited sporulation	2 (2)

* Culture morphology type (Table 5), brackets refer to morphology of original sector from which line was subbed

7.3.3.2 Fluorogenic enzyme assays

The enzyme activities of replicates from each line are presented in Table 4.

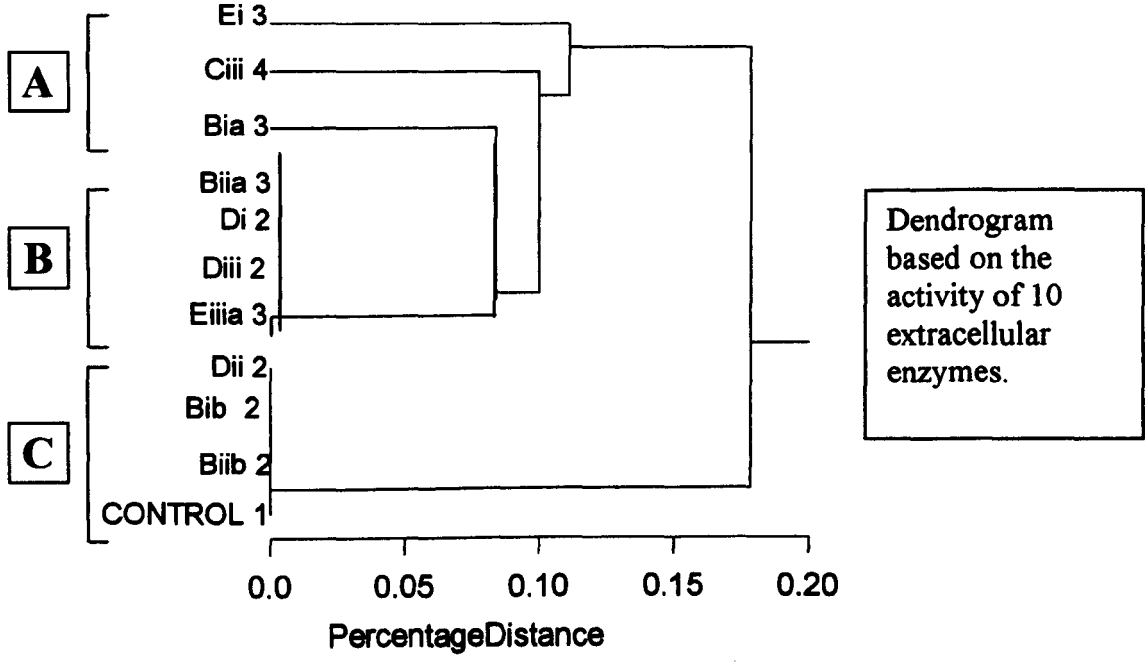
Table 4. Enzyme activities of lines and replicates derived from sectors.

	ENZYME ASSAYED FOR									
Line (replicate)	β-glucosamidase	α-arabinofuranosidase	β-chitobiosidase	α-fucosidase	β-glucosidase	β-galactosidase	β-glucuronidsidase	α-mannosidase	β-xylosidase	esterase
Bia 3	●	x	●	●	●	●	●	x	x	●
Bib 2	●	x	●	●	●	●	●	x	x	●
Biia 3	●	x	●	●	●	●	x	x	x	●
Ciii 4	●	x	●	●	x	●	x	x	x	●
Biib 2	●	x	●	●	●	●	●	x	x	●
Di 2	●	x	●	x	●	●	x	x	x	●
Dii 2	●	x	●	●	●	●	●	x	x	●
Diii 2	●	x	●	●	●	●	x	x	x	●
Ei 3	x	x	●	x	●	●	x	x	x	●
Eiii 3	●	x	●	x	●	●	x	x	x	●
Control	●	x	●	●	●	●	●	x	x	●

● enzyme activity detected; x enzyme activity not detected; 2,3,4 see Table 5.

Three replicates (Bib 2, Biib 2, Dii 2) retained the enzyme profile that was characteristic of the original isolate, shown as group C on the dendrogram (Fig 12). However, the others did not (groups A and B). Of these, 3 of the morphological variants failed to produce 1 or more enzymes and clustered together on the dendrogram as group A (Fig 12). Replicate Ciii4 did not produce β-galactosidase. α-Fucosidase activity was only retained by replicates Bib 1, Biib 2 and Biia 3 and Dii 2. β-glucosamidase activity was only retained by replicates Bib 2, Biib 2, Dii 2.

Fig 12: Dendrogram produced from extracellular enzyme profiles from replicates of lines cultured from sectors of *Metarhizium* isolate M4.



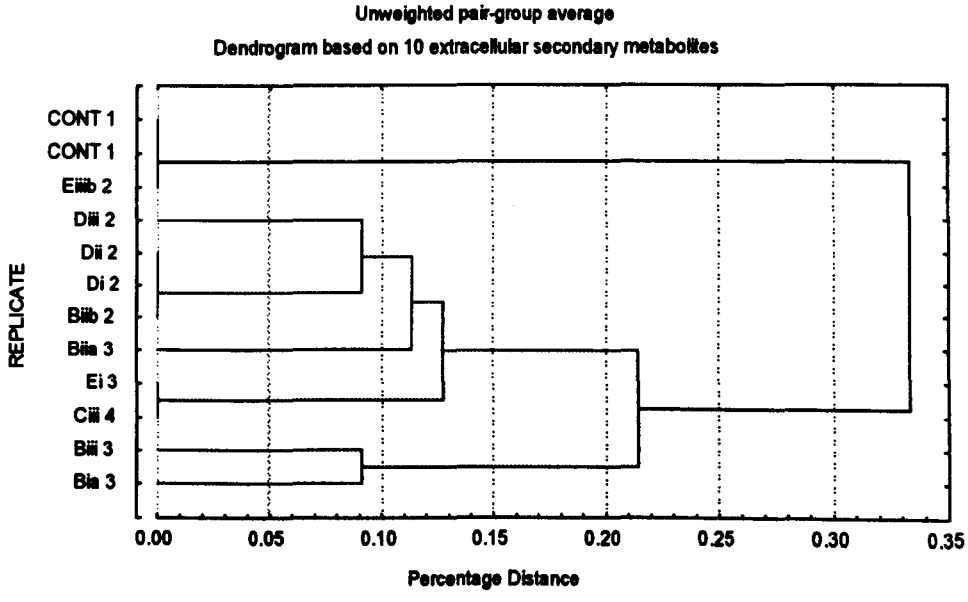
Key: B,C,D,E, = line; i, ii, iii = replicate number; Control = original M4 isolate; a, further sub-culture from non-sectoring mycelium, b. further sub-culture from a sector exhibiting a limited recovery in the ability to sporulate; 1-4, culture morphology (see Table 5).

7.3.3.3 Secondary metabolite profiling

The characteristic secondary metabolite profiles of *Metarhizium* isolate M4 is presented in chapter 4, section 4.2.2. Only 1 replicate Eiiib, exhibited the extracellular secondary metabolite profile that was characteristic of the original

isolate. All of the other replicates lost or gained additional extracellular secondary metabolites. Three replicates (Biib 3, Bib 3 and Ciii 4) gained 2 extra metabolites (Rf $_{(X100)}$ 59.3, 61.3). The remaining replicates lost either 1 or 2 metabolites from the original extracellular secondary metabolite profile exhibited by the original isolate.

Fig. 13: Dendrogram compiled from extracellular secondary metabolites of replicates of lines derived from sectors produced by *Metarhizium* isolate M4



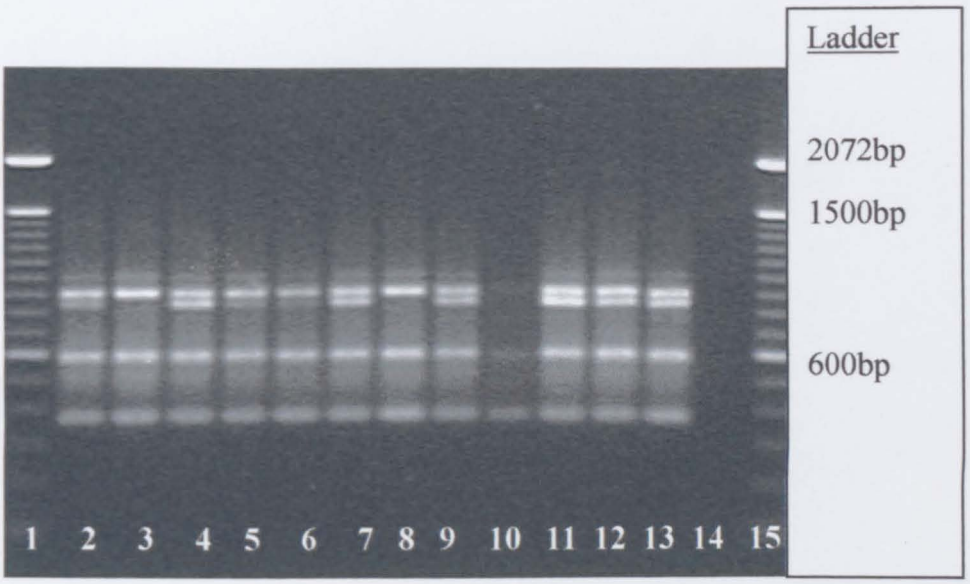
Key: B,C,D,E, = line; i, ii, iii = replicate number; Control = original M4 isolate; a, further sub-culture from non-sectoring mycelium, b. further sub-culture from a sector exhibiting a limited recovery in the ability to sporulate; 1-4, culture morphology (see Table 5).

7.3.3.4 Assessment of genetic stability

Polymorphisms were detected amongst replicates of the lines originally subbed from sectors. After PCR fingerprinting with GACA primer (Fig 14), 5 replicates exhibited a fingerprint of 4 strong bands characteristic of the fingerprint of the original M4 isolate. However, polymorphisms appeared in the fingerprints of 4

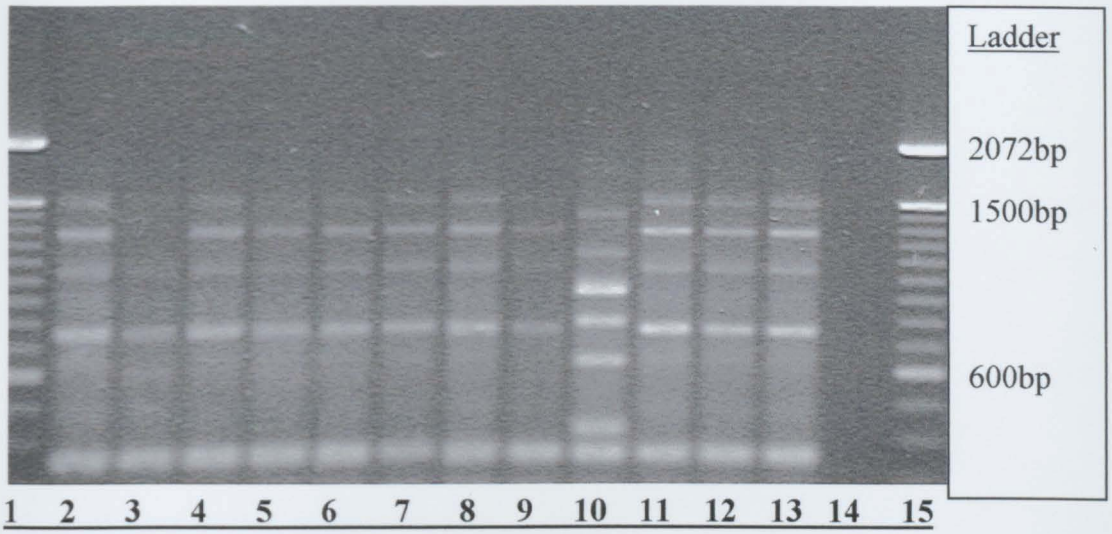
replicates and only 3 bands were visible (Bia, Ciii, Dii and Ei). A band of 850bp was completely lost. After PCR fingerprinting with MR primer (Fig 15), a polymorphism was detected in a replicate Ei that had exhibited non-sporulating typical culture morphology (type3) culture morphology. Out of 7 bands only 2 were homologous to the fingerprint of the original isolates. However, the distance between the bands appearing at approx. 1100bp and approx. 1400bp were spaced by the same distance on the gel as the bands at approx. 1000bp and approx.1300bp appearing in the fingerprints of the other replicates. All other replicates exhibited fingerprints that were characteristic of the original culture of isolate M4.

Fig 14. PCR fingerprints with GACA primer of replicates of lines derived from sectors produced by *Metarhizium* isolate M4



Key, lanes L to R: 1,100bp ladder; 2, Bib 2; 3, Bia 3; 4, Biib 2; 5, Biia 3; 6, Ciii 4; 7, Di 2; 8, Dii 2; 9, Diii 2; 10, Ei 3; 11, Eiiib 2; 12, M4 positive control; 13, M4 positive control; 14, control, 15 100bp ladder; bp= base pairs

Fig 15. PCR fingerprints with MR primer of replicates of lines derived from sectors produced by *Metarhizium* isolate M4



Key, lanes L to R: 1, 100bp ladder; 2, Bib 2; 3, Bia 3; 4, Biib 2; 5, Biia 3; 6, Ciii 4; 7, Di 2; 8, Dii 2; 9, Diii 2; 10, Ei 3; 11, Eiiib 2; 12, M4 positive control; 13, M4 positive control; 14, control, 15, 100bp ladder; bp= base pairs.

7.3.3.5 Discussion

The formation of sectors in cultures of *Metarhizium* isolate M4 is a degenerative process. Sectors differed from the parent culture in morphology, secondary metabolite production, enzyme production and genetic profile. Culture morphology can be classified into 1 of 4 types based on cultural morphology and sporulation. Type 4 represents the most severe form of degeneration as no spores or aerial conidiophores were produced. Sporulation was reduced and completely absent in some lines. Once the ability to sporulate was lost it was rarely recovered. However, replicates of some lines that exhibited a complete loss of sporulation, formed further sectors, some of which recovered limited sporulation. Kim (1997) found that sector morphology in *Fusarium* remained stable during subsequent sub-cultures. In this investigation, although cultural morphology usually remained unchanged or degenerated further on sub-culture, the formation of further sectors could result in a limited recovery of cultural morphology.

Sectorisation also resulted in changes in secondary metabolite production. Only 1 replicate from the lines originally cultured from a sector exhibited an extracellular secondary metabolite profile that was characteristic of the original isolate. Analysis of the dendrogram compiled from the extracellular TLC system (Fig 12) suggests that changes in the extracellular secondary metabolite profiles were associated with the degenerative states of cultural morphology. All 5 of the replicates examined that exhibited type 3 or type 4 degenerative cultural morphology grouped together on the dendrogram and differed most from the metabolite profile exhibited by the original isolate.

Sectorisation also resulted in changes in enzyme production. Only 3 replicates of line B and 1 replicate of line D exhibited enzyme profiles that were characteristic of that produced by the original isolate. All other replicates lost enzyme activities relative to the expected profile. One replicate (Ei3) lost the activities of 3 enzymes relative to the expected profile, which indicates more serious changes in physiological activity. The results of the enzyme tests and TLC of secondary metabolites suggested that the formation of sectors was associated with physiological instability. Increasing instability was associated with deterioration of cultural morphology as exemplified on the dendrograms.

Sectors also exhibited genetic changes from the original isolate. The polymorphisms obtained with GACA primer were present in 4 replicates, all of which exhibited type 3 or 4 degenerative culture morphology. The polymorphism obtained with MR primer was in a replicate that exhibited type 3 cultural morphology (and that had also exhibited a polymorphism with GACA primer). Only 1 band in the fingerprint of this replicate corresponded to the bands of the original isolates, suggesting that the genome had been substantially disrupted. The potential mechanisms of genetic change have been discussed in chapter 6. The implications of genetic change are important if the isolate is to be used as a biological control agent. If a replicate forms sectors and undergoes a genetic change after a duplicate replicate has been deposited in a culture collection as a patent deposit or as a reference for environmental release there could be legal complications. Additional problems may occur if polymorphism results in an incorrect grouping allocation in population studies. Similarly, isolates that formed sectors and were then used in molecular

research programmes could invalidate results and have wider scientific and financial implications.

If an isolate has failed to sporulate, it may also fail to produce blastospores that are essential in inducing pathogenesis. Passaging experiments could be undertaken to establish whether an isolate that has undergone changes in cultural morphology as a result of the formation of sectors retains its pathogenicity. The characteristics of a fungus may recover after passaging (Jenkins pers.comm.). From the results in this experiment, degeneration of cultural morphology was associated with changes in physiology and genetic stability. Cultures derived from sectors may affect the ability of a line to initiate and maintain pathogenesis in the target organism, as there were substantial changes in the enzyme and secondary metabolite profiles. Successful pathogenesis would require the synthesis of cuticle degrading enzymes and secondary metabolites such as the destruxins. Therefore, disruption in the synthesis of these compounds may prevent successful pathogenesis. The results suggest that scientists should not use isolates that perpetually form sectors in their research. Because inexperienced technicians could inadvertently transfer atypical sectors during sub-culture, research aimed at identifying and developing new biological control agents should focus on isolates that are found to be relatively stable in plate culture. The proposed method of analysing cultural morphology using a four-point scale (Table 5, page 199) would allow scientists to assess the physiological, genetic and cultural stability of an isolate. However, the characteristic morphology and physiology should be recorded when an isolate is first isolated from the environment so that future comparisons can be made.

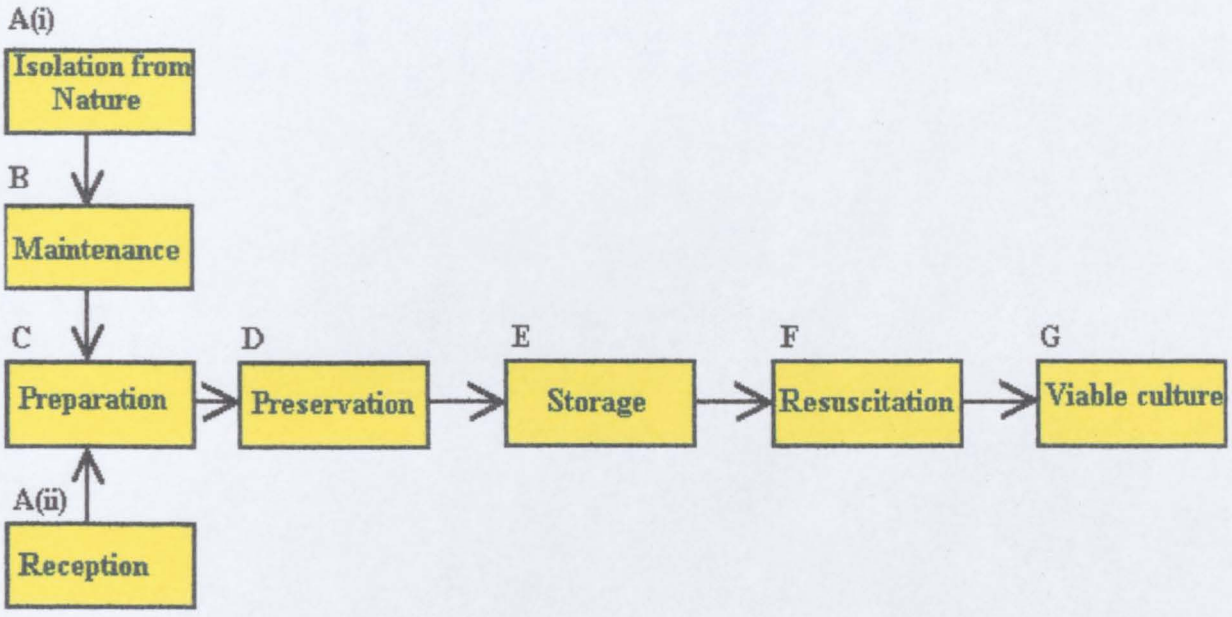
CHAPTER 8: DISCUSSION

The aim of this research was to establish the effects of five different preservation regimes on the physiological and genetic stability of three economically important fungi over a two year testing period. Two subsidiary experiments aimed to establish the suitability of some of the protocols and procedures used in culture collections. The major conclusion has been that fungal preservation regime (preservation method + length of storage + resuscitation) affected the cultural characteristics, secondary metabolite production, extracellular enzyme production and genetic profile of economically important fungi.

8.1 Mechanisms of change

There are several stages either before or during the preservation process or during storage when the stability of an isolate could be compromised (Fig 1). In a public service, academic or industrial culture collection, changes could result at any stage during handling and storage, but in this investigation, the likelihood of change before preservation was very small, as strict procedures and aseptic technique were observed. Because fungi are highly adaptive organisms, as soon as an organism is isolated from the environment and maintained on an artificial nutrient substrate it will begin to adapt to the conditions imposed upon it (Smith and Waller 1992). With longer periods in culture, the fungus may continue to adapt and may become less like the original isolate. The conditions of the preservation regime may facilitate this. In this study it has been shown that changes in culture morphology, physiology or genetic stability can occur in storage. Of the replicates that exhibited changed characters from the original isolate, a number exhibited changes in both enzyme production and secondary metabolism. Some replicates were viable but exhibited poor retention of cultural and physiological characters. There was no direct correlation between changes in enzyme production and secondary metabolism. For example, some replicates would exhibit both secondary metabolite and enzyme profiles that were different to the original profiles, other replicates would exhibit enzyme profiles that were typical of the original isolate but secondary metabolite profiles that were not. Genetic changes were not necessarily correlated with changes in physiological attributes. In this investigation each analysis was established individually, only if each test was undertaken at the same time after growth on the same medium could direct comparisons be made. However, each method of analysis used in this investigation required preparation under specific conditions to allow an

Fig. 1: Progress of an isolate through a culture collection



Key, A to G:

Part A - *Isolation from nature*: When an isolate is received into collection, it is essential that it is a pure culture, that it has been correctly identified and has been sub-cultured as little as possible.

Part B - *Maintenance*: Great care must be taken during maintenance to prevent contamination by mites and other microorganisms or culture degeneration. Transfer of degenerative sectors may result in unintentional selection of characters.

Part C - *Preparation*: Preparation for preservation often involves replicating the isolate many times and the same caution that is applied during maintenance should be observed.

Part D - *Preservation*: Preservation is protocol dependent. Freezing and drying processes may cause stresses at the cellular level. Other protocols may not induce stress.

Part E – *Storage*: The length of storage is protocol specific and depends on the intensity of metabolic suspension.

Part F: *Resuscitation*: Resuscitation from the frozen or dried states must be tightly controlled. All cultures should be inoculated onto a medium that promotes good vegetative growth and sporulation.

Part G – *Viable culture*: Viable cultures should be maintained, checked for purity and cultural stability and then dispatched in a healthy state.

optimal assessment of the desired characters to be made at each testing time. Because some secondary metabolites can suppress the synthesis of some enzymes, an in-depth study on the effect of preservation on physiological stability is warranted. Further research could be aimed at the effects of preservation regimes on specific enzymes or secondary metabolites or groups of secondary metabolites derived from specific pathways. Expression of enzymes, specific secondary metabolites or their precursors could be monitored using molecular methods. The full extent of preservation-induced genetic instability needs to be established.

8.2 Length of storage

The length of time that an isolate can be stored is dependent on the preservation protocol used. This investigation, undertaken over 2 years, was relatively short. In a culture collection, replicates will be preserved for storage periods of many years. However, changes were apparent in the physiological attributes of replicates of the test fungi, even after very short storage periods (<1 week). Increasing storage time resulted in increased changes from the original isolates in the physiological attributes of replicates maintained by continual sub-culture, stored by mycelial plugs in water and stored at –20°C. It is probable that longer storage periods (>2 years) will result in further deterioration of the viability, physiology and genetic stability of isolates. Replicates preserved by cryopreservation and lyophilisation are generally assumed to remain stable during longer-term storage (Smith and Thomas 1998; Tan 1997). However, this is only possible if storage conditions remain constant throughout the storage period. Any changes in the immediate storage environment may invoke changes. Nevertheless, of the preservation regimes assessed, cryopreservation and lyophilisation were the best for long-term preservation.

8.3 Species and strain specificity

The results obtained indicate that species-specific criteria should be applied when considering appropriate preservation protocols. However, the intra-specifc variation evident among the test isolates in response to preservation and storage, suggests that individual strains will respond differently to preservation procedures. Therefore, before important isolates are preserved, optimal preservation procedures

should be determined for each strain, even if species-specific protocols have already been established.

8.4 Assessment of continual sub-culture as a maintenance method

Continual sub-culture, although not a method of preservation, is best described as a maintenance method (Smith pers.comm.). During this investigation, it was relatively easy to set up the required number of replicates for examination. However, problems were experienced as the investigation progressed and each line gradually differed with time. Contamination by other fungal spores was a problem in two of the *Metarhizium* isolates and some of the *Serpula* isolates. Recovery of contaminated lines would have been possible using a combination of careful sub-culturing, single spore inoculations, hyphal tip transfers or growth on selective media. However, restorative techniques would have meant an increased chance of artificial selection and would have affected the validity of this investigation, as change could have resulted from the restoration as well as the preservation regimes. Although mite infestations can be problematical during sub-culture (Smith and Onions 1994), no problems were encountered during this investigation. The method of mite prevention was successful (cultures sealed with tape and original slope cultures, sealed with cigarette papers). Although most replicates remained viable after maintenance by continual sub-culture, there was no guarantee that what was transferred actually resembled the original isolate despite superficial resemblance. Viability was not indicative of the cultural and physiological stability of a culture. The culture characteristics of replicates maintained by continual sub-culture changed from those exhibited by the original isolates and varied as the investigation progressed. The effect of sub-culture on secondary metabolism and enzyme production was strain-specific. As the investigation progressed, enzyme production was changed in most replicates that had been maintained by continual sub-culture. Very few replicates exhibited the enzyme profile typical of the original isolate. The technique induced changes in secondary metabolite profile throughout the investigation. The recovery period allowed some of the replicates to regain a metabolite profile typical of the original isolate. There appeared to be no correlation between cultural degeneration and instability of secondary metabolite production. For example, replicates of isolate F2 maintained by continual sub-culture all retained the cottony morphology of the original isolate, but their metabolite profiles were

different from that exhibited by the original isolate. After 2 years of preservation all replicates of isolate F1 exhibited slimy cultural morphology, but 2 out of 5 replicates maintained the extracellular secondary metabolite profile characteristic of the original isolate. Contrasting reports exist in the literature as to the effects of sub-culture on the relationship between morphology and secondary metabolite production. Wing *et al.* (1995) found that dramatic changes in culture morphology where not necessarily associated with loss of toxigenicity in cultures of *Fusarium acuminatum* and *F. compactum*. Indeed, degenerate cultures were able to sustain secondary metabolite production. In contrast, Awuah & Lorbeer (1988) found that pionnotal (slimy) cultures of *F.oxysporum* were less pathogenic than wild-type cultures. The overall results agreed with those obtained by Kale *et al.* (1994), who found that after 5-12 mycelial transfers of *Aspergillus parasiticus* cultures, variants were formed that exhibited degenerative culture morphology, reduced sporulation and changes in secondary metabolite production of polyketides. Kale *et al.* (1994) found that after 10 transfers, morphological variants were stable and did not revert to previous forms. However, in this investigation some replicates of *Fusarium* and *Metarhizium* regained an ability to produce secondary metabolite profiles more typical of the original isolate after further transfer. It is not known whether the changes in secondary metabolite profiles and enzyme production would affect the pathogenicity of the test fungi. Despite the physiological and cultural instability of the replicates of *Fusarium* isolates that were maintained by continual sub-culture, only 2 genetic polymorphisms were detected after PCR fingerprinting. The genetic change was not necessarily associated with changes in secondary metabolite production or enzyme activity. No polymorphisms were detected in replicates of *Metarhizium* isolates that had been subject to continual sub-culture. It is possible that more frequent sub-cultures or a longer period of maintenance may increase the chances of polymorphisms. Shinohara *et al* (1995) found minor changes in the karyotype of *Saccharomyces cerevisiae* after 150 transfers over 450 days.

A potential problem with continual sub-culture is the chance of inadvertent transfer of polymorphic mycelium. Within one plate, it may appear that the culture consists of only one stable population, but if two or more populations exist, unintentional selection may occur during sub-culture (Smith and Waller 1992). It may be entirely due to chance whether a population typical of the original isolate or a

polymorphic form is transferred. When a plate has clearly formed a sector, it may be clear to the researcher what should be transferred, but to an inexperienced worker it may not be entirely obvious what should be sub-cultured. The presence of sectors should indicate that the fungus is unstable in culture. As demonstrated in the sectorisation experiment (Chapter 7), the cultural morphology of cultures derived from sectors is degenerative and there may be changes in the physiology and genetic stability of an isolate.

The choice of nutrient medium is very important. In this investigation media were selected for continual sub-culture that did not promote excessive sporulation but maintained adequate mycelial growth. Media such as potato sucrose agar (PSA), promote excellent growth of *Fusarium oxysporum*. However, it should not be used for continual-culture purposes as it promotes rapid cultural deterioration (Brayford pers.comm.). The results from this investigation suggest that because of the problems culturing *Metarhizium*, alternative nutrient substrates for continual-sub-culturing in place of malt agar should be sought. Malt agar is a nutrient-rich medium that may not be ideal for continual culturing. Smith and Waller (1992) suggested low nutrient media to be most suitable for culturing fungi. The use of SDA and other nutrient-rich agars as culture media is widely reported (Moore *et al.* 1995; Sim and Perry 1995; Chandler 1997). However, because of the cultural instability exhibited by *Metarhizium* on SDA, and its high nutrient content it should not be used. For *Serpula lacrymans*, maintenance on MEA provided good maintenance of viability. However, MEA is also nutrient-rich, but *Serpula* will not grow well on other nutrient sources. Some replicates of *Fusarium* and *Metarhizium* isolates regained some of the characteristics after a recovery period. This may have been because the sub-culture was effectively taken from younger mycelium after the recovery period (<28 days old), whereas the mycelium taken for the initial investigative procedures was older (>92 days). In this investigation mycelial transfer was used as inoculum, as it is the method that is most widely applied in culture collections and academia. However, some workers propose that different types of inoculum may improve the stability of replicates maintained by continual sub-culture. Burgess *et al.* (1988) suggest that a single microcondium or hyphal tip transfer may reduce the chances of cultural deterioration. Wing *et al.* (1994) found that hyphal tip transfer may be the preferred method of sub-culture (compared to singe spore transfer) as it reduced the chances of

culture degeneration in *Fusarium compactum* and *F.acuminatum*. Despite the simple methodology and low cost, continual sub-culture should not be used to maintain important fungi for long periods. It should only be used as a method for preparing cultures for preservation or experimentation and must otherwise be avoided. Where an absolute requirement exists for continual sub-culture, cultures should be transferred infrequently as it is the transfer procedure that initiates problems. Covering the cultures with mineral oil may reduce the chances of strain deterioration, but may in itself create a selective environment (Smith pers.comm.).

8.5 Assessment of lyophilisation as a preservation method

Lyophilisation, along with cryopreservation, is considered to be one of the best methods for preserving fungi. However, the results obtained during this investigation suggest that physiological and genetic profiles of some isolates, may be changed after lyophilisation and storage. No isolates of *Serpula lacrymans* survived lyophilisation. This agrees with the findings of other workers, who found that mycelial formulations of non-sporing fungi are notoriously difficult to lyophilise (Tan 1997; Tan *et al.* 1991). Inducement of fruiting would allow the formation of basidiospores, which may survive the lyophilisation process. However, this would correspond with meiotic crossing over and genetic variation and would not be suitable for maintaining the characteristics of the original isolate. All replicates of *Metarhizium* and *Fusarium* isolates were viable after lyophilisation throughout the investigation. Both fungi produce large numbers of conidia, which helps the fungus survive lyophilisation. The stimulation of asexual sporulation is a mitotic process that should not result in genetic exchange. The conidial wall may provide protection from the stresses encountered during the cooling stage of the lyophilisation process (Tan *et al.* 1994). The effect of lyophilisation on cultural characteristics, physiology and genetic stability was strain-specific. Most replicates that had been lyophilised retained secondary metabolite profiles typical of the original isolates. However, some replicates exhibited profiles that were different from that exhibited by the original isolate. After a recovery period, most of the replicates exhibited profiles characteristic of the original isolate. Enzyme production was also affected by lyophilisation. Some isolates retained enzyme activity better than others of the same species. From the data obtained, it was apparent that lyophilised replicates take longer to recover after storage than replicates preserved by other methods. All

replicates that had been lyophilised, displayed a delay in the onset of growth and exhibited changed culture characteristics from those exhibited by the original isolate. Therefore, it may take longer for the physiological activity to return to pre-preservation levels compared to replicates preserved by other treatments. The delay may be due to the time taken for the conidia to rehydrate and attain a state of biochemical stability. Thus when the biochemical analysis was carried out, the replicates that had been lyophilised may not have achieved the same point of post-preservation development. However, a high number of replicates still exhibited changed cultural and physiological characteristics after the recovery period, suggesting that more serious damage can be induced by lyophilisation. Genetic polymorphisms after PCR fingerprinting were detected among replicates of 2 isolates of *Metarhizium anisopliae* that had been lyophilised. Polymorphisms were detected after both 1 and 2 years of storage suggesting that the change was caused as a result of the preservation regime rather than the length of storage. This is the first report of lyophilisation-induced changes in genetic profiles of filamentous fungi. No polymorphisms were detected among replicates of *Fusarium* isolates that had been lyophilised.

The mechanisms of damage induced by the freezing stage of lyophilisation have been well documented (Tan 1997; Tan *et al.* 1994; Smith and Onions 1994). The solution effects that occur during the cooling process may change the biochemical equilibrium. Cellular organisation may become compromised, changes in pH and ionic stability may cause damage to the membranes surrounding organelles and may even damage nucleic acids. The genetic changes evident in some replicates may represent a response to the biochemical stresses that occur during the lyophilisation process. Movement of transposons within the genome has been reported to occur as a response to stress caused by biochemical imbalance (Anaya and Roncero 1996). Intracellular ice-damage and damage during the drying stage may also occur. Long-term stability may only be guaranteed if the residual moisture content following lyophilisation is low. Excess moisture content may allow limited metabolism under restricted growth conditions that may induce selection. Cracked or improperly sealed ampoules may allow moisture and pressure to return to environmental levels allowing uncontrolled growth. Storage in the dark or in metal containers may prevent the possibility of mutations caused by UV light or

background ionising radiation respectively. The precise mechanism of lyophilisation-induced changes necessitates further research. Improvements in methodology may also be warranted.

Although lyophilisation provided good viability of sporing fungi, the physiological and genetic stability of isolates cannot be guaranteed, although some replicates were unaffected. There is a need to develop species-specific protocols. The lyophilisation protocol is a time-consuming process but, once preserved, ampoules are easy to handle and can be stored for long periods, which makes it a preferred preservation technique throughout the world. The results from this investigation suggest that lyophilisation may damage the physiology and genetic stability of some replicates. Therefore, the appropriateness of preservation of important isolates by lyophilisation should be carefully considered. The physiological and genetic characters of isolates should be fully determined before lyophilisation and storage, so that the characteristics of resuscitated isolates can be compared to those exhibited by the original isolate. Enough replicates should be lyophilised to compensate for any that do not maintain the characteristics of the original isolate before preservation.

8.6 Assessment of mycelial plugs in water as a preservation method

Many authors suggest that storage in water is a suitable method for the preservation of some fungi (Qiangqiang *et al.* 1998; Boeswinkel 1976; Burdsall 1994; Onions and Smith 1984; Figueredo and Pimental 1975). The results from this investigation suggested that storage of mycelial plugs in water was good for ensuring the viability of *Fusarium* replicates over a two-year period. However, the viability of *Metarhizium* replicates stored by this method was variable and the process was lethal for replicates of *Serpula lacymans*. Maintenance of viability does not guarantee the stability of culture characteristics. After 2 years of storage by mycelial plugs in water, most replicates of both *Fusarium* and *Metarhizium* exhibited cultural degeneration. Preservation by mycelial plugs in water affected radial growth rate and conidial production, which was changed from the original isolates after preservation. Storage of mycelial plugs in water was not a good method for ensuring stability of physiological characters. Very few replicates of any isolate retained the secondary metabolite profiles that were characteristic of the original isolate. Enzyme production was also changed after storage in most replicates. The recovery period allowed some

replicates to regain the secondary metabolite and enzymes profiles that were exhibited by the original isolates. Despite the changes in culture characteristics and physiology, no genetic changes were detected in any replicates after PCR fingerprinting.

The results indicated that the physiological stability of replicates was very poor. In contrast, Burdsall (1994) reported that water storage did not significantly affect growth rate or viability in 155 isolates of Basidiomycota stored for 7 years. Storage in water may only provide a partial suppression of dormancy, cellular metabolism may not be totally suppressed, allowing restricted growth under stressful conditions that may result in selection. Slow deterioration of preserved material may be associated with a breakdown of cellular organisation and biochemical stability. As cellular organisation and biochemical stability becomes compromised, toxic compounds may be produced which may induce further damage to cell structures and biochemical integrity. Conidia, which often have thicker walls than hyphae, may be better equipped to withstand the pressures of storage in water; many fungi use water as a natural dispersal method. Storage of fungi in water may also induce intracellular osmotic stresses. Adding solutes to the water before the cells are added may protect osmotic equilibrium.

Despite the simple methodology and low cost, storage of mycelial plugs in water should not be used to preserve important fungi for long periods. Spore suspensions may survive water storage better than plugs of mycelium alone. Smith and Onions (1994) considered the method to be outdated, only suitable for short-term preservation (2-5 years) and not suitable for important organisms, where alternative methods may be available. The results from this investigation suggest that it should only be used in extreme circumstances where there are no alternative preservation methods available.

8.7 Assessment of storage at –20°C as a preservation method

Storage at –20°C is commonly used as a convenient method for the short-term preservation of fungi, and in some laboratories it is the only method available. The method gave mixed results with the test fungi used in the investigation, and physiological stability after storage appeared to be strain-specific. All *Fusarium* and

Metarhizium replicates were viable after 2 years of storage. However, few replicates of *Serpula lacrymans* retained viability, even after relatively short storage periods (<1 week). Some *Fusarium* replicates exhibited degenerative culture morphology and variation in conidial production increased as the investigation progressed. After 2 years of storage, degenerative culture morphology was exhibited by most of the *Metarhizium* replicates. Physiological stability deteriorated after 1 year of storage. Very few replicates of *Metarhizium* or *Fusarium* isolates maintained enzyme profiles of the original isolate after 2 years of storage. The effect of storage at –20°C on secondary metabolite production appeared to be species-specific. Very few *Fusarium* replicates exhibit secondary metabolite profiles typical of the original isolates. However, some replicates of *Metarhizium* isolates stored at –20°C exhibited metabolite profiles more typical of the original isolate throughout the investigation. The recovery period allowed some replicates to recover characters more typical of the original isolate. Despite the poor stability of cultural and physiological culture characteristics, no polymorphisms were detected after PCR fingerprinting in any of the replicates.

Storage at –20°C is a crude method of preservation, cryoprotectants are not used, controlled cooling rates are not applied and temperatures may fluctuate when people access the freezers. Additional problems may occur if electrical supplies are compromised. Metabolic activity may not be totally suppressed and metabolic activity could change as temperatures fluctuate. Ice damage and solution effects may cause damage at the cellular level. Storage at –20°C is a simple and low cost method of preservation. It may be suitable for short-term (<1 year) storage of replicates of little importance. Isolates of scientific or industrial importance should not be preserved at –20°C as there is a high risk of physiological deterioration.

8.8 Assessment of Cryopreservation as a preservation method

Cryopreservation is the most widely used preservation technique in developed countries. It has long been considered to be the best method for preserving the integrity of isolates. However the results from this investigation suggested that changes in physiology and genetic profiles may occur during preservation, storage and resuscitation. All replicates of *Fusarium* and *Metarhizium* isolates preserved by cryopreservation were viable throughout the investigation. However, very few

replicates of *Serpula lacrymans* retained viability. The poor viability may have been due to the nature of the preserved material. *Serpula* does not sporulate, so only mycelial suspensions can be preserved. Hyphae are particularly vulnerable to ice damage, ice can spread down the open-ended fragments during cooling. The application of a different cooling protocol could improve the viability of *Serpula* isolates. Smith and Onions (1994) claimed that no morphological or physiological changes were found among 7354 fungal strains stored in liquid nitrogen. However, the physiology stability of replicates of *Metarhizium* and *Fusarium* isolates was not guaranteed after cryopreservation and storage. Although the majority of replicates continued to exhibit the physiology and genetic characteristic of the original isolates, a number of replicates of most isolates exhibited characteristics that were changed from the original isolates. Secondary metabolite profiles were changed following cryopreservation throughout the investigation, and occasionally all replicates that had been cryopreserved exhibited secondary metabolite profiles that were different than that exhibited by the original isolate. After the recovery periods, the majority of replicates recovered to exhibit profiles typical of the original isolate. However, some did not regain profiles characteristic of the original isolate, which may indicate more significant physiological change. Cryopreservation proved to be the best method available for maintaining enzyme production, although some replicates showed changed enzyme activities from the original isolate. Replicates of some isolates preserved by cryopreservation showed increased variation at later testing times. Any changes may have occurred during resuscitation, although variation of temperatures within liquid nitrogen refrigerators could also induce changes with longer storage periods. It has been established that liquid nitrogen refrigerator temperatures can fluctuate quite widely, either as a result of opening and closing or as a result of fluctuations in the supply of liquid nitrogen (Smith pers.comm.). Genetic stability was compromised among replicates of 2 isolates of *Metarhizium* that had been cryopreserved. This is the first report of changes at the genetic level occurring in *Metarhizium* spp. after cryopreservation. Gaylarde and Kelley (1995) previously observed changes in the genetic stability of replicates of *Fusarium merismoides* after cryopreservation. Hubalek (1996) claimed that liquid nitrogen refrigeration preserves safely both the phenotype and genotype of microorganisms and that it does not stimulate mutagenesis compared to other maintenance procedures. This was not so in this study. The changes in physiology and genetic profiles may have been induced

during the cooling/thawing procedure of the cryopreservation procedure. Solution effects result in changes in osmolarity and pH changes, which may alter the biochemical integrity of cells. Cell membranes may also be damaged, either as a result of the biochemical changes that could alter the fluidity of the membranes after resuscitation or by physical ice damage during the cooling process. Extracellular ice may puncture the cell walls, compromising cell viability. Alternatively, intracellular ice crystals may form which may damage internal membrane structures such as the mitochondrial membranes, nuclear membrane and endoplasmic reticulum. Changes in the genetic stability of *Saccharomyces cerevisiae* have been attributed to damage of the nuclear membrane, which then allowed extracellular DNAse to enter the nuclear region and decompose the nuclear DNA (Komatsu *et al.* 1987). This could be the cause of genetic change in replicates that had been cryopreserved during this investigation. However, changes in osmotic equilibrium may equally promote conformational change in the DNA. The theories of genetic change have been discussed in Chapter 6. Damage to membranes may also alter physiology, as many enzymes are located within membranes, and damage to membrane-bound enzymes may impair the physiological recovery of the fungus during resuscitation. Smith and Thomas (1998) advocated the use of species-specific cooling regimes. If the prospects of cryoinjury can be reduced by the optimisation of cooling protocols, the chance of physiological and genetic damage may be reduced. However, the results from this investigation would suggest that the use of strain-specific cryopreservation protocols (cooling rate and cryoprotectants) should be examined. A study of the effects of preservation regime on cell ultrastructure would allow an assessment of stability following preservation and storage at the cellular level.

Despite changes in the genetic stability of some *Metarhizium* replicates, cryopreservation proved to be the best preservation regime for maintaining the physiological stability of the test fungi. This would confirm the view of many authors who consider that cryopreservation is probably the best method for the preservation of living fungi (Hubalek 1996; Smith and Thomas 1998). However, extreme caution should be exercised because of the changes in physiology and genetic characters of some replicates. It should not be taken for granted that cryopreservation will always maintain the stability of important isolates. To compensate for the possibility of damage, an optimal cooling regime should be

established, numerous replicates of important isolates should be preserved and back-ups should be preserved by other methods or deposited in other collections

8.9 The role and future development of culture collections

The comparisons of cultures obtained from different culture collections indicated that replicates of the same isolate deposited in different culture collections will often exhibit different physiological and genetic attributes. The result from the main investigation would suggest that changes can be caused by preservation regime, although procedures within culture collections may be different and this may, in itself, be damaging. The effect of the formation of sectors on the physiology and genetic stability of cultures illustrates the importance of good training. If staff are inexperienced, atypical mycelium may be transferred during sub-culture, the characters of which may be different from the original isolate, this may remain unnoticed until it is too late. As far as reasonably practical, collections will supply the correctly named organism (Smith pers.comm.). However, there can be no definitive guarantee that the culture received from a collection is physiologically and genetically similar to the original isolate. Therefore, there is a need for a high standard of training, harmonisation of procedures and standardisation of techniques, so that collections can assure their customers that they are getting good service and a guarantee of the quality of the isolate. When considering what preservation method is to be used, scientists should consider a number of practical and financial criteria (see appendix G). However, as a quality assurance measure, procedures could be applied to monitor the stability of an isolate after preservation. This would provide reassurance to the depositor and allow the collection to monitor the effectiveness of its own procedures. A series of standard analytical tests, that can be used to characterise the physiological, cultural characteristics and genetic identity of an isolate when it is first deposited in a culture collection. Tests could include an assessment of culture characteristics, an enzyme assay, a secondary metabolite profile or a PCR fingerprint(s) with a suite of primers. On resuscitation, the same series of tests could be applied to ensure that physiological and genetic stability has been retained.

The role of culture collections as service providers to the scientific community is important and must be maintained, but improvements are required to

methods and procedures to ensure optimum preservation of isolates and to improve the standard of service. In the future, associated with widespread habitat destruction, collections may take on new roles to preserve the fungal diversity of the world. Without them, potentially important isolates may be lost forever.

REFERENCES

Adams T.H. 1995. Asexual sporulation in higher fungi. In: *The Growing Fungus (Gow N.A.R. & Gadd G.M. eds), Chapman & Hall, U.K.* pp. 367-382

Addy H. D., Boswell E.P. & Koide R.T. 1998. Low temperature acclimitisation and freezing resisitance of extraradical VA mycorrhizal fungi. *Mycological Research* **102:** 582-586

Akins R.A., Kelly R.L. & Lambowitz A.M. 1986. Mitochondrial plasmids of *Neurospora*: integration into mitochondrial DNA and evidence for reverse transcription in mitochondria. *Cell* **47:** 505-516

Al Airdroos K. 1980. Demonstration of parasexual cycle in the entomopathogenic fungus *Metarhizium anisopliae. Canadian Journal of Genetics and Cytology* **22:** 309-314

Al Doory Y. 1968. Survival of dermatophyte cultures maintained on hair. *Mycologia* **60**: 720-723

Anaya N. & Roncero M.I.G. 1996. Stress-induced rearrangement of *Fusarium* retrotransposon sequences. *Molecular General Genetics* **253**: 89-94

Anderson C. & Solomoms G.L. 1984. Primary metabolism and biomass production from *Fusarium.* In: *British Mycological Society Symposium Series- Applied Mycology of Fusarium, (Moss M.O and Smith J.E eds), Cambridge University Press, U.K.* pp. 231-250

Anon. 1999. CABRI (common access to biotechnological resource information). **http:\\www.cabri.org**

Archer D.B. & Wood D.A 1995. Fungal Exoenzymes. In: *The Growing Fungus (Gow N.A.R & Gadd G.M eds), Chapman & Hall, U.K.* pp.137-162

Azevedo J.L., Messias C.L., & Silveira W.D. 1987. Genetics and breeding of the entomopathogenic fungus *Metarhizium anisopliae*: parasexuality and protoplast fusion. In: *Biotechnology of Plants and Microorganisms, (Crocomo O.J., Sharp E.R., Evans D.A., Bravo J.E., Tavares F.C.A. & Padock E.P eds), Ohio State University Press, U.S.A.* pp. 305-318

Awuah R.T. & Lorbeer J.W. 1988. Nature of variability in *Fusarium oxysporum* f.sp. *apii* race 2. *Phytopathology* **78:** 385-389

Ball C. 1984. Filamentous fungi. In: *Genetics and Breeding of Industrial Microorganisms, (Ball C. ed), CRC Press, Florida U.S.A.* pp. 159-188

Barnet H.L. & Hunter B.B. 1998. Illustrated Genera of Imperfect Fungi. *4th edition APS Press, Minnesota U.S.A*

Bartman C.D., Doerfler D.L., Bird B.A., Remaley A.T., Peace J.N. & Campbell I.M. 1981. Mycophenolic acid production by *Penicillium breviocompactum* on solid media. *Applied & Environmental Microbiology* **41:** 729-736

Barth M.G.M. & Bridge P.D. 1989. 4-methylumbelliferyl substituted compounds as fluorogenic substrates for extracellular enzymes. *Letters in Applied Microbiology* **9**: 9-12

Bateman R.P. 1992. Controlled droplet application of mycopesticides to locusts. In: *Biological Control of Locusts and Grasshoppers, (Lomer C.J & Prior C eds), CAB International, Wallingford, U.K.* pp. 249-254

Bateman R.P., Carey M., Moore D. & Prior C. 1993. The enhanced infectivity of *Metarhizium flavoviride* in oil formulations to dessert locusts at low humidities. *Annals of Applied Biology* **122:**145-152

Bentley S., Pegg K.G. & Dale J.L. 1994. Optimization of RAPD-PCR fingerprinting to analyse genetic variation within populations of *Fusarium oxysporum* f.sp *cubense. Journal of Phytopathology* **142:** 62-78

Bentley S., Pegg K.G. & Dale J.L. 1995. Genetic variation among a world-wide collection of isolates of *Fusarium oxysporum* f.sp. *cubense* analysed by RAPD-PCR fingerprinting. *Mycological Research* **99:** 1378-1384

Boesewinkel H.J. 1976. Storage of fungal cultures in water. *Transactions of the British Mycological Society* **66:** 183-185

Bogo M.R., Queiroz M. V., Silva D.M., Gimenez M.P., Azevedo J.L. & Schrank A. 1996. Double-stranded RNA and isometric virus-like particles in the entomopathogenic fungus *Metarhizium anisopliae. Mycological Research* **100**: 1468-1472

Booth C. 1971a. The Genus *Fusarium. Commonwealth Mycological Institute, U.K.*

Booth C. 1971b. *Fusarium oxysporum. CMI Descriptions of Pathogenic Fungi and Bacteria, CAB International, Wallingford, U.K.*

Booth C. 1984. The *Fusarium* problem, historical, ecological and taxonomic aspects. In: *British Mycological Society - Applied Mycology of Fusarium. (Moss M.O and Smith J.E eds), Cambridge University Press. U.K.* pp. 1-13

Bridge P.D. & Arora D.K. 1998. Interpretation of PCR methods for species defination. In: *Applications of PCR in Mycology, (Bridge P.D., Arora D.K., Reddy C.A. & Elander R.P. eds), CAB International, Wallingford, U.K.* pp.1-20

Bridge P.D., Hawksworth D.L., Kozakiewicz Z., Onions A.H.A., Paterson R.R.M., Sackin M.J. & Sneath P.H.A. 1989. A reappraisal of the terverticillate Penicillia using biochemical, physiological and morphological features. 1. Numerical Taxonomy. *Journal of General Microbiology* **135:** 2941-2966

Bridge P.D., Hopkinson L.A. & Rutherford M.A. 1995. Rapid mitochondrial probes for analysis of polymorphisms in *Fusarium oxysporum* special forms. *Letters in Applied Microbiology* **21**: 198-201

Bridge P.D., Pearce D.A., Rivera A., & Rutherford M.A. 1997. VNTR derived oligonucleotides as PCR primers for population studies in filamentous fungi. *Letters in Applied Microbiology* **24:** 426-430

Bridge P.D. & May J.W. 1988. Change from homothallism to heterothallism in *Schizosaccharomyces malidivorans*: biochemical and taxonomic consequences. *Transactions of the British Mycological Society* **91:** 439-442

Bridge P.D., Williams M.A.J., Prior C. & Paterson R.R.M. 1993. Morphological, biochemical and molecular characteristics of *Metarhizium anisopliae* and *Metarhizium flavoviridae. Journal of General Microbiology* **139:** 1163-1169

Buck K.W. 1998. Molecular variability of viruses of fungi. In: *Molecular Variability of Fungal Pathogens, (Bridge P.D., Couteaudier Y. & Clarkson J.M. eds), CAB International, Wallingford, U.K.* pp. 53-72

Bu'lock J.D. 1961. Intermediary metabolism and antibiotic synthesis. *Advances in Applied Microbiology* **3:** 293-342

Bu'lock J.D. 1984. Useful metabolites of *Fusarium*. In: *British Mycological Society Symposium Series- Applied Mycology of Fusarium, (Moss M.O and Smith J.E. eds), Cambridge University Press. U.K.* pp. 215-229

Burdsall H.H. & Dorworth E.B. 1994. Preserving cultures of wood decaying Basidiomycotina using sterile distilled water in cryovials. *Mycologia* **86:** 275-280

Burgess L.W., Liddell C.M. & Summerell B.A. 1988. *Laboratory Manual for Fusarium Research. The University of Sydney, Australia.*

Burnett J.H. 1984. Aspects of *Fusarium* genetics. In: *British Mycological Society - Applied Mycology of Fusarium, (Moss M.O and Smith J.E. eds), Cambridge University Press. U.K.* pp. 39-66

Buxton E.W. 1956. Heterokaryosis and parasexual recombination in pathogenic strains of *Fusarium oxysporum. Journal of General Microbiology* **15:** 133-139

Campbell M.A., Medd R.W. & Brown J.F. 1996. Growth and sporulation of *Pyrenophora semeniperda in vitro*: effects of culture media, temperature and pH. *Mycological Research* **100:** 311-317

Castro H.P., Teixeira P.M. & Kirby R. 1995. Storage of lyophilised cultures of *Lactobacillus bulgaricus* under different relative humidities. *Applied Microbiology and Biotechnology* **44:** 172-176

Chandler D. 1997. Selection of an isolate of the insect pathogenic fungus *Metarhizium anisopliae* virulent to the lettuce root aphid. *Pemphigus bursarius. Biocontrol Science and Technology* **7:** 95-104

Chvostova V., Nerud F & Homolk A. 1995. Viability of wood inhabiting Basidiomycetes following cryogenic preservation. *Folia Microbiologica* **40:** 193-197

Clapp J.P., Young J.P.W., Merryweather J.W. & Fitter A.H. 1995. Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. *New Phytologist* **130:** 259-265

Clutterbuck A.J. 1995. Genetics of fungi. In: *The Growing Fungus, (Gow N.A.R. & Gadd G.M. eds), Chapman and Hall, London U.K.* pp. 239-254

Crowhurst R.N., King F.K., Hawthorne B.T., Sanderson F.T. & Choi-Pheng Y. 1995. RAPD characterisation of *Fusarium oxysporum* associated with wilt of angsana (*Pterocarpus indicus*) in Singapore. *Mycological Research* **99:**14-18

Coulson G.E., Morris G.J. & Smith D. 1987. A cryomicroscopic study of *Penicillium expansum* hyphae during freezing and thawing. *Journal of General Microbiology* **132:** 183-190

Couteaudier Y., Viaud M. & Neuveglise C. 1998. Combination of different independent molecular markers to understand the genetic structure of *Beauveria* populations. In: *Molecular Variability of Fungal Pathogens, (Bridge P.D., Couteaudier Y. & Clarkson J.M. eds), CAB International, Wallingford, U.K.* pp. 95-104

Corbery Y. & Le Tacon F. 1997. Storage of ectomycorrhizal fungi by freezing. *Annales des Sciences Forestières* **54:** 211-217

Daboussi M.J. 1997. Fungal transposable elements and genome evolution. *Genetica* **100:** 253-260

Daboussi M.J. & Langin T. 1994. Transposable elements in the fungal plant pathogen *Fusarium oxysporum. Genetica* **93:** 49-59

Daoust R.A., Ward M.G. & Roberts D.W. 1993. Effect of formulation on the viability of *Metarhizium anisopliae* conidia. *Journal of Invertebrate Pathology* **41:** 151-160

Debets A.J.M. 1998. Parasexuality in fungi: Mechanisms and significance in wild populations. In: *Molecular Variability of Fungal Pathogens, (Bridge P.D., Couteaudier Y. & Clarkson J.M. eds), CAB International, Wallingford, U.K.* pp. 41-52

Desenzo R.A. & Harrington T.C. 1994. Use of $(CAT)_5$ as a DNA fingerprinting probe for fungi. *Phytopathology* **84:** 534-540

Dipietro A., Anaya N. & Roncero G. 1994. Occurrence of a retrotransposon like sequence among different formae speciales and races of *Fusarium oxysporum. Mycological Research* **98:** 993-996

Doi S. 1988. Studies on the damages of wooden constructions from *Serpula lacrymans* (Fr). *Report of the Hokkaido Forest Products Research Institute, No 78*

Donnelly D.P., Boddy L, & Wilkins M.F. 1999. Image analysis – a valuable tool for recording and analysing development of mycelial systems. *Mycologist* **13:** 120:125

Donnison L. (1994) Molecular biology of *Serpula lacrymans. MSc thesis, University of Kent.*

Drysdale R.B. 1984. The production and significance in phytopathology of toxins produced by species of *Fusarium* In: *British Mycological Society Symposium Series, Applied Mycology of Fusarium, (Moss M.O and Smith J.E. eds), Cambridge University Press.U.K.* pp. 95-104

Edel V. 1998. Polymerase chain reaction in mycology: an overview. In: *Applications of PCR in Mycology, (Bridge P.D., Arora D.K., Reddy C.A. & Elander R.P. eds), CAB International, Wallingford, U.K.* pp. 1-20

Ericksson K.E.L & Kirk T.K. 1990. *Micobial Degredation of Wood and Wood components. Springer Verlag, Berlin*

Figueredo M.B. & Pimental C.P.V. 1975. Metodos utilizados para conservacao de fungos na micoteca da Secao de Micologia Fitopatologica de Instituto Biologico. *Summa Phytopathogica* **1:** 229-302

Foster L.M., Kozak K.R., Loftus M.G., Stevens J.J. & Ross I.I. 1993. The polymerase chain reaction and its application to filamentous fungi. *Mycological Research* **97:** 769-781

Fox R.T.V & Sanson S. 1996. Lethal effects of an electrical field on *Armillaria melleia* in culture. *Mycological Research* **100:** 318-320

Garraway M.O & Evans R.C. 1991. Secondary metabolism. In: *Fungal Nutrition and Physiology, Kriegar Publishing Company, Florida, U.S.A.* pp. 336-367

Glockling S.L. & Shimazu M. 1997. Culturing of three species of endoparasitic fungi infecting nematodes. *Mycological Research* **101:** 55-60

Gramss G. 1991a. Definitive senescence in stock cultures of basidiomycetous wood decaying fungi. *Journal of Basic Microbiology* **31:** 107-112

Gramss G. 1991b. Appearance of senescent sectors in the aging vegetative thallus of several basidiomycetous fungi held in pure culture. *Journal of Basic Microbiology* **31:** 113-120

Grout B.W.W & Morris G.J. 1987. The Effect of Low Temperature on Biological Systems. *Edward Arnold, London, U.K.*

Gullino M.L., Migheli Q. & Mezzalama M. 1995. Risk analysis in the release of biological control agents. *Plant Disease* **79:**1193-1201

Hajek A.E., Shimazu M. & Kumber R.A. 1995. Instability in pathogenicity of *Entomophaga maimniga* after long term preservation. *Mycologia* **87:** 483-489

Hall R.A., Peterkin D.D., Ali B. & Lopez V.F. 1994. Influence of culture age on rate of conidiospore germination in four deuteromycetous entomogenous fungi. *Mycological Research* **98:** 763-768

Hallsworth J.E. and Magan N. 1996. Culture age, temperature and pH affect the polyol and trehalose content of fungal propagules. *Applied and Environmental Microbiology* **62:** 2435-2442

Hajek A.E., Shimazu M. & Kumber R.A. 1995. Instability in pathogenicity of *Entomophaga maimniga* after long term preservation. *Mycologia* **87:** 483-489

Hamlyn P.F. & Temple J. 1997. Growing gourmet mushrooms - The stamets seminars. *Mycologist* **11:**157-158

Hawksworth D.L., Sastramihardja KL, Kokke R. & Stevenson R. 1990. Guidelines for the establishment and operations of collections of cultures of microorganisms. *WFCC Secretariat, Brazil:WFCC*

Hawksworth D.L., Kirk P.M, Sutton B.C., Pegler D.N. 1996. Ainsworth and Bisby`s Dictionary of the Fungi, 8th Edition, *CAB International, Wallingford, U.K.*

Horgen P., Carvalho D., Sonnenberg A., Li A. & Van Griensven L.J.L.D. 1996. Chromosomal abnormalities associated with strain degeneration in the cultivated mushroom, *Agaricus bisporus. Fungal Genetics and Biology* **20:** 229-241

Hubalek Z. 1996. Cryopreservation of Microorganisms at Ultra-low Temperatures. *Academia Praha, Czech Republic*

Hwang S.W. 1966. Long term preservation of fungus cultures with liquid nitrogen refrigeration. *Applied Microbiology* **14:** 784-788

Isaac S. 1997. How are the activities of enzymes from fungi utilised in cheese production? *Mycologist* **3:**11 122-123

Jennings D.H. 1990. Osmophiles In: *Microbiology of Extreme Environments, (C. Edwards ed), Open University, Milton Keynes* p.117

Jennings D.H. 1991. The vegatative mycelium. In: *Serpula lacrymans: Fundametal Biology and Control Stratergies. (Jennings D.H., Bravery A.F eds), John Willey and Sons. Chichester, U.K.*

Kelly A., Alcala-Jimenez A.R., Bainbridge B.W., Heale J.B., Perez-Artes E. & Jimenez-Diaz R.M. 1995. Use of genetic fingerprinting and random amplified

polymorphic DNA to characterise pathotypes of *Fusarium oxysporum* f. sp. *ciceris* infecting chickpea. *Molecular Plant Pathology* **84:** 1293-1298

Kim D.H. 1997. Induced change in DNA methylation of *Fusarium oxysporum* f.sp. *niveum* due to successive transfer. *Journal of Biochemistry and Molecular Biology* **30:** 216-221

Kleespies R.G. & Zimmerman G. 1992. Production of blastospores by three strains of *Metarhizium anisopliae* (Metch.) Sorokin in submerged culture. *Biocontrol Science and Technology* **2:** 127-135

Kobayashi T. 1984. Maintaining cultures of Basidiomycetes by mineral oil method 1. *Bulletin of Forestry and Forestry Product Research Institute* **325:** 141-147

Kolkowski J.A. & Smith D. 1995. Cryopreservation and freeze drying of fungi. In: *Methods in Molecular Biology 38, Cryopreservation and Freeze-Drying Protocol. (Day J.G & Mclellan eds), Humana Press, NJ U.S.A.* pp. 49-61

Kuhls K., Liekfeldt E. & Borner T. 1995. PCR fingerprinting used for comparison of ex type strains of *Trichoderma* species deposited in different culture collections. *Microbiological Research* **150:** 363-371

Langley D. 1997. Exploiting the fungi – Novel leads to new medicines. *Mycologist* **11:**165-167

Larsen T.O. & Frisvad J.C. 1995. Chemosystematics of *Penicillium* based on profiles of volatile metabolites. *Mycological Research* **99:** 1167-1174

Latge J.P., Debeaupuis J., Sarfati J. & Paris S. 1998. Variability in the human oppurtunistic fungal pathogen *Aspergillus fumigatus.* In: *Molecular Variability of Fungal Pathogens, (Bridge P.D., Couteaudier Y. & Clarkson J.M. eds), CAB International, Wallingford, U.K.* pp.121-132

Liang Z.Q., Liu A.Y. & Liu J.L. 1991. A new species of the genus *Cordyceps* and its *Metarhizium* anamorph. *Acta Mycologia Sinica* **10:** 257-262

Lodwig E., Bridge P.D., Rutherford M.A., Kung'u J.N. & Jeffries P. 1999. Molecular differences distinguish clonal lineages within East African populations of *Fusarium oxysporum* f.sp. *cubense. Journal of Applied Microbiology* **86:** 71-77

Loomis P. & Leung H. 1995. Low temperature storage of monosporidial cultures of the wheat bunt fungi. *Canadian Journal of Botany* **73:** 758-760

Lowe D.A. 1992. Fungal enzymes. In: *Handbook of Applied Mycology, vol 4 (Arora D.K.,Elander R.P. & Mukerji K.I. eds), Marcel Dekker, New York. U.S.A.* pp. 681-706

Madhosingh C. 1994. Production of intraspecific hybrids of *Fusarium oxysporum* f.sp. *radicis lycopersici* and *Fusarium oxysporum* f.sp. *lycopersici* by protopast fusions. *Journal of Phytopathology* **142:** 301-309

Martin J.F. & Demain A.L. 1978. Fungal development and metabolite formation. In: *The Filamentous Fungi, (Smith J.E & Berry D.R eds), Vol 3, Wiley, New York U.S.A.* pp. 226-259

Mauperin C.H., Mortier F., Garbaye F., Le Taycon F. & Carr G. 1987. Viability of an ectomycorrhizal inoculum produced in a liquid medium and entrapped in a calcium alginate gel. *Canadian Journal of Botany* **65:** 2326-2329

Marx D. H & Daniel W. J. 1976. Maintaining cultures of ectomycorrhizal and plant pathogenic fungi in sterile cold water storage. *Canadian Journal of Microbiology* **22:** 338-341

Mazur P. 1970. Cryobiology: The freezing of biological systems. *Science* **168:** 939-949

Mazur P. 1977. Slow freezing injury in mammalian cells. In: *The Freezing of Mammalian Embyos, Ciba Foundation Symposium, (Elliot K. & Whelan J. eds),* **52:** 19-48

McClatchi G.V., Moore D., Bateman R.P. & Prior C. 1994. Effects of temperature on the viability of the conidia of *M.flavoviridae* in oil formulations. *Mycological Research* **98:** 749-756

McGrath J.J. 1985. Temperature controlled cryogenic light microscopy. In: *The Effects of Low Temperatures in Biological Systems, (Grout B.W.W and Morris G.J. eds), Edward Arnold, London. U.K.* pp. 234-267

Merryman H.T., Williams R.J. & Douglas M. St. J. 1977. Freezing injury from "solution effects" and its prevention by natural or artificial cryoprotection. *Cryobiology* **14:** 287-302

Migheli Q., Berio T., Gullini M.L. & Garibaldi A. 1995. Electrophoretic karyotype variation among pathotypes of *Fusarium oxysporum* f.sp. *dianthi. Plant Pathology* **44:** 362-370

Moore D., Bateman R.P., Carey M. & Prior C. 1995. Long-term storage of *Metarhizium flavoviride* conidia in oil formulations for the control of locusts and grasshoppers. *Biocontrol Science and Technology* **5:** 193-199

Moore D., Douro-Kpindo O.K., Jenkins N.E. & Lomer C.J. 1996. Effects of moisture content and temperature on storage of *Metarhizium flavoviride* conidia. *Biocontrol Science and Technology* **6:** 51-61

Morris G.J., Smith D. & Coulson G.E. 1988. A comparative study of the morphology of hyphae during freezing with the viability upon thawing of 20 species of fungi. *Journal of General Microbiology* **134:** 2897-2906

Moss M.O. 1984. Biosynthesis of *Fusarium* mycotoxins. In: *British Mycological Society Symposium Series, Applied Mycology of Fusarium, (Moss M.O and Smith J.E eds), Cambridge University Press, U.K.* pp.197-213

Nandan R. & Raisuddin S. 1992. Fungal degredation of industrial wastes and wastewater. In: *Handbook of Applied Mycology, vol 4, (Arora D.K.,Elander R.P. & Mukerji K.I. eds), Marcel Dekker, New York. U.S.A.* pp. 931-961

Nash T. 1966. Freezing and freeze drying of microorganisms. *Cryobiology* **1:** 87-93

Nielson R.I. & Oxenboll K.1998. Enzymes from fungi: their technology and uses. *Mycologist* **12:**69-71

Nuss I., Jennings D.H. & Veltkamp C.J. 1991. Morphology of *Serpula lacrymans. In: Serpula lacrymans: Fundemental Biology and Control Statergies, (Jennings D.H. and Bravery A.F. eds) John Wiley & Sons Ltd, U.K.* pp. 9-38

Okuda T. 1994. Variation in colony characteristics of *Penicillium* strains resulting from minor variations in culture conditions. *Mycologia* **86:** 259-262

Ozino O.I., Piatti P. & Cravanzola F. 1998. RAPD-PCR for the molecular characterisation of *Beauveria brongniartii* isolates useful as biocontrol agents of the European cockchafer *Melolontha melolontha.* In: *Molecular Variability of Fungal pathogens, (Bridge P.D., Couteaudier Y. & Clarkson J.M. eds), CAB International, Wallingford, U.K.* pp. 239-252

Onions A.H.S & Smith D. 1984. Current status of culture preservation technology. In: *Critical Problems of Culture Collections. Symposium in IMC3. Institute of Fermentation, Osaka, Japan.* pp.41-47

Palagyi Z.S., Nagy A., Vastag M., Ferenczy L. & Vagvolgyi C.S. 1997. Maintenance of fungal strains on cryopreservative-immersed porous ceramic beads. *Biotechnology Techniques* **11:** 249-250

Paterson R.R.M. 1986. Standardized one-and two-dimensional thin layer chromatographic methods for the identification of secondary metabolites in *Penicillium* and other fungi. *Journal of Chromatography* **368:** 249-264

Paterson R.R.M. & Kemmelmeier C. 1989. Gradient high performance liquid chromatograpghy using alkylphenone retention indices of insecticidal extracts of *Penicillium* strains. *Journal of Chromatography* **483:** 153-168

Paterson R.R.M. & Kemmelmeier C. 1990. UV, alkaline and difference spectra of secondary metabolites from *Penicillium* and other fungi, and comparisons to published maxima from diode array detection and gradient HPLC. *Journal of Chromatography* **511:** 195-221

Pegler D.N. 1991. Taxonomy, identification and recognition of *Serpula lacrymans.* In: *Serpula lacrymans: Fundamental Biology and Control Statergies (Jennings D.H. and Bravery A.F. eds) John Wiley & Sons Ltd, U.K.* pp.1-7

Pereira R.M & Roberts D.W. 1991. Alginate and cornstarch mycelial formulations of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae. Journal of Economic Entomology* **86:** 1657-1661

Pertot E., Puc A. & Kresmer M. 1977. Lyophilization of non-sporulating strains of the fungus *Claviceps. European Journal of Applied Microbiology* **4:** 289-294

Pipe N.D., Chandler D., Bainbridge B.W. & Heale J.B. 1995. Restriction fragment length polymorphism in the ribosomal RNA gene complex of isolates of the entomopathogenic fungus *Metarhizium anisopliae. Mycological Research* **99:** 485- 491

Polge C., Smith A.U. & Parkes S. 1949. Revival of spermatozoa after dehydration at low temperatures. *Nature* **164:** 666

Pringle M.J. & Chapman D. 1981. Biomembrane structure and effects of temperature. In: *Effects of Low Temperature on Biological Membranes, (Morris G.J & Clarke A. eds), London Academic Press, U.K.* pp. 21-37

Priest F.G. 1984. Extracellular enzymes. In: *Aspects of Microbiology vol. 9. Van Nostrand Reinhold, Wokingham, U.K.*

Prior C. 1992. Discovery and characteristics of fungal pathogens for locust and grasshopper control. In: *Biocontrol of Locusts and Grasshoppers. (Lomer C.J & Prior C. eds) CAB International, Wallingford, U.K.* pp. 159-180

Prosser J.I. 1993. Growth kinetics of mycelial colonies and aggregations of ascomycetes. *Mycological Research* **97:** 513-528

Qiangqiang Z., Jiajun W & Li L. 1998. Storage of fungi using distilled water or lyophilisation: comparison after 12 years. *Mycoses* **41:** 255-257

Rath A.C., Carr C.J. & Graham B.R. 1995. Characterization of *Metarhizium anisopliae* strains by carbohydrate uitilisation (API50CH). *Journal of Invertebrate Pathology* **65:** 152-161

Riedy, M.C. Timm Jr. E.A. and Stewart C.C. 1995. Quantitative RT-PCR for measuring gene expression. *BioTechniques* **18**: 84-90.

Reynalopez G.E., Simpson J. & RuizHerrera J. 1997. Differences in DNA methylation patterns are detectable during the dimorphic transition of fungi by

amplification of restriction polymorphisms. *Molecular and General Genetics* **253:** 703-710

Riba G., Bouvier-Fourcade L & Caudal A. 1986. Isoenzymes polymorphism in *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) entomogenous fungi. *Mycopathologia* **96:** 161-169

Rindler V., Schwindkle P., Heschel I, Rau G. Technical description of a new low-temperature freeze-drying device. *International Journal of Refrigeration-Revue Internationale Du Froid* **21:** 535-541

Roger C. & Tivoli B. 1996. Effect of culture medium, light and temperature on sexual and asexual reproduction of four strains of *Mycosphaerella pinodes*. *Mycological Research* **100:** 304-306

Romaine C.P. & Schlagnhauffer B. 1992. Characteristics of a hydrated, alginate-based delivery system for cultivation of button mushroom. *Applied and Environmental Microbiology* **58:** 3060-3066

Rombach M.C, Humber R.A. & Evans H.C. 1987. *Metarhizium album* a fungal pathogen of leaf and planthoppers of rice. *Transactions of the British Mycological Society* **88**: 451-459

Roquebert M.F & Bury E. 1993. Effects of freezing and thawing on cell membrane of *Lentinus edodes*, the Shiitake mushroom. *World Journal of Microbiology and Biotechnology* **9:** 641-647

Samson R.A., Evans H.C. & Latge J.P. 1988. Atlas of Entomopathogenic Fungi. *Springer Verlag, Berlin.*

Schlick A., Kuhls K., Meyer W., Leickfeldt E., Borber T. & Messner K. 1994. Fingerprinting reveals gamma-ray induced mutations in fungal DNA: implications for identification of patent strains of *Trichoderma harzianum*. *Current Genetics* **26:** 74-78

Schmidt O. & Morton Kerbenik U. 1991. A simple method for producing basidiomes of *Serpula lacrymans* in culture. *Mycological Research* **95:** 375-376

Shinohara T., Yanagida F. & Goto S. 1995. Biological and enological stability of wine yeasts during preservation by periodic transfer. *Microbiology Culture Collections* **11:** 1-9

Silviera W.D. & Azevedo J.L. 1997. Protoplast fusion and genetic recombination in *Metarhizium anisopliae*. *Enzyme and Microbial Technology* **9:** 149-151

Sim K.L. & Perry D. 1995. Swainsonine production by *Metarhizium anisopliae* determined by means of an enzymatic assay. *Mycological Research* **99:** 1078-1082

Singleton. P & Sainsbury D. 1993. Dictionary of Microbiology and Molecular Biology, 2nd Edition. *Wiley, London, U.K.* 367-368

Smith D. 1993. Cryoprotectants and the cryopreservation of fungi. *Transactions of the British Mycological Society.* **80:** 360-363

Smith D. 1998. The use of cryopreservation in the ex-situ conservation of fungi. *Cryo-Letters* **19:** 79-90

Smith D. & Allsopp D. 1993. Preservation and maintenance of biotechnological important microorganisms. In: *Industrial Biotechnology International, (R.N Greenshields ed), Stirling Press, U.K.* pp. 103-107

Smith D. & Onions A.H.S. 1983. A comparison of some preservation techniques for fungi. *Transactions of the British Mycological society* **81:** 535-540

Smith D. & Onions A.H.S. 1984. The preservation and maintenance of living fungi. *Commonwealth Mycological Institute. CAB International, Wallingford, U.K.*

Smith D. & Onions A.H.S. 1994. The preservation and maintenance of living fungi. Second Edition, IMI Technical Handbooks No 2, *CAB International, Wallingford, U.K.*

Smith D. & Thomas V.E. 1998. Cryogenic light microscopy and the development of cooling protocols for the cryopreservation of filamentous fungi. *World Journal of Microbiology and Biotechnology* **14:** 49-57

Smith S.N., Jeffers D., DeVay J. E. 1994. Effect of glucose and biotin on the growth and sporulation of *Fusarium* spp., especially pathogenic and non-pathogenic isolates of *Fusarium oxysporum. Mycologia* **85:** 547 - 554

Stathers T.E., Moore D. & Prior C. 1993. The effect of different temperatures on the viability of *Metarhizum flavoviride* conidia stored in vegetable and mineral oils. *Journal of Invertebrate Pathology* **62:** 111-115

Steponkus P.L. 1984. Role of the plasma membrane in freezing injury and cold acclimitisation. *Annual Review of Plant Physiology* **35:** 543-543

St Leger R.J., Butt T.M., Staples R.C. & Roberts D.W. 1989. Synthesis of proteins including a cuticle-degrading protease during differentiation of the entomopathogenic fungus *Metarhizium anisopliae. Experimental Mycology* **13:** 253-262

St Leger R.J., Charnley A.K. & Cooper R.M. 1986. Cuticle degrading enzymes of entomopathogenic fungi. Synthesis in culture on cuticle. *Journal of Invertebrate Pathology* **48:** 85-95

St Leger R.J., Charnley A.K. & Cooper R.M. 1986. Enzymatic characterization of entomopathogens with the APIZYM system. *Journal of Invertebrate Pathology* **48:** 375-376

St Leger R.J., Joshi L., Bidochka M.J. & Roberts D.W. 1995. Protein synthesis in *Metarhizium anisopliae* grown on host cuticle. *Mycological Research* **99:** 1034-1040

St Leger R.J., Joshi L., Bidochka M.J. & Roberts D.W. 1996. Construction of an improved mycoinsecticide overexpressing a toxic protease. *Proceedings of the National Academy of Sciences of the United States of America* **93:** 6349-6354

St Leger R.J., May B., Aclee L.C., Frank D.L., Staples R.L. & Roberts D.W. 1992. Genetic differences in allozymes and in formation of infection structures among isolates of the entomopathogenic fungus *Metarhizium anisopliae. Journal of Invertebrate Pathology* **60:** 89-01

Svensen A. & Frisvad J.C. 1994. A chemotaxonomic study of the terverticillate penicillia based on high performance liquid chromatography of secondary metabolites. *Mycological research* **98:** 1317-1328

Talbot N.J. 1998. Molecular variability of fungal pathogens: Using the rice blast fungus as a case study. In: *Molecular Variability of Fungal Pathogens, (Bridge P.D., Couteaudier Y. & Clarkson J.M. eds), CAB International, Wallingford, U.K.* pp.1-18

Tamura S. & Takahashi N. 1971. Destruxins and piericidinds. In: *Naturally Occuring Insecticides, (Jacobsen M.J. & Crosby D.G eds), Marcel Dekker, N.Y. U.S.A.* pp. 499-539

Tan C.S. 1997. Preservation of fungi. *Cryptogamic Mycology* **18:** 157-163

Tan C.S. & Stalpers J.A. 1996. Vitrification of Fungi. In: *Biodiversity, International Biodiversity Seminar ECCO XIV meeting, (Cimerman A. & Gunde-Cimerman N. eds), Ljubljana, Slovenia* 189-193

Tan C.S., Stalpers J.A. & Van Ingen C.W. 1991a. Freeze drying of fungal hyphae. *Mycologia* **83:** 654-657

Tan C.S., Van Ingen C.W. & Stalpers J.A. 1991b. Freeze-drying of fungal hyphae and stability of the product. In: *Genetics and Breeding of Agarics, (Van Griensben A. ed), Wageningen.*

Tan C.S., Van Ingen C.W.,Talsma H.,Van Miltenburg J.C,Steffensen C.L,Vlug A. & Stalpers J.A. 1995. Freeze drying of fungi: Influence of composition and glass transition temperature of the protectant. *Cryobiology* **32:** 60-67

Tan C.S., Vlug L.J.A., Stalpers J.A. & Van Ingen C.W. 1994. Microscopical observations on the influence of cooling rate during freeze drying of conidia. *Mycologia* **86:** 281-289

Tian S.P. & Bertolini P. 1996. Changes in conidial morphology and germinability of *Botrytis alli* and *Penicillium hirsutum* in response to low temperature incubation. *Mycological Research* **100:** 591-596

Timberlake W.E. 1980. Developmental gene regulation in *Aspergillus nidulans, Developmental Biology* **78:** 497-510

Tisserent B., Brenac V., Requena N., Jeffries P. & Dodd J.C. 1998. The detection of *Glomus* spp. (arbuscular mycorrhizal fungi) in three plants at different stages of seedling development, using mycorrhiza-specific isoenzymes. *New Phytologist* **133:** 225-239

Tommerup I.C. 1988. Long term preservation by L-drying and storage of vesicular arbuscular mycorrhizal fungi. *Transactions of the British Mycological Society* **90:** 585-591

Torres P. & Honrubia M. 1994 Basidiospore viability in stored slurries. *Mycological Research* **98:** 527-530

Tulloch M. 1976. The genus *Metarhizium. Transactions of the British Mycological Society* **66:** 407-411

Trinci A.P.J. 1992. Myco-protein, a 20-year overnight success story. *Mycological Research* **96:** 1-13

UKNCC 1999. The United Kingdom National Culture Collection, Information leaflet. http://www.ukncc.co.uk

Wing N., Burgess L.W. & Bryden T. 1995. Cultural degeneration in two *Fusarium* species and its effects on and its effects on toxigenicity and cultural morphology. *Mycological Research* **99:** 615-620

Woo S.L., Noviello C. & Lorito M. 1998. Sources of molecular variability and applications in characterisation of the plant pathogen *Fusarium oxysporum.* In: *Molecular Variability of Fungal Pathogens, (Bridge P.D., Couteaudier Y. & Clarkson J.M. eds), CAB International, Wallingford, U.K.* pp.187-208

Yang D.Q & Rossignol L. 1998. Survival of wood inhabiting fungi in liquid nitrogen storage. *Mycologist* **12:** 121-123

Zolan M. E. 1995. Chromosome-length polymorphism in fungi. *Microbiological Reviews* **59:** 686-696

Zolan M.E & Pukkila P.J. 1986. Inheritance of DNA methylation in *Coprinus cinerus. Molecular and Cellular Biology* **6:** 195-200

APPENDIX A

1) Media recipes

(All chemicals supplied by BDH/MERCK unless otherwise stated)

GLUCOSE YEAST MEDIUM (GYM

Glucose	10g
$NH_4H_2PO_4$	1g
KCl	0.2g
Mg SO_4 $7H_2O$	0.5g
1% w/v $ZnSO_4$ $7H_2O$ solution	1ml
0.5% w/v Cu SO_4 5 H_2O solution	1ml
Distilled water	to1 litre

Autoclaved at 121°C for 15 minutes

MALT AGAR (MA)

Toffee barley malt extract (Difco)	20g
Agar No3 (Oxoid)	20g
Distilled water	to 1 litre

pH 6.5 (HCl/NaOH)

Autoclaved at 121°C for 20 minutes

MALT BROTH (MB)

Malt extract (Amersham)	30g
Mycological Peptone (Oxoid)	5g
Distilled water	to1litre

Autoclaved at 121°C for 20 minutes

MALT EXTRACT AGAR (MEA)

Malt extract (Amersham)	30g
Mycological Peptone (Oxoid)	5g
Agar No3 (Oxoid)	16g
Distilled water	to 1litre

Autoclaved at 121°C for 20 minutes

POTATO SUCROSE AGAR (PSA)

Potato water	500ml
Sucrose	20g
Agar No 3 (Oxoid)	20g
Distilled water	to 500ml

pH 6.5 (HCl/NaOH)

Autoclaved at 121°C for 15 minutes

SABOURAUD'S DEXTROSE AGAR (SDA)

Mycological Peptone (Oxoid)	10g
Dextrose	20g
Agar No3 (Oxoid)	15g
Distilled Water	to 1 litre

Autoclaved at 121°C for 15 minutes

SYNTHETIC NUTRIENT AGAR (SNA

KH_2PO_4	1g
KNO_3	1g
$MgSO_47H_20$	0.5g
KCl	0.5g
Glucose analar	0.2g
Sucrose analar	0.2
Agar No 3 (Oxoid)	20g
Distilled water	to1 litre

pH 6.5 (HCl/NaOH)

Autoclaved at 121°C for 20 minutes

YEAST EXTRACT SUCROSE (YES)

Yeast extract	20g
Sucrose	150g
Agar No3 (Oxoid)	20g
Distilled water	to 1 litre

Autoclaved at 121°C for 20 minutes

2. Buffers, solvents and reagents

(All chemicals supplied by BDH/MERCK unless otherwise stated)

Agarose gel: 1.4% LE agarose (Seakem, FMC Bioproducts) in TAE buffer

p-Anisaldehyde: 0.5% (v/v) p-anisaldehyde (Sigma) in ethanol : acetetic acid : conc. sulphuric acid (17:2:1 v/v/v)

CAP Solvent: Chloroform : Acetone : Propan 2-ol (85:15:20 v/v/v)

Chloroform : Iso-amyl alcohol: Chloroform : Iso-amyl alcohol (24:1 v/v)

Chloroform:Methanol: Chloroform : Methanol (2:1 v/v)

CTAB Solution: 700 mM NaCl (40.9 g/l)
50 mM Tris HCl (pH 8) (6g/l)
10 mM EDTA (3.72 g/l)
2 % Cetryltrimethyl Ammonium Bromide
Autoclaved at 121°C for 15 minutes
Before use made up with 1% β-mercaptoethanol (Sigma)

Deoxyribonucleoside-5`-triphosphatase (dNTPs):
2.5 mM each, (Pharmacia Biotech) in sterile distilled water

Griseofulvin: Griseofulvin (Sigma) 5mg in chloroform : methanol (2:1)

Ribonuclease A: 0.8 units ribonuclease A (Calbiochem) μl^{-1} TE buffer

Stop Solution (Loading buffer):
EDTA - 3.72 g
Sucrose - 40.00 g
Bromophenol Blue - 0.05 g
Distilled water – 1 litre

TAE Buffer: Tris base 4.84g
Sodium acetate (dihydrate) 2.72g
EDTA 0.38g
Distilled water to 1 litre
Adjusted to pH 7.2 with glacial acetic acid

TBE Buffer: Tris HCl 6.05 g
Boric acid 3.085 g
EDTA 0.37 g
Distilled water to 1 litre
Adjusted to pH 8.3 with HCl

TE Buffer: 10 mM Tris HCL (1.2 g/l)
1 mM EDTA (0.38 g/l)
Ajusted to pH 8 with HCl

TEF Solvent: Toluene : Ethyl acetate : 90% Formic acid (5:4:1 v/v/v)

Zym A: (Biomeriux ™ France):
Tris-hydroxylmethyl-aminomethane 25g
Sodium lauryl sulphate 10g
Hydrochloric acid (37%) 11ml
Water to 8ml

Zym B: (Biomeriux ™ France):
Fast Blue BB 0.35g
2 methoxyethanol 100ml

APPENDIX B

Statistical data for analysis of culture characteristics (chapter 3)

Radial growth analysis - ANOVA OF REGRESSION, SYSTAT DATA FILES
***Metarhizium anisopliae* isolate M1**
i) After 1 year of storage

REGRESSION EQUATIONS
CS y=2.778x -4.251
FD y=2.392x -7.889
MP y=1.748x -0.103
LN y=2.004x -0.283

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	118.742	4	29.685	39.151	0.119
Residual	0.758	1	0.758		

ii) After 2 years of storage

REGRESSION EQUATIONS
FD y=1.502x -3.284
MP y=1.825x -2.332
LN y=1.362x -2.618

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	146.730	3	48.910	948.791	0.001
Residual	0.103	2	0.052		

iii) After 2 years of storage and a recovery period

REGRESSION EQUATIONS
FD y=1.730x -0.595
MP y=1.604x -0.376
LN y=1.70x -1.417

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	365.738	3	121.913	529.742	0.000
Residual	1.151	5	0.230		

***Metarhizium anisopliae* isolate M2**
Radial growth analysis - ANOVA OF REGRESSION, SYSTAT DATA FILES.

i) After 1 year of storage
REGRESSION EQUATIONS
CS y=1.892x -2.469
FD y=2.247x -6.072
MP y=2.024x -2.258
FZ y=2.067x -1.873
LN y=2.008x -3.185

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	166.840	5	33.368	1979.393	0.017
Residual	0.017	1	0.017		

ii) After 2 years of storage

REGRESSION EQUATIONS
FD y=1.709x -0.890
MP y=1.490x -1.089
FZ y=1.689x -3.121
LN y=1.346x -1.096

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	218.776	4	54.694	1351.586	0.001
Residual	0.081	2	0.040		

iii) After 2 years of storage and a recovery period

REGRESSION EQUATIONS
FD y=1.251x -0.711
MP y=1.480x -0.836
FZ y=1.304x -0.4417
LN y=1.450x -0.887

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	366.252	4	91.563	574.708	0.001
Residual	0.637	4	0.159		

***Metarhizium anisopliae* isolate M4**
Radial growth analysis - ANOVA OF REGRESSION, SYSTAT DATA FILES.
i) After 1 week of storage

REGRESSION EQUATIONS
CS y=1.719x -0.651
FD y=1.388x -1.770
MP y=1.471x +0.346
FZ y=1.383x -0.714
LN y=1.480x -0.780

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	292.390	5	58.478	241.038	0.004
Residual	0.485	2	0.243		

ii) After 1 week of storage and a recovery period
(ANALYSIS USING MINITAB not SYSTSAT)

REGRESSION EQUATIONS
CS y=1.794x +0.588
FD y=1.499x +0.756
MP y=1.4661 +0.432
FZ y=1.551x -0.147
LN y=1.571x -0.306

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	173.214	34.643	69.29	0.091
Error	1	0.500	0.500		
Total	6	173.714			

iii) After 16 weeks of storage

REGRESSION EQUATIONS
CS y=2.068x -2.000
FD y=1.672x -2.040
MP y=1.931x -1.828
FZ y=1.525x -2.253
LN y=1.904x -0.829

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	171.989	34.398	3095.69	0.014
Error	1	0.011	0.011		
Total	6	172.000			

iv) After 16 weeks and a recovery period

REGRESSION EQUATIONS
CS y=1.689x -1.787
FD y=1.620x -1.974
MP y=1.849x -2.644
FZ y=1.742x -2.004
LN y=1.835x -2.453

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	98.83333	19.76667	-	>0.05
Error	0	*	*		
Total	5	98.83333			

v) After 1 year of storage

REGRESSION EQUATIONS
CS y=1.669x -4.226
FD y=1.533x -5.018
MP y=1.980x -3.128
FZ y=1.687x -2.868
LN y=1.162x -1.983

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	243.884	5	48.777	840.823	0.001
Residual	0.116	2	0.058		

v) After 1 year of storage and a recovery period

REGRESSION EQUATIONS
CS y=1.326x -1.252
FD y=1.293x -0.489
MP y=1.319x -0.115
FZ y=1.530x -1.401
LN y=1.280x -1.073

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regres	336.725	5	67.345	900.161	0.001
Residual	0.150	2	0.075		

Conidial production
***Metarhizium anisopliae* isolate M1**

i) After 1 year of storage

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	3	7426751.568	2475583.856	6.29511E+11
FD	3	4650530.785	1550176.928	6.12785E+11
MP	5	1125435.244	225087.0488	14103440250
LN	3	4665817.986	1555272.662	1.92018E+11

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.02986E+13	3	3.43286E+12	11.73611716	0.001294432	3.708265695
Within Groups	2.92504E+12	10	2.92504E+11			
Total	1.32236E+13	13				

ii) After 2 years of storage

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
FD	5	5760127.16	1152025.432	37832789835
MP	5	4094728.801	818945.7601	3.12274E+11
LN	4	6098803.157	1524700.789	4.11082E+11

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.10808E+12	2	5.54039E+11	2.314042715	0.144936576	3.982307817
Within Groups	2.63367E+12	11	2.39425E+11			
Total	3.74175E+12	13				

iii) After 2 years of storage and a recovery period

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
FD	4	5169340.463	1292335.116	38481335470
MP	3	3460656.991	1153552.33	33155378545
LN	4	4833206.009	1208301.502	4428930862

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	34553088380	2	17276544190	0.708630285	0.520788756	4.458968306
Within Groups	1.95042E+11	8	24380194511			
Total	2.29595E+11	10				

Metarhizium anisopliae isolate M2
Conidial production

i) After 1 year of storage

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	3	986802.547	328934.1823	66448446790
FD	3	4676687.897	1558895.966	3.96592E+11
MP	5	1656998.725	331399.745	21774136668
F-20	3	5133248.418	1711082.806	2.4673E+11
LN	3	1766114.648	588704.8827	57929576502

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6.09296E+12	4	1.52324E+12	11.26589527	0.000496264	3.259160053
Within Groups	1.6225E+12	12	1.35208E+11			
Total	7.71546E+12	16				

ii) After 2 years of storage

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
FD	5	3855360.321	771072.0642	4.50948E+11
MP	2	1583906.29	791953.145	12969049.48
F-20	4	6282149.223	1570537.306	65672571666
LN	5	6822001.548	1364400.31	2.34235E+11

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.91029E+12	3	6.36762E+11	2.601005411	0.99988	3.490299605
Within Groups	2.93776E+12	12	2.44814E+11			
Total	4.84805E+12	15				

iii) After 2 years of storage and a recovery period

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
FD	5	3524318.819	704863.7638	27729127676
MP	5	4784823.02	956964.604	2.3919E+11
F-20	5	3771326.263	754265.2526	14876791485
LN	5	3132161.955	626432.391	39805259384

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.98533E+11	3	99511096816	1.237697591	0.328758621	3.238866952
Within Groups	1.2864E+12	16	80400170064			
Total	1.58494E+12	19				

Metarhizium spp. isolate M4
Conidial production

i) After 1 Week of storage

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
csc	4	5107770.4	1276942.6	1.83581E+11
fd	3	11352866.4	3784288.8	25474377480
mp	5	9910488	1982097.6	66829507511
f-20	5	13240309.8	2648061.96	1.33938E+12
ln	4	9198405.6	2299601.4	8.30173E+11

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1.18946E+13	4	2.97365E+12	5.458077603	0.005748231	3.006917382
Within Groups	8.71706E+12	16	5.44816E+11			
Total	2.06117E+13	20				

ii) After 16 weeks of storage

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
CSC	5	5143887.76	1028777.55	2.0285E+11
FD	5	16554660.6	3310932.11	1.4319E+10
MP	4	3465758.04	866439.51	2.0535E+11
F-20	5	8857927.51	1771585.5	1.4782E+11
LN	5	10450750.2	2090150.04	8.9814E+11

ANOVA

rce of Varia	SS	df	MS	F	P-value	F crit
Between G	1.8232E+13	4	4.558E+12	15.2776794	9.54E-06	2.895106
Within Grou	5.6686E+12	19	2.9835E+11			
Total	2.3901E+13	23				

iii) After 16 weeks of storage and a recovery period

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
CSC	5	3740127.388	748025.4776	3.51172E+11
FD	5	4834923.568	966984.7136	4.39401E+11
MP	2	1989808.918	994904.459	94903647290
F-20	5	9050318.473	1810063.695	3.97688E+11
LN	4	18617827.07	4654456.768	5.44987E+13

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	4.28825E+13	4	1.07206E+13	1.018924305	0.427149994	3.006917382
Within Groups	1.68344E+14	16	1.05215E+13			
Total	2.11226E+14	20				

iv) After 1 year of storage

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	5	7615286.63	1523057.33	6.8366E+11
FD	5	7747770.7	1549554.14	4.5318E+11
MP	2	2170700.65	1085350.33	4.4913E+11
F-20	5	7674789.81	1534957.96	1.8491E+11
LN	5	5332484.08	1066496.82	1.5549E+11

ANOVA

rce of Varia	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between G	1.0297E+12	4	2.5742E+11	0.68828744	0.60995921	2.964711
Within Grou	6.3581E+12	17	3.7401E+11			
Total	7.3878E+12	21				

v) After 1 year of storage and a recovery period

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	5	3740127.388	748025.4776	3.51172E+11
FD	5	4834923.568	966984.7136	4.39401E+11
MP	2	1989808.918	994904.459	94903647290
F-20	5	9050318.473	1810063.695	3.97688E+11
LN	4	4494827.071	1123706.768	4.36835E+11

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.20461E+12	4	8.01152E+11	2.081436889	0.130942174	3.006917382
Within Groups	6.15846E+12	16	3.84904E+11			
Total	9.36307E+12	20				

RADIAL GROWTH DATA
Regression Analysis
***Fusarium oxysporum* isolate F1**
i) After 1 day of storage

REGRESSION EQUATION
CS y=3.686x -0.585
FD y=3.743x -1.124
MP y=3.730x +1.899
FZ y=3.608x -0.651
LN y=3.970x -2.028

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	79.697	15.939	939.77	0.025
Error	1	0.017	0.017		
Total	6	79.714			

ii) After 1 year of storage

REGRESSION EQUATIONS
CS y=4.593x -2.709
FD y=3.709x -4.308
MP y=4.345x -3.692
FZ y=3.408x -2.528
LN y=3.549x -1.498

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	67.417	13.483	1169.78	0.022
Error	1	0.012	0.012		
Total	6	67.429			

iii) After 1 year of storage and a recovery period

REGRESSION EQUATIONS
CS y=3.742x -2.535
FD y=3.764x -2.469
MP y=3.305x -2.427
FZ y=3.796x -1.745
LN y=3.966x -2.235

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	53.712	10.742	5416.28	0.010
Error	1	0.002	0.002		
Total	6	53.714			

iv) After 2 years of storage

REGRESSION EQUATIONS
CS y=5.310x -2.622
FD y=5.609x -8.041
MP y=4.827x -3.115
FZ y=6.191x -13.558
LN y=5.352x -9.470

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	53.714	10.743	8.460E+06	<0.0001
Error	1	0.000	0.000		
Total	6	53.714			

v) After 2 years of storage and a recovery period

REGRESSION EQUATIONS
CS y=4.671x -2.322
FD y=4.421x -1.872
MP y=4.900x -2.300
FZ y=4.756x -2.210
LN y=5.291x -2.870

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	111.899	22.380	23.36	0.156
Error	1	0.958	0.958		
Total	6	112.857			

***Fusarium oxysporum* isolate F2**

i) After 1 day of storage

REGRESION EQUATIONS
CS y=5.167x -5.500
FD y=4.945x -3.555
MP y=5.233x -1.733
FZ y=5.333x -6.111
LN y=5.222x -5.443

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	42.0000	8.4000	4.255E+06	<0.0005
Error	1	0.0000	0.0000		
Total	6	42.0000			

ii) After 1 year of storage

REGRESSION EQUATIONS
CS y=3.934x -2.919
FD y=3.783x -5.309
MP y=3.930x -2.530
FZ y=3.618x -3.552
LN y=3.652x -2.126

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	104.682	20.936	217.03	0.005
Error	2	0.193	0.096		
Total	7	104.875			

iii) After 1 year of storage and a recovery period

REGRESSION EQUATIONS
CS y=4.027x -3.089
FD y=4.021x -3.662
MP y=4.155x -3.393
FZ y=4.229x -3.593
LN y=4.078x -2.247

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	77.675	15.535	391.75	0.038
Error	1	0.040	0.040		
Total	6	77.714			

iv) After 2 years of storage

REGRESSION EQUATIONS

CS y=6.575x -2.181
FD y=4.000x -4.250
MP y=5.583x -2.679
FZ y=5.302x -5.324
LN y=6.398x -5.676

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	119.428	23.886	53996.86	0.003
Error	1	0.000	0.000		
Total	6	119.429			

V) After 2 years of storage and a recovery period

REGRESSION EQUATIONS

CS y=5.032x -3.070
FD y=5.758x -3.517
MP y=5.178x -3.005
FZ y=4.907x -3.083
LN y=4.800x -3.261

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	27.8644	5.5729	41.10	0.118
Error	1	0.1356	0.1356		
Total	6	28.0000			

***Fusarium oxysporum* isolate F3**

i) After 1 week of storage

REGRESSION EQUATIONS

CS y=5.283x -2.419
FD y=5.128x -3.311
MP y=5.263x -1.589
FZ y=5.098x -2.037
LN y=4.920x -4.713

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	59.720	11.944	85.34	0.012
Error	2	0.280			
Total	7	60.00			

ii) After 1 week of storage and recovery period

REGRESSION EQUATION

CS y=5.559x -4.305
FD y=5.497x -4.552
MP y=5.493x -4.330
FZ y=5.406x -3.676
LN y=5.522x -4.327

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	57.869	11.574	38.44	0.181
Error	1	0.131	0.131		
Total	6	58.000			

iii) After 16 weeks of storage

REGRESSION EQUATIONS
CS y=5.461x -1.955
FD y=4.488x -3.229
MP y=5.617x -2.788
FZ y=4.039x -3.734
LN y=3.991x -3.086

Analyis of Variance

Source	DF	SS	MS	F	P
Regression	5	81.559	16.312	74.00	0.013
Error	2	0.441	0.220		
Total	7	82.00			

iv) After 16 weeks of storage and a recovery period

REGRESSION EQUATIONS
CS y=5.971x -2.293
FD y=5.843x -2.536
MP y=6.186x -2.521
FZ y=5.929x -2.251
LN y=6.357x -2.464

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	41.3545	8.2709	22.99	0.157
Error	1	0.3598	0.3598		
Total	6	41.714			

v) After 1 year of storage

REGRESSION EQUATIONS
CS y=3.722x -1.674
FD y=3.345x -3.179
MP y=3.307x -1.501
FZ y=3.910x -4.302
LN y=4.248x -4.329

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	91.499	18.300	59358.02	0.0001
Error	2	0.001	0.000		
Total	7	91.500			

vi) After 1 year of storage and a recovery period

REGRESSION EQUATIONS
CS y=3.909x -1.399
FD y=3.862x -0.796
MP y=3.676x -0.473
FZ y=3.940x -1.586
LN y=3.963x -0.737

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	53.714	10.743	37.22	0.245
Error	1	0.001	0.001		
Total	6	53.71			

Conidial production

***Fusarium oxysporum* isolate F1**

i) After 1 year of storage

A.Microconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	5	18894.262	3778.8524	3379861.441
FD	3	10877.562	3625.854	1432449.101
MP	5	10657.45	2131.49	442485.2729
F-20	3	4527.074	1509.024667	154184.0242
LN	3	4904.334	1634.778	505047.0372

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	17557574.47	4	4389393.616	3.155769962	0.048025259	3.112248237
Within Groups	19472747.18	14	1390910.513			
Total	37030321.64	18				

B.Macroconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	5	2546.139	509.2278	56008.23521
FD	3	754.512	251.504	6918.434908
MP	5	1349.018	269.8036	8173.242875
F-20	3	378.132	126.044	7001.309764
LN	3	132.039	44.013	276.741169

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	502509.6982	4	125627.4245	6.168598582	0.004452492	3.112248237
Within Groups	285118.884	14	20365.63457			
Total	787628.5822	18				

ii) After 1 year of storage and a recovery period

A.Microconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	5	13632.77	2726.554	2847610.099
FD	3	5067.508	1689.169333	1979313.098
MP	4	8912.702	2228.1755	1449738.23
F-20	3	4558.21	1519.403333	244685.4436
LN	3	2291.837	763.9456667	27883.7421

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8282679.221	4	2070669.805	1.329750997	0.31064345	3.179117414
Within Groups	20243419.65	13	1557186.127			
Total	28526098.87	17				

B.Macroconidia

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
CSC	4	1196.85	299.2125	14860.52729
FD	3	636.621	212.207	4106.884852
MP	4	1375.101	343.77525	19237.5682
F-20	3	509.297	169.7656667	4106.952758

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	65069.75502	3	21689.91834	1.826950804	0.205945107	3.708265695
Within Groups	118721.9617	10	11872.19617			
Total	183791.7167	13				

iii) After 2 year of storage

A.Microconidia

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
CSC	5	12860.262	2572.0524	249184.8569
FD	5	13078.603	2615.7206	1225520.673
MP	5	5676.857	1135.3714	23121.25622
F-20	5	14497.813	2899.5626	3141723.649
LN	5	11593.875	2318.775	316643.0807

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	9447677.211	4	2361919.303	2.382795683	0.085817318	2.866080706
Within Groups	19824774.06	20	991238.7031			
Total	29272451.27	24				

B.Macroconidia

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
CSC	5	5174.672	1034.9344	11346.10142
FD	5	4344.982	868.9964	53011.42144
MP	5	3231.441	646.2882	31130.3386
F-20	5	3624.454	724.8908	114986.1584
LN	5	2030.566	406.1132	23740.92698

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1120232.205	4	280058.0512	5.978654544	0.002474454	2.866080706
Within Groups	936859.7873	20	46842.98936			
Total	2057091.992	24				

iv) After 2 years of storage and a recovery period

A.Microconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	5	8209.698	1641.9396	191039.7812
FD	5	6179.04	1235.808	491647.7129
MP	5	3668.122	733.6244	21834.12445
F-20	5	4825.333	965.0666	113796.1202
LN	5	4563.318	912.6636	349774.0097

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2498258.1	4	624564.525	2.673439504	0.061868071	2.866080706
Within Groups	4672366.994	20	233618.3497			
Total	7170625.094	24				

***Fusarium oxysporum* isolate F2**

i) After 1 year of storage

A.Microconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	5	9305.65	1861.13	1240080.568
FD	3	15324.523	5108.174333	6399209.253
MP	5	58877.158	11775.4316	44814415.75
F-20	3	2955.175	985.0583333	723801.7373
LN	3	3238.118	1079.372667	233588.2384

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	371825149.1	4	92956287.29	6.541900559	0.003474218	3.112248237
Within Groups	198931183.7	14	14209370.27			
Total	570756332.9	18				

B.Macroconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	5	785.95	157.19	5435.913142
FD	3	1006.017	335.339	12189.7282
MP	5	4181.228	836.2456	376367.8201
F-20	3	220.066	73.35533333	2306.144969
LN	3	377.256	125.752	3953.391376

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1748823.042	4	437205.7604	3.913322655	0.024549037	3.112248237
Within Groups	1564113.462	14	111722.3901			
Total	3312936.504	18				

ii) After 1 year of storage and a recovery period

A. Microconidia

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
CSC	5	3389.233	677.8466	57116.7712
FD	3	5878.912	1959.637333	1270358.441
MP	5	12386.586	2477.3172	2041633.078
F-20	3	3866.874	1288.958	730394.5584
LN	3	1446.149	482.0496667	231603.4002

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	11907942.62	4	2976985.654	3.240958936	0.044407749	3.112248237
Within Groups	12859712.19	14	918550.871			
Total	24767654.81	18				

B.Macroconidia

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
CSC	5	785.95	157.19	5435.913
FD	3	1006.017	335.339	12189.73
MP	5	4181.228	836.2456	376367.8
F-20	3	220.066	73.35533	2306.145
LN	3	377.256	125.752	3953.391

ANOVA

rce of Varia	SS	df	MS	F	P-value	F crit
Between G	1748823	4	437205.8	3.913323	0.024549	3.112248
Within Grou	1564113	14	111722.4			
Total	3312937	18				

iii) After 2 years of storage

A.Microconidia

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
CSC	5	14126.837	2825.3674	3174112.057
FD	4	20545.852	5136.463	325246.109
MP	5	32620.092	6524.0184	6495242.16
F-20	5	22161.572	4432.3144	3559237.392
LN	5	14082.97	2816.594	771105.1444

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	49487938.33	4	12371984.58	4.125838797	0.014265154	2.895106377
Within Groups	56974525.34	19	2998659.229			
Total	106462463.7	23				

B. Macroconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	5	1419.215	283.843	61020.92084
FD	4	2510.917	627.72925	29676.19784
MP	5	4061.136	812.2272	91626.78711
F-20	5	3144.104	628.8208	48006.51624
LN	5	1615.721	323.1442	47291.21693

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1002165.411	4	250541.3527	4.40436721	0.010940237	2.895106377
Within Groups	1080810.358	19	56884.75569			
Total	2082975.769	23				

iv) After 2 years of storage and a recovery period

A. Microconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	4	6288.21	1572.0525	1134926.502
FD	4	24541.485	6135.37125	5855152.776
MP	5	19388.646	3877.7292	4721163.025
F-20	5	16331.876	3266.3752	1855561.18
LN	5	11441.047	2288.2094	979529.504

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	50703492.94	4	12675873.24	4.456774922	0.011173575	2.92774871
Within Groups	51195252.67	18	2844180.704			
Total	101898745.6	22				

B. Macroconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	4	1069.868	267.467	24273.32723
FD	4	4323.143	1080.78575	277931.4125
MP	5	2467.25	493.45	95488.26538
F-20	5	2249.909	449.9818	24276.84848
LN	5	1069.869	213.9738	28222.20482

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1982862.195	4	495715.5487	5.954288833	0.003107696	2.92774871
Within Groups	1498563.494	18	83253.52744			
Total	3481425.689	22				

***Fusarium oxysporum* isolate F3**

i) After 1 week of storage

A. Microconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	5	21314.089	4262.8178	4088080.758
FD	5	9702.1114	1940.42228	209257.8573
MP	5	11026.284	2205.2568	138965.0082
F-20	5	11581.044	2316.2088	153322.1251
LN	5	15150.992	3030.1984	3112677.208

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	17537300.37	4	4384325.092	2.846113115	0.051108498	2.866080706
Within Groups	30809211.83	20	1540460.591			
Total	48346512.19	24				

B. Macroconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	5	687.5506	137.51012	2788.386433
FD	5	127.319	25.4638	648.3898327
MP	5	509.297	101.8594	648.4407603
F-20	5	356.499	71.2998	778.2205872
LN	5	458.364	91.6728	4733.798351

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	34083.79142	4	8520.947855	4.439271831	0.009917839	2.866080706
Within Groups	38388.94385	20	1919.447193			
Total	72472.73527	24				

ii) After 16 weeks of storage

A.Microconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	4	42921.641	10730.41025	6230091.555
FD	4	44537.205	11134.30125	5073615.605
MP	3	47942.892	15980.964	5568785.526
F-20	4	54427.085	13606.77125	1161837.295
LN	4	28807.277	7201.81925	13063790.02

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	154791527.5	4	38697881.87	6.175740077	0.004431054	3.112248237
Within Groups	87725574.49	14	6266112.463			
Total	242517102	18				

B.Macroconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	4	2117.701	529.42525	76698.57775
FD	4	1026.114	256.5285	5202.310942
MP	3	2205.025	735.0083333	367164.1574
F-20	4	1790.22	447.555	476.636224
LN	4	1288.086	322.0215	18231.24096

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	483391.1408	4	120847.7852	1.632834494	0.221181464	3.112248237
Within Groups	1036154.612	14	74011.04375			
Total	1519545.753	18				

iii) After 16 weeks of storage and a recovery period

A. Microconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	5	58247.877	11649.5754	1099618.057
FD	5	35630.924	7126.1848	15653769.97
MP	5	47768.334	9553.6668	13859248.47
F-20	5	40148.978	8029.7956	6872451.428
LN	5	30783.069	6156.6138	5827336.616

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	93149540.16	4	23287385.04	2.688303101	0.060852822	2.866080706
Within Groups	173249698.2	20	8662484.909			
Total	266399238.3	24				

B.Macroconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	5	5436.159	1087.2318	34651.29193
FD	5	2903.651	580.7302	194609.7079
MP	5	4191.737	838.3474	149806.0971
F-20	5	3601.764	720.3528	54624.70856
LN	5	3687.601	737.5202	59238.12628

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	710213.485	4	177553.3712	1.801000099	0.168284353	2.866080706
Within Groups	1971719.727	20	98585.98636			
Total	2681933.212	24				

iv) After 1 year of storage

A.Microconidia

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
CSC	5	24668.088	4933.6176	8749849.891
FD	5	15283.841	3056.7682	168522.4539
MP	5	21943.22	4388.644	4280060.538
F-20	4	13078.603	3269.65075	646877.6938
LN	5	20087.336	4017.4672	446930.5172

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	11660295.19	4	2915073.796	0.979907243	0.441834162	2.895106377
Within Groups	56522086.68	19	2974846.667			
Total	68182381.87	23				

B.Macroconidia

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
CSC	5	3799.126	759.8252	415562.3146
FD	5	1572.053	314.4106	14921.4473
MP	5	3864.297	772.8594	360145.2016
F-20	4	661.572	165.393	11817.70639
LN	5	4104.793	820.9586	196556.1615

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1677969.912	4	419492.4779	2.000494414	0.135341336	2.895106377
Within Groups	3984193.619	19	209694.401			
Total	5662163.531	23				

v) After 1 year of storage and a recovery period

A. Microconidia

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
CSC	5	17576.419	3515.2838	2739030.038
FD	5	16113.537	3222.7074	559342.8814
MP	4	17423.58	4355.895	7695790.068
F-20	5	20655.022	4131.0044	803616.9966
LN	4	15174.673	3793.66825	1090868.889

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3846636.021	4	961659.0053	0.404739239	0.802762424	2.92774871
Within Groups	42767936.53	18	2375996.474			
Total	46614572.55	22				

B.Macroconidia

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
CSC	5	2663.755	532.751	109313.2594
FD	5	2314.41	462.882	38948.46518
MP	4	1441.049	360.26225	58637.44135
F-20	5	2052.403	410.4806	93533.69007
LN	4	764.192	191.048	33490.01176

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	289338.4413	4	72334.61033	1.047009215	0.410722244	2.92774871
Within Groups	1243564.018	18	69086.88989			
Total	1532902.459	22				

APPENDIX C

Appendix to chapter 4: Secondary metabolite profiles obtained using thin layer chromatography and binary matrices used to compile dendrograms. (1=metabolite detected,0 = metabolite not detected). EXT =Extracellular, INT= Intracellular.

1)*Fusarium oxysporum*
Extracellular secondary metabolite profile
Fusarium oxysporum **isolate F1:**

METABOLITE	Properties	Mean Rf	Number	S.E.
1	W L/UV	7.6	78	0.2
2	W L/UV, T A/S	11.1	77	0.2
3	Y L/UV	16.9	76	0.3
4	BL A/S	25.3	72	0.3
5	BL A/S	32.6	77	0.1
6	P A/S	38.8	78	0.1
7	W L/UV	39.0	53	0.3
8	W/Y L/UV	49.0	78	0.3
9	Y L/UV	56.7	76	0.2
10	P A/S	90.2	63	0.3
Extra 1*	O L/UV	31.8	6	0.2
Extra 2*	O L/UV	33.3	2	0

BL= blue, W= white, Y= yellow, T= Turquoise, P= purple, O = orange, L/UV= long wave ultraviolet light, A/S= after spray

i) After 1 year of storage

Isolate F1, EXT	RF 1	RF 2	RF 3	RF 4	RF 5	RF E1*	RF E2*	RF 6	RF 7	RF 8	RF 9	RF 10
Preservation treatment												
CS1	1	1	1	1	1	0	0	1	1	1	1	1
CS2	1	1	1	1	1	0	0	1	1	1	1	1
CS3	1	1	1	1	1	0	0	1	1	1	1	1
CS4	1	1	1	1	1	0	0	1	1	1	1	1
CS5	1	1	1	1	1	0	0	1	1	1	1	1
FD1	1	1	1	1	1	0	0	1	1	1	1	1
FD2	1	1	1	1	1	0	0	1	1	1	1	1
FD5	1	1	1	1	1	0	0	1	1	1	1	1
MP1	1	1	1	1	1	0	0	1	1	1	1	1
MP2	1	1	1	1	0	0	0	1	1	1	1	1
MP3	1	1	1	1	1	0	0	1	1	1	1	1
MP4	1	1	1	1	1	0	0	1	1	1	1	1
MP5	1	1	1	1	1	0	0	1	1	1	1	1
FZ1	1	1	1	1	1	0	0	1	1	1	1	1
FZ2	1	1	1	1	1	0	0	1	1	1	1	1
FZ3	1	1	1	1	1	0	0	1	1	1	1	1
LN1	1	1	1	1	1	0	0	1	1	1	1	0
LN2	1	1	1	1	1	0	0	1	1	1	1	1
LN3	1	1	1	1	1	0	0	1	1	1	1	1

ii) After 1 year of storage and a recovery period

Isolate F1, EXT	RF1	RF2	RF3	RF4	RF5	RF E1*	RF E2*	RF6	RF7	RF 8	RF 9	RF 10
Preservation treatment												
CS1	1	1	1	1	1	0	0	1	1	1	1	1
CS2	1	1	1	1	1	0	0	1	1	1	1	1
CS3	1	1	1	1	1	0	0	1	1	1	1	1
CS4	1	1	1	1	1	0	0	1	1	1	1	1
CS5	1	1	1	1	1	0	0	1	1	1	1	1
FD1	1	1	1	1	1	0	0	1	1	1	1	1
FD2	1	1	1	1	1	0	0	1	1	1	1	1
FD5	1	1	1	1	1	0	0	1	1	1	1	1
MP1	1	1	1	1	1	0	0	1	1	1	1	1
MP2	1	1	1	1	1	0	0	1	1	1	1	1
MP3	1	1	1	1	1	0	0	1	1	1	1	1
MP4	1	1	1	1	1	0	0	1	1	1	1	1
MP5	1	1	1	1	1	0	0	1	1	1	1	1
FZ1	1	1	1	1	1	0	0	1	1	1	1	1
FZ2	1	1	1	1	1	0	0	1	1	1	1	1
FZ3	1	1	1	1	1	0	0	1	1	1	1	1
LN1	1	1	1	1	1	0	0	1	1	1	1	1
LN2	1	1	1	1	1	0	0	1	1	1	1	1
LN3	1	1	1	1	1	0	0	1	1	1	1	1

E= extra metabolite not detected in the profile of the original isolate

iii) After 2 years of storage

Isolate F1, EXT	RF1	RF2	RF3	RF4	RF5	RFE 1*	RFE 2*	RF6	RF7	RF 8	RF 9	RF 10
Preservation treatment												
CS1	1	0	1	1	1	0	0	1	1	1	1	1
CS2	1	1	1	1	1	0	0	1	1	1	1	1
CS3	1	1	0	1	1	0	0	1	0	1	1	1
CS4	1	1	1	1	1	0	0	1	0	1	1	1
FD1	1	1	1	1	1	1	0	1	1	1	1	1
FD2	1	1	1	1	1	1	0	1	1	1	1	1
FD3	1	1	1	1	1	0	0	1	1	1	1	1
FD4	1	1	1	1	1	0	0	1	1	1	1	1
MP1	1	1	1	1	1	0	0	1	1	1	1	1
MP2	1	1	1	1	1	0	0	1	0	1	1	1
MP3	1	1	1	0	1	0	0	1	0	1	1	1
MP4	1	1	1	1	1	0	0	1	0	1	1	1
FZ1	1	1	1	1	1	0	0	1	1	1	1	1
FZ2	1	1	1	0	1	0	0	1	0	1	1	1
FZ3	1	1	1	1	1	1	1	1	1	1	1	1
FZ4	1	1	1	1	1	1	1	1	1	1	1	1
LN1	1	1	1	1	1	0	0	1	1	1	1	1
LN2	1	1	1	1	1	0	0	1	1	1	1	1
LN3	1	1	1	1	1	0	0	1	1	1	1	1
LN4	1	1	1	1	1	0	0	1	1	1	1	1

iv) After 2 years of storage and a recovery period

Isolate F1, EXT	RF1	RF2	RF3	RF4	RF5	RF E1*	RF E2*	RF6	RF7	RF 8	RF 9	RF 10
Preservation treatment												
CS1	1	1	1	1	1	0	0	1	1	1	1	1
CS2	1	1	1	1	1	0	0	1	1	1	1	1
CS3	1	1	1	1	1	0	0	1	1	1	1	1
CS4	1	1	1	1	1	0	0	1	0	1	1	1
FD1	1	1	1	1	1	0	0	1	1	1	1	1
FD2	1	1	1	1	1	0	0	1	1	1	1	1
FD3	1	1	1	1	1	0	0	1	1	1	1	1
FD4	1	1	1	1	1	0	0	1	0	1	1	1
MP1	1	1	1	0	1	0	0	1	1	1	1	1
MP2	1	1	1	0	1	0	0	1	0	1	1	1
MP3	1	1	1	0	1	0	0	1	1	1	1	1
MP4	1	1	1	0	1	0	0	1	0	1	1	1
FZ1	1	1	1	1	1	0	0	1	1	1	1	1
FZ2	1	1	1	1	1	0	0	1	1	1	1	1
FZ3	1	1	1	1	1	0	0	1	1	1	1	1
FZ4	1	1	1	1	1	0	0	1	1	1	1	1
LN1	1	1	1	1	1	0	0	1	1	1	1	1
LN2	1	1	1	1	1	0	0	1	1	1	1	1
LN3	1	1	1	1	1	0	0	1	1	1	1	1
LN4	1	1	1	1	1	0	0	1	1	1	1	1

Intracellular secondary metabolite profile of *Fusarium oxysporum* isolate F1:

METABOLITE	Properties	Mean Rf	Number	S.E.
1	W/Y L/UV	4.6	77	0.1
2	W/Y L/UV	6.5	51	0.1
3	Y L/UV	9.7	76	0.2
4	Y L/UV	17.2	73	0.2
5	Y L/UV	21.3	34	0.2
6	Y L/UV	38.0	46	0.2
7	Y L/UV	62.0	55	0.6
8	Y L/UV	82.2	78	0.8

W= white, Y= yellow, L/UV= long wave ultraviolet light

i) After 1 year of storage

Isolate F1, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	0	1	1	0	0	0	1
CS2	1	0	1	1	0	1	1	1
CS3	1	0	1	1	0	1	1	1
CS4	1	0	1	1	0	1	1	1
CS5	1	0	1	1	0	1	0	1
FD1	1	0	1	1	0	0	0	1
FD2	1	0	1	1	0	0	0	1
FD5	1	0	1	1	0	1	0	1
MP1	1	0	1	1	0	1	0	1
MP2	1	0	1	1	0	0	0	1
MP3	1	0	1	1	0	0	0	1
MP4	1	0	1	1	0	1	0	1
MP5	1	0	1	1	0	0	0	1
FZ1	1	1	1	1	1	1	1	1
FZ2	1	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	1	1	1
LN1	1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1

ii) After 1 of storage and a recovery period

Isolate F1, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1	1
CS3	1	1	1	1	1	1	1	1
CS4	1	1	1	1	1	1	1	1
CS5	1	1	1	1	1	1	1	1
FD1	1	1	1	1	1	1	1	1
FD2	1	1	1	1	1	1	1	1
FD5	1	1	1	1	1	1	1	1
MP1	1	1	1	1	1	1	1	1
MP2	1	1	0	0	0	1	1	1
MP3	1	1	1	1	1	1	1	1
MP4	1	1	1	1	1	1	1	1
MP5	1	1	1	1	1	1	1	1
FZ1	1	1	0	1	1	1	1	1
FZ2	1	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	1	1	1
LN1	1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1

iii) After 2 years of storage

Isolate F1, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	1	0	0	0	1
CS2	1	1	1	1	0	0	0	1
CS3	1	1	1	1	0	0	0	1
CS4	1	1	1	1	0	0	0	1
FD1	1	0	1	1	0	0	0	1
FD2	1	1	1	1	0	0	0	1
FD3	1	1	1	1	0	0	0	1
FD4	1	1	1	1	0	0	0	1
MP1	1	1	1	0	0	1	0	1
MP2	1	1	1	0	0	1	0	1
MP3	1	1	1	1	0	0	0	1
MP4	1	1	1	1	0	0	0	1
FZ1	1	1	1	1	0	0	0	1
FZ2	0	1	1	1	0	0	1	1
FZ3	1	0	1	1	1	0	1	1
FZ4	1	0	1	1	1	1	1	1
LN1	1	0	1	1	1	1	1	1
LN2	1	0	1	1	1	1	1	1
LN3	1	0	1	1	1	1	1	1
LN4	1	0	1	1	1	1	1	1

iv) After 2 years of storage and a recovery period

Isolate F1, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	1	0	0	1	1
CS2	1	1	1	1	0	0	1	1
CS3	1	1	1	1	0	0	1	1
CS4	1	1	1	1	0	0	1	1
FD1	1	1	1	1	0	0	1	1
FD2	1	1	1	1	0	0	1	1
FD3	1	1	1	1	0	0	1	1
FD4	1	1	1	1	0	0	1	1
MP1	1	1	1	1	0	0	1	1
MP2	1	1	1	0	0	0	1	1
MP3	1	1	1	0	0	0	1	1
MP4	1	1	1	1	0	0	1	1
FZ1	1	1	1	1	0	0	1	1
FZ2	1	0	1	1	0	1	1	1
FZ3	1	0	1	1	0	1	1	1
FZ4	1	0	1	1	1	1	1	1
LN1	1	0	1	1	1	1	1	1
LN2	1	0	1	1	1	1	1	1
LN3	1	0	1	1	1	1	1	1
LN4	1	0	1	1	0	1	1	1

Extracellular secondary metabolite profile of *Fusarium oxysporum* isolate F2

METABOLITE	Properties	Mean Rf	Number	S.E.
1	W L/UV	6.1	77	0.1
2	W L/UV	9.3	78	0.7
3	Y L/UV	14.7	78	0.1
4	BL A/S	21.6	53	0.5
5	Y L/UV	34.1	77	0.3
6	Y L U/V	51.3	55	0.1
7	Y L/UV	61.8	67	0.3
8	P A/S	95.3	70	0.5

BL= blue, W= white, Y= yellow, P= purple, O = orange, L/UV= long wave ultraviolet light, A/S= after spray

i) After 1 year of storage

Isolate F2, EXT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1	1
CS3	1	1	1	1	1	1	1	1
CS4	0	1	1	1	1	1	1	1
CS5	1	1	1	1	1	1	1	1
FD1	1	1	1	0	1	1	1	0
FD2	1	1	1	0	0	1	1	0
FD3	1	1	1	0	1	1	1	1
MP1	1	1	1	0	1	1	1	0
MP2	1	1	1	0	1	1	1	1
MP3	1	1	1	0	1	1	1	0
MP4	1	1	1	1	1	1	1	1
MP5	1	1	1	1	1	1	1	1
FZ1	1	1	1	1	1	1	1	1
FZ2	1	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	1	1	1
LN1	1	1	1	0	1	1	1	1
LN2	1	1	1	1	1	1	1	1
LN3	1	1	1	0	1	1	1	1

ii) After 1 year of storage and a recovery period

Isolate F2, EXT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1	1
CS3	1	1	1	1	1	1	1	1
CS4	1	1	1	0	1	1	1	1
CS5	1	1	1	1	1	1	1	1
FD1	1	1	1	1	1	1	1	1
FD2	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1
MP1	1	1	1	1	1	1	1	0
MP2	1	1	1	1	1	1	1	1
MP3	1	1	1	1	1	1	1	0
MP4	1	1	1	1	1	1	1	1
MP5	1	1	1	1	1	1	1	1
FZ1	1	1	1	1	1	1	1	1
FZ2	1	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	1	1	1
LN1	1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1

iii) After 2 years of storage

Isolate F2, EXT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	0	1	0	0	0
CS2	1	1	1	0	1	0	1	1
CS3	1	1	1	1	1	0	0	0
CS4	1	1	1	1	1	0	1	1
FD1	1	1	1	1	1	0	0	1
FD2	1	1	1	0	1	0	0	1
FD3	1	1	1	0	1	1	1	1
FD4	1	1	1	0	1	1	1	1
MP1	1	1	1	1	1	0	0	1
MP2	1	1	1	0	1	1	1	1
MP3	1	1	1	0	1	0	1	1
MP4	1	1	1	1	1	0	1	1
FZ1	1	1	1	0	1	0	0	1
FZ2	1	1	1	0	1	0	0	1
FZ3	1	1	1	0	1	0	0	1
FZ4	1	1	1	1	1	0	0	1
LN1	1	1	1	1	1	1	1	1
LN2	1	1	1	0	1	1	1	1
LN3	1	1	1	0	1	1	1	1
LN4	1	1	1	0	1	1	1	1

iv) After 2 years of storage and a recovery period

Isolate F2, EXT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	1	1	0	1	1
CS2	1	1	1	1	1	0	1	1
CS3	1	1	1	1	1	1	1	1
CS4	1	1	1	1	1	0	1	1
FD1	1	1	1	1	1	0	0	1
FD2	1	1	1	1	1	0	1	1
FD3	1	1	1	0	1	0	1	1
FD4	1	1	1	1	1	1	1	1
MP1	1	1	1	0	1	0	1	1
MP2	1	1	1	1	1	0	0	1
MP3	1	1	1	0	1	1	1	1
MP4	1	1	1	1	1	1	1	1
FZ1	1	1	1	1	1	1	1	1
FZ2	1	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	0	1	1
FZ4	1	1	1	1	1	0	1	1
LN1	1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1
LN4	1	1	1	1	1	1	1	1

Intracellular secondary metabolite profile of *Fusarium oxysporum* isolate F2:

METABOLITE	Properties	Mean Rf	Number	S.E.
1	Y L/UV	6.3	76	0.3
2	W/Y L/UV	11.2	62	0.2
3	BL/W S/UV	41.2	71	0.2
4	Y L/UV	64.4	75	0.2
5	Y L/UV	89.6	68	0.5

W= white, Y= yellow, BL= blue L/UV= long-wave ultraviolet light, S/UV= Short-wave ultraviolet light.

i) After 1 year of storage

Isolate F2, INT	RF1	RF2	RF3	RF4	RF5
Preservation treatment					
CS1	1	1	1	1	0
CS2	1	1	1	1	1
CS3	1	1	1	1	1
CS4	1	0	0	0	1
CS5	1	1	1	1	0
FD1	1	1	1	0	1
FD2	1	1	1	1	1
FD3	1	1	1	1	1
MP1	1	1	0	1	1
MP2	1	1	1	1	1
MP3	1	1	0	1	1
MP4	1	0	1	1	1
MP5	1	0	1	1	1
FZ1	1	1	1	1	1
FZ2	1	0	1	1	1
FZ3	1	0	1	1	1
LN1	1	1	1	1	1
LN2	1	1	1	1	1
LN3	1	1	1	1	1

ii) After 1 year of storage and a recovery period

Isolate F2, INT	RF1	RF2	RF3	RF4	RF5
Preservation treatment					
CS1	1	1	1	1	1
CS2	1	1	1	1	1
CS3	1	1	1	1	1
CS4	1	1	1	1	1
CS5	1	1	1	1	1
FD1	1	0	1	1	1
FD2	1	0	1	1	1
FD3	1	0	1	1	1
MP1	1	1	1	1	1
MP2	1	1	1	1	1
MP3	1	1	1	1	1
MP4	1	1	1	1	1
MP5	1	1	1	1	1
FZ1	1	1	1	1	1
FZ2	1	1	1	1	1
FZ3	1	1	1	1	1
LN1	1	1	1	1	1
LN2	1	1	1	1	1
LN3	1	1	1	1	1

iii) After 2 years of storage

Isolate F2, INT	RF1	RF2	RF3	RF4	RF5
Preservation treatment					
CS1	1	1	1	1	1
CS2	1	1	1	1	1
CS3	1	1	1	1	0
CS4	1	1	1	1	1
FD1	1	1	1	1	0
FD2	1	1	1	1	0
FD3	1	0	1	1	1
FD4	1	1	1	1	1
MP1	1	1	1	1	0
MP2	1	1	1	1	1
MP3	1	1	0	1	1
MP4	1	1	0	1	1
FZ1	1	1	1	1	1
FZ2	1	1	1	1	0
FZ3	1	1	1	1	0
FZ4	1	1	1	1	1
LN1	1	1	1	1	1
LN2	1	1	1	1	1
LN3	1	1	1	1	1
LN4	1	1	1	1	1

iv) After 2 years of storage and a recovery period

Isolate F2, INT	RF1	RF2	RF3	RF4	RF5
Preservation treatment					
CS1	1	1	1	1	1
CS2	1	1	1	1	1
CS3	1	0	1	1	1
CS4	1	1	1	1	1
FD2	0	1	1	1	1
FD3	1	1	1	1	1
FD4	1	1	1	1	1
MP1	1	0	1	1	1
MP2	1	0	1	1	1
MP3	1	1	1	1	1
MP4	1	0	1	1	1
FZ1	1	1	1	1	1
FZ2	1	1	1	1	1
FZ3	1	1	1	1	1
FZ4	1	1	1	1	1
LN1	1	1	1	1	1
LN2	1	0	1	1	1
LN3	1	1	1	1	1
LN4	1	0	1	1	1

Extracellular secondary metabolite profiles of *Fusarium oxysporum* isolate F3

METABOLITE	Properties	Mean Rf	Number	S.E.
1	Y L/UV P A/S	6.5	120	0.2
2	W S/UV-L/UV	8.9	120	0.2
3	Y L/UV BL A/S	13.0	120	0.2
4	FL P L/UV	20.0	4	0.2
5	Y L/UV P/AS	27.5	115	0.3
6	Y L/UV	36.2	116	0.2
7	Y L/UV	40.7	117	0.4
8	P A/S	96.3	120	0.2

W= white, Y= yellow, BR= brown, L/UV= long-wave ultraviolet light, S/UV= Short-wave ultraviolet light, VIS= visible

i) After 1 week of storage

Isolate F3, EXT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1	1
CS3	1	1	1	1	1	1	1	1
CS4	1	1	1	1	1	1	1	1
FD1	1	1	1	0	1	1	1	1
FD2	1	1	1	0	1	0	1	1
FD3	1	1	1	0	1	0	1	1
FD4	1	1	1	0	1	0	1	1
MP1	1	1	1	0	1	1	1	1
MP2	1	1	1	0	1	1	1	1
MP3	1	1	1	0	1	1	1	1
MP4	1	1	1	0	1	1	1	1
FZ1	1	1	1	0	1	1	1	1
FZ2	1	1	1	0	1	1	1	1
FZ3	1	1	1	0	1	1	1	1
FZ4	1	1	1	0	1	1	1	1
LN1	1	1	1	0	1	1	1	1
LN2	1	1	1	0	1	1	1	1
LN3	1	1	1	0	1	1	1	1
LN4	1	1	1	0	1	1	1	1

ii) After 1 week of storage and a recovery period

Isolate F3, EXT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	0	1	1	1	1
CS2	1	1	1	0	1	1	1	1
CS3	1	1	1	0	1	1	1	1
CS4	1	1	1	0	1	1	1	1
FD1	1	1	1	0	1	1	1	1
FD2	1	1	1	0	1	1	1	1
FD3	1	1	1	0	1	1	1	1
FD4	1	1	1	0	1	1	1	1
MP1	1	1	1	0	1	1	1	1
MP2	1	1	1	0	1	1	1	1
MP3	1	1	1	0	1	1	1	1
MP4	1	1	1	0	1	1	1	1

FZ1	1	1	1	0	1	1	1	1
FZ2	1	1	1	0	1	1	1	1
FZ3	1	1	1	0	1	1	1	1
FZ4	1	1	1	0	1	1	1	1
LN1	1	1	1	0	1	1	1	1
LN2	1	1	1	0	1	1	1	1
LN3	1	1	1	0	1	1	1	1
LN4	1	1	1	0	1	1	1	1

iii) After 16 weeks of storage

Isolate F3, EXT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	0	1	1	1	1
CS2	1	1	1	0	1	1	1	1
CS3	1	1	1	0	1	1	1	1
CS4	1	1	1	0	0	1	1	1
FD1	1	1	1	0	0	1	1	1
FD2	1	1	1	0	0	1	1	1
FD3	1	1	1	0	1	1	1	1
FD4	1	1	1	0	0	1	1	1
MP1	1	1	1	0	1	1	1	1
MP2	1	1	1	0	1	1	1	1
MP3	1	1	1	0	1	0	1	1
MP4	1	1	1	0	1	1	1	1
FZ1	1	1	1	0	1	1	1	1
FZ2	1	1	1	0	1	1	1	1
FZ3	1	1	1	0	1	1	1	1
FZ4	1	1	1	0	1	1	1	1
LN1	1	1	1	0	1	1	1	1
LN2	1	1	1	0	1	1	1	1
LN3	1	1	1	0	1	1	1	1
LN4	1	1	1	0	1	1	1	1

iv) After 16 weeks of storage and a recovery period

Isolate F3, EXT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	0	1	1	1	1
CS2	1	1	1	0	1	1	1	1
CS3	1	1	1	0	1	1	1	1
CS4	1	1	1	0	1	1	1	1
FD1	1	1	1	0	1	1	1	1
FD2	1	1	1	0	1	1	1	1
FD3	1	1	1	0	1	1	1	1
FD4	1	1	1	0	1	1	1	1
MP1	1	1	1	0	1	1	1	1
MP2	1	1	1	0	1	1	1	1
MP3	1	1	1	0	1	1	1	1
MP4	1	1	1	0	1	1	1	1
FZ1	1	1	1	0	1	1	1	1
FZ2	1	1	1	0	1	1	1	1
FZ3	1	1	1	0	1	1	1	1
FZ4	1	1	1	0	1	1	1	1
LN1	1	1	1	0	1	1	1	1

LN2	1	1	1	0	1	1	1	1
LN3	1	1	1	0	1	1	1	1
LN4	1	1	1	0	1	1	1	1

v) After 1 year of storage

Isolate F3, EXT	RF 1	RF 2	RF 3	RF 4	RF 5	RF 6	RF 7	RF 8
Preservation treatment								
CS1	1	1	1	0	1	1	0	1
CS2	1	1	1	0	0	1	0	1
CS3	1	1	1	0	1	1	0	1
CS4	1	1	1	0	1	1	0	1
FD1	1	1	1	0	1	1	0	1
FD2	1	1	1	0	1	1	0	1
FD3	1	1	1	0	1	1	0	1
FD4	1	1	1	0	1	1	1	1
MP1	1	1	1	0	1	1	1	1
MP2	1	1	1	0	1	1	1	1
MP3	1	1	1	0	1	1	1	1
MP4	1	1	1	0	1	1	1	1
FZ1	1	1	1	0	1	1	1	1
FZ2	1	1	1	0	1	1	1	1
FZ3	1	1	1	0	1	1	1	1
FZ4	1	1	1	0	1	1	1	1
LN1	1	1	1	0	1	1	1	1
LN2	1	1	1	0	1	1	1	1
LN3	1	1	1	0	1	1	1	1
LN4	1	1	1	0	1	1	1	1

vi) After 1 year of storage and a recovery period

Isolate F3, EXT	RF 1	RF 2	RF 3	RF 4	RF 5	RF 6	RF 7	RF 8
Preservation treatment								
CS1	1	1	1	0	1	1	1	1
CS2	1	1	1	0	1	1	1	1
CS3	1	1	1	0	1	1	1	1
CS4	1	1	1	0	1	1	1	1
FD1	1	1	0	0	1	1	1	1
FD2	1	1	0	0	1	1	1	1
FD3	1	1	1	0	1	1	1	1
FD4	1	1	1	0	1	1	1	1
MP1	1	1	1	0	1	1	1	1
MP2	1	1	1	0	1	1	1	1
MP3	1	1	1	0	1	1	1	1
MP4	1	1	1	0	1	1	1	1
FZ1	1	1	1	0	1	1	1	1
FZ2	1	1	1	0	1	1	1	1
FZ3	1	1	1	0	1	1	1	1
FZ4	1	1	1	0	1	1	1	1
LN1	1	1	1	0	1	1	1	1
LN2	1	1	1	0	1	1	1	1
LN3	1	1	1	0	1	1	1	1
LN4	1	1	1	0	1	1	1	1

Intracellular secondary metabolite profile of *Fusarium oxysporum* isolate F3:

METABOLITE	Properties	Mean Rf	Number	S.E.
1	Y L/UV	5.9	120	0.1
2	Y L/UV	11.2	102	0.1
3	Y L/UV	16.0	112	0.2
4	AS P	41.0	81	0.6
5	AS P	46.6	90	0.4
6	Y L/UV	66.3	116	0.5
7	W/Y L/UV	91.8	96	0.3
8	TQ A/S	94.7	100	0.3
9	AS P	97.5	120	0.2

W= white, Y= yellow, P= purple, TQ= turquoise L/UV= long-wave ultraviolet light, A/S= after spray

i) After 1 week of storage

Isolate F3, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9
Preservation treatment									
CS1	1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1	1	1
CS3	1	1	1	1	1	1	1	1	1
CS4	1	1	1	0	0	1	1	1	1
FD1	1	1	1	1	1	1	1	1	1
FD2	1	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1	1
FD4	1	1	1	1	1	1	1	1	1
MP1	1	1	1	1	1	1	1	1	1
MP2	1	1	1	1	1	1	1	1	1
MP3	1	0	1	1	1	1	1	1	1
MP4	1	0	1	1	1	1	1	1	1
FZ1	1	1	1	1	1	1	1	1	1
FZ2	1	1	1	1	1	1	1	1	1
FZ3	1	0	1	1	1	1	1	1	1
FZ4	1	1	1	1	1	1	1	1	1
LN1	1	1	1	0	0	1	1	1	1
LN2	1	1	1	0	0	1	1	1	1
LN3	1	1	1	1	1	1	1	1	1
LN4	1	1	1	0	0	1	1	1	1

ii) After 1 week of storage and a recovery period

Isolate F3, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9
Preservation treatment									
CS1	1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1	1	1
CS3	1	1	1	1	1	1	1	1	1
CS4	1	1	1	1	1	1	1	1	1
FD1	1	1	1	1	1	1	1	1	1
FD2	1	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1	1

FD4	1	1	1	1	1	1	1	1	1
MP1	1	1	1	1	1	1	1	1	1
MP2	1	1	1	1	1	1	1	1	1
MP3	1	1	1	1	1	1	1	1	1
MP4	1	1	1	1	1	1	1	1	1
FZ1	1	1	1	1	1	1	1	1	1
FZ2	1	1	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	1	1	1	1
FZ4	1	1	1	1	1	1	1	1	1
LN1	1	1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1	1
LN4	1	1	1	1	1	1	1	1	1

iii) After 16 weeks of storage

Isolate F3, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9
Preservation treatment									
CS1	1	1	1	0	0	1	1	1	1
CS2	1	1	1	0	0	1	1	1	1
CS3	1	1	1	0	0	1	1	1	1
CS4	1	1	1	0	0	1	1	1	1
FD1	1	1	1	0	0	1	1	1	1
FD2	1	1	1	0	0	1	1	1	1
FD3	1	1	1	0	0	1	1	1	1
FD4	1	1	0	1	0	1	1	1	1
MP1	1	1	1	0	0	1	1	1	1
MP2	1	1	1	0	0	1	1	1	1
MP3	1	1	1	0	0	1	1	1	1
MP4	1	1	1	0	0	1	1	1	1
FZ1	1	1	0	1	0	0	0	1	1
FZ2	1	1	0	1	0	0	0	1	1
FZ3	1	1	0	1	0	0	0	1	1
FZ4	1	1	0	1	0	0	0	1	1
LN1	1	1	1	0	0	1	1	1	1
LN2	1	1	1	0	0	1	1	1	1
LN3	1	1	1	0	0	1	1	1	1
LN4	1	1	1	0	0	1	1	1	1

iii) After 16 weeks of storage and a recovery period

Isolate F3, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9
Preservation treatment									
CS1	1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1	1	1
CS3	1	1	1	1	1	1	1	1	1
CS4	1	1	1	1	1	1	1	1	1
FD1	1	1	1	1	1	1	1	1	1
FD2	1	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1	1
FD4	1	1	1	1	1	1	1	1	1
MP1	1	1	1	1	1	1	1	1	1

MP2	1	1	1	1	1	1	1	1	1
MP3	1	1	1	1	1	1	1	1	1
MP4	1	1	1	1	1	1	1	1	1
FZ1	1	1	1	1	1	1	1	1	1
FZ2	1	1	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	1	1	1	1
FZ4	1	1	1	1	1	1	1	1	1
LN1	1	1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1	1
LN4	1	1	1	1	1	1	1	1	1

v) After 1 year of storage

Isolate F3, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9
Preservation treatment									
CS1	1	0	1	0	1	1	0	0	1
CS2	1	0	1	0	1	1	0	0	1
CS3	1	0	1	0	1	1	0	0	1
CS4	1	0	1	0	1	1	0	0	1
FD1	1	0	1	0	1	1	0	0	1
FD2	1	0	1	0	1	1	0	0	1
FD3	1	0	1	0	1	1	0	0	1
FD4	1	0	1	0	1	1	0	0	1
MP1	1	0	1	0	1	1	0	0	1
MP2	1	0	0	0	0	1	0	0	1
MP3	1	0	1	0	1	1	0	0	1
MP4	1	0	1	0	1	1	0	0	1
FZ1	1	0	1	0	1	1	0	0	1
FZ2	1	0	0	0	0	1	0	0	1
FZ3	1	1	1	0	0	1	0	0	1
FZ4	1	0	0	0	1	1	0	0	1
LN1	1	1	1	0	1	1	0	0	1
LN2	1	1	1	0	0	1	0	0	1
LN3	1	1	1	0	0	1	0	0	1
LN4	1	1	1	0	0	1	0	0	1

vi) After 1 year of storage and a recovery period

Isolate F3, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9
Preservation treatment									
CS1	1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1	1	1
CS3	1	1	1	1	1	1	1	1	1
CS4	1	1	1	1	1	1	1	1	1
FD1	1	1	1	1	1	1	1	1	1
FD2	1	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1	1
FD4	1	1	1	1	1	1	1	1	1
MP1	1	1	1	1	1	1	1	1	1
MP2	1	1	1	1	1	1	1	1	1

MP3	1	1	1	1	1	1	1	1	1
MP4	1	1	1	1	1	1	1	1	1
FZ1	1	1	1	1	1	1	1	1	1
FZ2	1	1	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	1	1	1	1
FZ4	1	1	1	1	1	1	1	1	1
LN1	1	1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1	1
LN4	1	1	1	1	1	1	1	1	1

2) Metarhizium anisopliae

Extracellular Secondary Metabolite Profile of *Metarhizium anisopliae* isolate M1

METABOLITE	Properties	Mean Rf	Number	S.E.
1	Y L/UV	9.7	54	0.3
2	W S/UV	15.1	31	0.2
3	Y/O L/UV	24.7	49	0.3
4	Y L/UV	34.9	53	0.6

W= white, Y= yellow, O= orange, L/UV= long-wave ultraviolet light, S/UV= Short-wave ultraviolet light.

i) After 1 year of storage

Isolate M1, EXT	RF1	RF2	RF3	RF4
Preservation treatment				
CS1	1	0	1	1
CS2	1	0	1	1
CS3	1	0	1	1
CS4	1	1	1	1
CS5	1	0	1	1
FD1	1	0	1	1
FD2	1	1	1	1
FD3	1	1	1	1
MP1	1	1	1	1
MP2	1	0	1	1
MP3	1	0	1	1
MP4	1	0	1	1
MP5	1	0	1	1
LN1	1	1	1	1
LN2	1	1	0	1
LN3	1	1	1	1

ii) After 1 year of storage and a recovery period

Isolate M1, EXT	RF1	RF2	RF3	RF4
Preservation treatment				
CS1	1	1	1	1
CS2	1	1	1	1
CS3	1	1	1	1
CS4	1	1	1	1
CS5	1	1	1	1
FD1	1	1	1	1
FD2	1	1	1	1
FD3	1	1	1	1
MP1	1	1	1	1
MP2	1	0	0	1
MP3	1	1	1	1
MP4	1	0	1	1
MP5	1	0	1	1
LN1	1	1	1	1
LN2	1	1	1	1
LN3	1	1	1	1

iii) After 2 years of storage

Isolate M1, EXT	RF1	RF2	RF3	RF4
Preservation treatment				
FD1	1	1	1	1
FD2	1	1	1	1
FD3	1	1	1	1
FD4	1	1	0	1
FD5	1	1	1	1
MP1	1	1	1	1
MP3	1	1	0	1
MP5	1	1	0	1
E2	1	1	1	1
E4	1	1	1	1
E5	1	1	1	1

iv) After 2 years of storage and a recovery period

Isolate M1, EXT	RF1	RF2	RF3	RF4
Preservation treatment				
FD1	1	1	1	1
FD2	1	1	1	1
FD3	1	1	1	1
FD4	1	1	0	1
FD5	1	1	1	1
MP1	1	1	1	1
MP3	1	1	1	1
MP5	1	1	1	1
E2	1	1	1	1
E4	1	1	1	1
E5	1	1	1	1

Intracellular Secondary Metabolite Profiles of *Metarhizium anisopliae* isolate M1

METABOLITE	Properties	Mean Rf	Number	S.E.
1	Y L/UV	9.3	45	0.5
2	W S/UV	14.8	34	0.9
3	Y VIS / BR L/UV	42.6	34	0.2
4	Y/W L/UV	49.5	39	0.6
5	Y VIS / BR L/UV	73.2	28	0.6
6	Y/W L/UV	87.9	54	0.6

W= white, Y= yellow, BR= brown, L/UV= long-wave ultraviolet light, S/UV= Short-wave ultraviolet light, VIS= visible

i) After 1 year of storage

Isolate M1, INT	RF1	RF2	RF3	RF4	RF5	RF6
Preservation treatment						
CS1	1	0	0	0	1	1
CS2	1	0	0	0	1	1
CS3	1	0	0	0	1	1
CS4	1	0	0	0	0	1
CS5	1	0	0	0	1	1
FD1	1	1	0	0	1	1
FD2	0	1	0	0	0	1
FD3	1	1	0	0	1	1
MP1	1	0	0	1	0	1
MP2	1	0	0	0	0	1
MP3	1	0	0	0	0	1
MP4	1	0	0	0	0	1
MP5	1	1	0	0	0	1
LN1	1	1	1	1	1	1
LN2	1	1	1	1	1	1
LN3	1	1	1	1	1	1

ii) After 1 year of storage and a recovery period

Isolate M1, INT	RF1	RF2	RF3	RF4	RF5	RF6
Preservation treatment						
CS1	0	0	1	1	1	1
CS2	1	0	1	1	1	1
CS3	1	0	1	1	1	1
CS4	1	0	1	1	0	1
CS5	1	1	1	1	1	1
FD1	1	1	1	1	1	1
FD2	1	1	1	1	0	1
FD3	1	1	1	1	1	1
MP1	1	1	1	1	1	1
MP2	1	0	1	1	0	1
MP3	1	0	1	1	0	1
MP4	1	0	1	1	0	1
MP5	1	1	0	1	0	1
LN1	1	1	1	1	1	1
LN2	1	1	1	1	1	1
LN3	1	1	1	1	1	1

iii) After 2 years of storage

Isolate M1, INT	RF1	RF2	RF3	RF4	RF5	RF6
Preservation treatment						
FD1	0	0	1	1	0	1
FD2	1	0	0	1	1	1
FD3	0	1	1	1	0	1
FD4	1	1	0	1	1	1
FD5	0	1	1	1	0	1
MP1	1	1	1	1	1	1
MP3	1	1	1	1	0	1
MP5	1	1	0	1	0	1
E2	0	1	0	1	0	1
E4	0	1	1	1	0	1
E5	0	1	1	1	0	1

iv) After 2 years of storage and a recovery period

Isolate M1, INT	RF1	RF2	RF3	RF4	RF5	RF6
Preservation treatment						
FD1	1	1	1	1	1	1
FD2	1	1	0	1	1	1
FD3	1	1	1	1	1	1
FD4	1	1	0	1	1	1
FD5	1	1	1	1	1	1
MP1	1	1	1	1	1	1
MP3	1	1	1	1	1	1
MP5	1	1	1	1	1	1
E2	1	1	1	1	0	1
E4	0	1	1	1	0	1
E5	1	1	1	1	0	1

Extracellular secondary metabolite profile of *Metarhizium anisopliae* isolate M2

METABOLITE	Properties	Mean Rf	Number	S.E.
1	W/Y L/UV P AS	8.4	78	0.1
2	Y L/UV	16.8	75	0.3
3	Y L/UV	23.5	71	0.2
4	O/Y L/UV	33.1	75	0.2
5	W/Y S/UV	43.3	73	0.3

W= white, Y= yellow, L/UV= long-wave ultraviolet light, S/UV= Short-wave ultraviolet light,

i) After 1 year of storage

Isolate M2, EXT	RF1	RF2	RF3	RF4	RF5
Preservation treatment					
CS1	1	1	0	1	1
CS2	1	1	0	1	1
CS3	1	1	0	1	1
CS4	1	1	0	1	1
CS5	1	1	0	1	1
FD1	1	1	1	1	1
FD2	1	1	1	1	1
FD5	1	1	1	1	1
MP1	1	1	1	1	1
MP2	1	1	1	1	1
MP3	1	1	1	1	1

MP4	1	1	1	1	1
MP5	1	1	0	1	1
FZ1	1	1	1	0	1
FZ2	1	1	1	1	1
FZ3	1	1	1	1	1
LN1	1	1	1	1	1
LN2	1	1	1	1	1
LN3	1	0	1	0	1

ii) After 1 year of storage and a recovery period

Isolate M2, EXT	RF1	RF2	RF3	RF4	RF5
Preservation treatment					
CS1	1	1	1	1	1
CS2	1	1	1	1	1
CS3	1	1	1	1	1
CS4	1	1	1	1	1
CS5	1	1	1	1	1
FD1	1	1	1	1	1
FD2	1	1	1	1	1
FD5	1	1	1	1	1
MP1	1	1	1	1	1
MP2	1	1	1	1	1
MP3	1	1	1	1	1
MP4	1	1	1	1	1
MP5	1	1	0	0	0
FZ1	1	1	1	1	1
FZ2	1	1	1	1	1
FZ3	1	1	1	1	1
LN1	1	1	1	1	1
LN2	1	1	1	1	1
LN3	1	1	1	1	1

iii) After 2 years of storage

Isolate M2, EXT	RF1	RF2	RF3	RF4	RF5
Preservation treatment					
FD1	1	1	1	1	1
FD2	1	1	1	1	1
FD4	1	1	1	1	1
FD3	1	1	1	1	1
FD5	1	1	1	1	1
MP1	1	1	1	1	0
MP2	1	1	1	1	0
MP3	1	1	1	1	1
MP4	1	1	1	1	1
MP5	1	1	1	1	1
FZ1	1	1	1	1	1
FZ2	1	1	1	1	1
FZ3	1	1	1	1	1
FZ4	1	1	1	1	1
FZ5	1	1	1	1	1
LN1	1	1	1	1	1
LN2	1	1	1	1	1
LN3	1	1	1	1	1
LN5	1	1	1	1	1

iv) After 2 years of storage and a recovery period

Isolate M2, EXT	RF1	RF2	RF3	RF4	RF5
Preservation treatment					
FD1	1	1	1	1	0
FD2	1	1	1	1	0
FD3	1	1	1	1	1
FD4	1	1	1	1	1
FD5	1	1	1	1	1
MP1	1	1	1	1	1
MP2	1	0	1	1	1
MP3	1	1	1	1	1
MP4	1	0	1	1	1
MP5	1	1	1	1	1
FZ1	1	1	1	1	1
FZ2	1	1	1	1	1
FZ3	1	1	1	1	1
FZ4	1	1	1	1	1
FZ5	1	1	1	1	1
LN1	1	1	1	1	1
LN2	1	1	1	1	1
LN3	1	1	1	1	1
LN5	1	1	1	1	1

Intracellular secondary metabolite profiles of *Metarhizium anisopliae* isolate M2

METABOLITE	Properties	Mean Rf	Number	S.E.
1	Y/W L/UV	6.4	75	0.3
2	Y VIS BR L/UV	36.7	72	0.3
3	Y VIS BR L/UV	45.0	74	0.4
4	Y/W S/UV	65.3	76	0.6
5	O L/UV	79.6	77	1.3
6	BR L/UV	89.3	76	0.4
7	T L/UV	98.5	77	0.1

W= white, Y= yellow, O= orange, T= turquoise, BR= brown, L/UV= long wave ultraviolet, S/UV= short wave ultraviolet, VIS = Visible in white light

i) After 1 year of storage

Isolate M2, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7
Preservation treatment							
CS1	1	0	0	1	1	1	1
CS2	1	0	1	1	1	1	1
CS3	0	1	1	1	1	1	1
CS4	1	0	1	1	1	1	1
CS5	1	1	1	1	1	1	1
FD1	1	1	1	1	1	1	1
FD2	1	1	1	1	1	1	1
FD5	1	1	1	1	1	1	1
MP1	1	1	1	1	1	1	1
MP2	1	1	1	1	1	1	1
MP3	1	1	1	1	1	1	1
MP4	1	1	1	1	1	0	1
FZ1	1	1	1	1	1	1	1

FZ2	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	1	1
LN1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1

ii) After 1 year of storage and a recovery period

Isolate M2, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7
Preservation treatment							
CS1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1
CS3	1	1	1	1	1	1	1
CS4	1	1	1	1	1	1	1
CS5	1	1	1	1	1	1	1
FD1	0	0	1	0	1	1	1
FD2	1	1	1	1	1	1	1
FD5	1	1	1	1	1	1	1
MP1	1	1	1	1	1	1	1
MP2	1	1	1	1	1	1	1
MP3	1	1	1	1	1	1	1
MP4	1	1	0	1	1	1	1
MP5	1	1	0	1	1	1	1
FZ1	1	1	1	1	1	1	1
FZ2	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	1	1
LN1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1

iii) After 2 years of storage

Isolate M2, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	M
Preservation treatment								
FD1	1	1	1	1	1	1	1	0
FD2	1	1	1	1	1	1	1	0
FD3	1	1	1	1	1	1	1	0
FD4	1	1	1	1	1	1	1	1
FD5	1	1	1	1	1	1	1	0
MP1	1	1	1	1	1	1	1	1
MP2	1	1	1	1	1	1	1	0
MP3	1	1	1	1	1	1	1	0
MP4	1	1	1	1	1	1	1	0
MP5	1	1	1	1	1	1	1	1
FZ1	1	1	1	1	1	1	1	0
FZ2	1	1	1	1	1	1	1	0
FZ3	1	1	1	1	1	1	1	1
FZ4	1	1	1	1	1	1	1	1
FZ5	1	1	1	1	1	1	1	0
LN1	1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1
LN5	1	1	1	1	1	1	1	1

iv) After 2 years of storage and a recovery period

Isolate M2, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF 7	M
Preservation treatment								
FD1	1	1	1	1	1	1	1	1
FD2	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1
FD4	1	1	1	1	1	1	1	1
FD5	1	1	1	1	1	1	1	1
MP1	1	1	1	1	1	1	1	1
MP2	1	1	1	1	1	1	1	1
MP3	1	1	1	1	1	1	1	1
MP4	1	1	1	1	1	1	1	1
MP5	1	1	1	1	1	1	1	1
FZ1	1	1	1	1	1	1	1	1
FZ2	1	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	1	1	1
FZ4	1	1	1	1	1	1	1	1
FZ5	1	1	1	1	1	1	1	1
LN1	1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1

Extracellular secondary metabolite profiles of *Metarhizium* spp. isolate M4

METABOLITE	Properties	Mean Rf	Number	S.E.
1	W L/S UV P A/S	7.6	93	0.1
2	Y/W L/UV	11.0	92	0.1
3	W L/UV	16.3	87	0.2
4	Y L U/V P A/S	30.3	80	0.3
5	Y L/UV	39.5	93	0.3
6	P L/UV	49.8	79	0.3
7	P L/UV	55.7	80	0.5
8	P A/S	96.5	93	1.6

W= white, Y= yellow, P= PURPLE L/UV= long-wave ultraviolet light, S/UV= Short-wave ultraviolet light.

i) After 1 week of storage

Isolate M4, EXT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1	1
CS3	1	1	1	1	1	1	1	1
FD1	1	1	1	1	1	1	0	1
FD2	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1
FD4	0	0	0	1	0	0	0	1
FD5	1	1	1	1	1	1	1	1
MP1	1	1	0	0	1	1	1	1
MP2	1	1	1	1	1	1	1	1
MP5	1	1	1	1	1	0	0	1
FZ1	1	1	1	0	1	1	1	1
FZ2	1	1	1	1	1	1	1	1
FZ3	1	1	1	0	1	0	0	1
LN1	1	1	1	0	1	0	0	1
LN2	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1
LN4	1	1	1	1	1	1	1	1

ii) After 1 week of storage and a recovery period

Isolate M4, EXT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	1	1	1	1	1
CS2	1	1	0	1	1	1	0	1
CS3	1	1	1	1	1	1	1	1
CS4	1	1	1	1	1	1	1	1
FD1	1	1	1	1	1	1	0	1
FD2	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1
FD4	1	1	1	1	1	0	0	1
MP1	1	1	1	1	1	1	1	1
MP2	1	1	1	1	1	1	1	1
MP4	1	1	1	1	1	1	0	1
FZ1	1	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	1	1	1
FZ5	1	1	1	1	1	1	1	1
LN1	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1
LN4	1	1	1	1	1	1	1	1
LN5	1	1	1	1	1	1	1	1

iii) After 16 weeks of preservation

NO RESULTS

iv) After 16 weeks of storage and a recovery period

Isolate M4, EXT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1	1
CS3	1	1	0	0	1	1	0	1
CS4	1	1	1	1	1	1	1	1
FD1	1	1	1	0	1	0	0	1
FD2	1	1	1	1	1	1	1	1
FD5	1	1	1	1	1	1	1	1
MP1	1	1	1	1	1	0	0	1
MP2	1	1	1	1	1	1	1	1
MP4	1	1	1	1	1	1	1	1
FZ1	1	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	1	1	1
FZ5	1	1	1	1	1	1	1	1
LN1	1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1
LN4	1	1	1	1	1	1	1	1

v) After 1 year of storage

Isolate M4, EXT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1	1
CS3	1	1	1	1	1	1	1	1
CS4	1	1	1	1	1	1	1	1
CS5	1	1	1	1	1	1	1	1
FD1	1	1	1	1	1	0	0	1
FD2	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1
FD4	1	1	1	1	1	1	1	1
FD5	1	1	1	1	1	1	1	1
MP2	1	1	1	1	1	0	0	1
MP5	1	1	1	1	1	0	1	1
FZ1	1	1	1	1	1	1	1	1
FZ2	1	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	1	1	1
FZ4	1	1	1	1	1	1	1	1
FZ5	1	1	1	1	1	1	1	1
LN1	1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1
LN4	1	1	1	1	1	1	1	0
LN5	1	1	1	0	1	1	1	0

vi) After 1 year of storage and a recovery period

Isolate M4, EXT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8
Preservation treatment								
CS1	1	1	1	0	1	1	1	1
CS2	1	1	1	1	1	1	1	1
CS3	1	1	1	1	1	1	1	1
CS4	1	1	1	1	1	1	1	1
CS5	1	1	1	1	1	1	1	1
FD1	1	1	1	1	1	1	0	1
FD2	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1
FD4	1	1	1	1	1	1	1	1
FD5	1	1	1	1	1	1	1	1
MP2	1	1	1	1	1	1	1	1
MP5	1	0	1	0	1	1	1	1
FZ1	1	1	1	1	1	1	1	1
FZ2	1	1	1	0	1	1	1	1
FZ3	1	1	1	1	1	1	1	1
FZ4	1	1	1	1	1	1	1	1
FZ5	1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1
LN4	1	1	1	1	1	1	1	0
LN5	1	1	1	1	1	1	1	1

Intracellular secondary metabolite profile of *Metarhizium* spp. isolate M4

METABOLITE	Properties	Mean Rf	Number	S.E.
1	Y L/UV	4.6	116	0.1
2	Y/W L/UV	7.2	112	0.1
3	Y L/UV G/AS	10.2	115	0.1
4	Y L/UV	32.8	93	0.4
5	P L/UV	68.6	111	0.2
6	P L/UV	74.7	104	0.2
7	L/UV	84.2	112	0.2
8	G A/S Y L/UV	90.6	109	0.1
9	T A/S	96.4	117	0.2

W= white, Y= yellow, P= purple, G=green L/UV= long-wave ultraviolet light, A/S=after spray,

i) After 1 week of preservation

Isolate M4, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9
Preservation treatment									
CS1	1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1	1	1
CS3	1	1	1	1	1	1	1	1	1
FD1	1	1	1	1	1	0	1	1	1
FD2	1	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1	1
FD4	1	1	1	0	0	0	0	1	1
FD5	1	1	1	1	1	1	1	1	1
MP1	1	1	1	1	1	1	1	1	1
MP2	1	1	1	1	1	0	1	1	1
MP5	1	1	1	1	1	0	1	1	1
FZ1	1	1	1	1	1	1	1	1	1
FZ2	1	1	1	1	1	0	1	1	1
FZ3	1	1	1	1	1	0	1	1	1
LN2	1	1	1	0	0	0	1	1	1
LN3	1	1	1	1	1	1	1	1	1
LN4	1	1	1	1	1	0	1	1	1
LN5	1	1	1	1	1	1	1	1	1

ii) After 1 week of storage and a recovery period

Isolate M4, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9
Preservation treatment									
CS1	1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	0	1	1	1	1
CS3	1	1	1	1	1	1	1	1	1
CS4	1	1	1	1	1	1	1	1	1
FD2	1	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1	1
FD4	1	1	1	1	1	1	1	1	1
FD5	1	1	1	1	1	1	1	1	1
MP1	1	1	1	1	1	1	1	1	1
MP2	1	1	1	1	1	1	1	1	1
MP4	1	1	1	1	0	1	1	1	1
FZ3	1	1	1	1	1	1	1	1	1
FZ4	1	1	1	1	1	1	1	1	1
FZ5	1	1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1	1
LN4	1	1	1	1	1	1	1	1	1
LN5	1	1	1	1	1	1	1	1	1

iii) After 16 weeks of preservation

Isolate M4, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9
Preservation treatment									
CS1	1	1	1	0	1	1	1	1	1
CS2	1	0	1	0	1	1	1	1	1
CS3	1	0	1	0	1	1	1	1	1
CS4	1	1	1	0	1	1	1	1	1
CS5	1	1	1	0	1	1	1	1	1
FD1	1	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1	1
FD5	1	1	1	1	1	1	1	1	1
MP2	1	1	1	1	1	1	1	1	1
MP4	1	1	1	0	1	1	1	1	1
MP5	1	1	1	0	1	1	1	1	1
FZ1	1	0	1	1	1	1	1	1	1
FZ2	1	1	1	1	1	1	1	1	1
FZ3	1	0	1	1	1	1	1	1	1
FZ4	1	1	1	1	1	1	1	1	1
LN1	1	1	1	0	1	1	1	1	1
LN2	1	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1	1
LN5	1	1	1	1	1	1	1	1	1

iv) After 16 weeks of storage and a recovery period

Isolate M4, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9
Preservation treatment									
CS1	1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1	1	1
CS3	1	1	1	1	1	1	1	1	1
CS4	1	1	1	1	1	1	1	1	1
CS5	1	1	1	1	1	1	0	1	1
FD1	1	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1	1
FD5	1	1	1	1	1	1	1	1	1
MP2	1	1	1	1	1	1	1	1	1
MP4	1	1	1	1	1	1	1	1	1
MP5	1	1	1	1	1	1	1	1	1
FZ1	1	0	1	1	1	1	1	1	1
FZ2	1	1	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	1	1	1	1
FZ4	1	1	1	1	1	1	1	1	1
LN1	1	1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1	1
LN5	1	1	1	1	1	1	1	1	1

v) After 1 year of storage

Isolate M4, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9
Preservation treatment									
CS1	1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1	1	1
CS3	1	1	1	0	1	1	1	1	1
CS4	1	1	1	0	1	1	1	1	1
CS5	1	1	1	0	1	1	1	1	1
FD1	1	1	1	0	1	1	1	1	1
FD2	1	1	1	0	1	1	1	1	1
FD3	1	1	1	0	1	1	1	1	1
FD4	1	1	0	0	1	1	1	1	1
FD5	1	1	1	1	1	1	1	1	1
MP2	1	1	1	0	1	0	1	1	1
MP5	1	1	1	0	1	0	1	1	1
FZ1	1	1	1	0	1	0	1	1	1
FZ2	1	1	1	1	1	1	1	1	1
FZ3	1	1	1	0	1	1	1	1	1
FZ4	1	1	1	0	1	1	1	1	1
FZ5	1	1	1	1	1	0	1	1	1
LN1	1	1	1	1	1	0	1	1	1
LN2	1	1	1	1	1	1	1	1	1
LN3	1	1	1	1	1	1	1	1	1
LN4	1	1	1	0	1	1	1	1	1
LN5	1	1	1	0	1	1	1	1	1

vi) After 1 year of storage and a recovery period

Isolate M4, INT	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9
Preservation treatment									
CS1	1	1	1	1	1	1	1	1	1
CS2	1	1	1	1	1	1	1	1	1
CS3	1	1	1	1	1	1	1	1	1
CS4	1	1	1	1	1	1	1	1	1
CS5	1	1	1	1	1	1	1	0	1
FD1	1	1	1	1	1	1	1	1	1
FD2	1	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1	1
FD4	1	1	1	1	1	1	0	0	1
FD5	1	1	1	1	1	1	1	1	1
MP2	1	1	1	1	1	1	1	1	1
MP5	1	1	1	1	1	1	1	1	1
FZ1	1	1	1	1	1	1	1	1	1
FZ2	1	1	1	1	1	1	1	1	1
FZ3	1	1	1	1	1	1	1	1	1
FZ4	1	1	1	1	1	1	1	1	1
FZ5	1	1	1	1	1	1	1	1	1
LN2	1	1	1	1	1	1	1	0	1
LN3	1	1	1	1	1	1	1	0	1
LN4	1	1	1	1	1	1	1	0	1
LN5	1	1	1	1	1	1	1	1	1

3) High Performance Liquid Chromatography

i) Secondary Metabolite profile compiled from HPLC analysis of Fusarium oxysporum isolate F3 after 16 weeks of preservation

Peak No	Time eluted from column (min)♦
1	8.5
2	20.0
3	23.0
4	24.0
5	25.0
6	26.5
7	29.0
8	29.5
9	32.0
10	34.5
11	35.5
12	38.0
13	42.0
14	43.5
15	53.5
16	57.5
17	59.0
18	65.5
19	68.0
20	69.0
21	70.0
22	72.5
23	75.0
24	76.0

♦ major peaks to the nearest half-minute

Binary matrix of HPLC profiles, 1= peak, 0= absent.

Peak	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Preservation treatment																								
CS2	1	1	1	1	1	1	0	1	1	1	0	0	0	1	0	1	0	0	1	0	0	0	1	1
CS3	1	1	1	1	0	1	0	0	1	0	0	0	0	1	1	1	0	1	1	0	0	0	1	1
CS4	1	1	1	1	0	1	1	1	1	1	0	0	0	1	1	1	1	0	1	1	1	0	1	1
FD1	1	1	1	1	1	1	0	1	1	1	0	0	0	1	0	1	0	1	1	0	0	0	1	1
FD2	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0	1	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	0	1	1	1	0	0	1	1	0	1	1	0	1	0	1	1	1	1
MP1	1	1	1	1	1	1	0	0	1	0	0	0	1	1	0	0	0	1	1	0	0	1	1	1
MP2	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	0	1	1	0	0	0	1	1
MP3	1	1	1	1	1	1	1	0	0	0	0	1	1	1	0	1	0	1	1	0	0	0	1	1
FZ1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	0	1	0	0	1	1	1
FZ2	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	0	0	1	0	0	0	1	1
FZ3	1	1	1	1	1	1	0	0	1	1	0	0	1	1	1	0	0	1	1	0	0	0	1	1
LN1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	1	1
LN2	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	0	1	0	0	0	1	1
LN3	1	1	1	1	1	1	0	0	1	1	1	0	1	1	0	1	0	0	1	0	0	0	1	1

ii) Secondary Metabolite profile compiled from HPLC analysis of *Metarhizium* spp. isolate M4 after 16 weeks of preservation

Peak No	Time eluted from column (min)◆
1	11.5
2	12.5
3	13.5
4	16.0
5	17.0
6	18.0
7	18.5
8	20.5
9	21.5
10	22.0
11	23.0
12	24.0
13	25.5
14	26.0
15	27.5
16	28.0
17	29.0
18	30.5
19	31.0
20	32.0
21	32.5
22	33.5
23	34.5
24	35.5
25	37.5
26	38.0
27	39.5
28	40.0
29	41.0
30	42.0
31	43.5
32	44.0
33	45.0
34	47.0
35	50.5
36	52.0
37	53.5
38	55.0
39	55.5

◆ major peaks to the nearest half-minute

Binary matrix of HPLC profiles, 1= peak, 0= absent

Metabolite no	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
Preservation treatment																																								
CS1	1	1	0	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1
CS3	1	1	0	1	1	0	1	0	0	1	0	1	1	0	1	1	1	0	0	1	1	1	0	1	1	1	0	1	1	1	1	0	1	0	1	1	1	1	1
CS5	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
FD2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
FD3	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
FD5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
MP2	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0
MP4	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	0	0	1	1
MP5	1	1	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0
FZ1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0
FZ3	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0
FZ4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	0
LN2	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0
LN3	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0
LN5	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0

APPENDIX D

Extracellular enzyme profiles

Binary matrices compiled from APIZYM and 4MU enzyme profiles

1) ***Fusarium oxysporum***: Pages lvi-lxii
2) ***Metarhizium anisopliae***: Pages lxii-lxvii

Where 1= enzyme detected, 0= enzyme not detected

I.D.	ENZYME
1	Alkaline phosphatase
2	Esterase
3	Esterase Lipase
4	Lipase
5	Leucine arylamidase
6	Valine arylamidase
7	Cysteine arylamidase
8	Trypsin
9	Chymotrypsin
10	Acid phosphatase
11	Phosphoamidase
12	α-galactosidase
13	β-galactosidase
14	β-glucuronidase
15	α-glucosidase
16	β-glucosidase
17	β-glucosamidase
18	α-mannosidase
19	α-fucosidase
20	α-arabinofuranosidase
21	β-chitobiosidase
22	β-xylosidase

1) *Fusarium oxysporum*

i) *Fusarium oxysporum* isolate F1: After 1 day of storage

ENZYME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Preservation treatment																			
CS1	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0
CS2	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0
CS3	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0
FD1	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0
FD2	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0
FD3	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0
MP1	0	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	0	1	0
MP2	1	1	1	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1	0
MP3	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0
FZ1	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0
FZ2	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0
FZ3	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0
LN1	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0
LN2	0	1	1	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1	0
LN3	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0

ii) *Fusarium oxysporum* isolate F1: After 1 year of storage

ENZYME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Preservation treatment																						
CS1	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	0
CS2	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	0
CS3	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	0
CS4	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	0
CS5	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	0
FD1	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	1	1
FD2	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	1	1
FD3	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	0
MP1	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	1
MP2	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	0
MP3	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	0
MP4	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	1	0
MP5	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	1	0
FZ1	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	0
FZ2	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	0
FZ3	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	1	1
LN1	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	1	0
LN2	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	1	1
LN3	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	1	1

iii) *Fusarium oxysporum* isolate F1: After 2 years of storage

ENZYME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Preservation treatment																						
CS1	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	0	0	1	1	1
CS2	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	0	0	1	1	1
CS3	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	0	0	1	1	0
CS4	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	0	0	1	1	0
CS5	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	0	0	1	1	1
FD1	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	1
FD2	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	0
FD3	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	1
FD4	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	1
FD5	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	1
MP1	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	0	0	1	1	0
MP2	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	0	0	1	1	0
MP3	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	0	0	1	1	0
MP4	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	0	0	1	1	0
MP5	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	0	0	1	1	0
FZ1	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	1
FZ2	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	0
FZ3	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	0
FZ4	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	0	0	0	1	1	0
FZ5	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	1
LN1	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	1
LN2	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	1
LN3	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	1
LN4	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	1
LN5	1	1	1	0	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	1

iv) *Fusarium oxysporum* isolate F2 : After 1 day of storage

ENZYME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Preservation treatment																			
CSC1	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0
CSC2	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0
CSC3	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0
FD1	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0
FD2	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0
FD3	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0
MP1	0	1	1	0	0	1	0	0	0	1	1	1	0	0	0	1	1	1	0
MP2	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0
MP3	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0
FZ1	0	1	1	0	0	1	0	0	0	1	1	1	0	0	0	1	1	1	0
FZ2	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0
FZ3	0	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0
LN1	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0
LN2	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0
LN3	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0

v) *Fusarium oxysporum* isolate F2 : After 1 year of storage

Enzyme	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Preservation treatment																						
CSC1	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
CSC2	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
CSC3	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
FD1	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
FD2	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
FD3	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
MP1	1	1	1	1	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	1	1	1
MP2	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
MP3	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
FZ1	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
FZ2	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
FZ3	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
LN1	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
LN2	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
LN3	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1

vi) *Fusarium oxysporum* isolate F2 : After 2 years of storage

ENZYME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Preservation treatment																						
CS1	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
CS2	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
CS3	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
CS4	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
CS5	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
FD1	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
FD2	1	1	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
FD3	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	0	1	0
FD4	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
FD5	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
MP1	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
MP2	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
MP3	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
MP4	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
MP5	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
FZ1	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	0	0	0
FZ2	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	0	0	1
FZ3	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	0	0	1
FZ4	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
FZ5	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
LN1	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
LN2	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
LN3	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
LN4	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1
LN5	1	1	1	0	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	1	1	1

vii) *Fusarium oxysporum* isolate F3 : After 1 week of storage

ENZYME	17	20	21	19	13	16	14	18	22	2
Preservation treatment										
CS1	1	0	1	0	0	1	0	1	1	1
CS2	1	0	1	0	0	1	0	1	1	1
CS3	1	0	1	0	0	1	0	1	1	1
CS4	1	0	1	0	0	1	0	1	1	1
CS5	1	0	1	0	0	1	0	1	1	1
FD1	1	0	1	0	0	1	0	1	1	1
FD2	1	0	1	0	0	1	0	1	1	1
FD3	1	0	1	0	0	1	0	1	1	1
FD4	1	0	1	0	0	1	0	1	1	1
FD5	1	0	1	0	0	1	0	1	1	1
MP1	1	0	1	0	0	1	0	1	1	1
MP2	1	0	1	0	0	1	0	1	1	1
MP3	0	0	1	0	0	1	0	1	1	1
MP4	1	0	1	0	0	1	0	1	1	1
MP5	1	0	1	0	0	1	0	1	1	1
FZ1	1	0	1	0	0	1	0	1	1	1
FZ2	1	0	1	0	0	1	0	1	1	1
FZ3	1	0	1	0	0	1	0	1	1	1
FZ4	1	0	1	0	0	1	0	1	1	1
FZ5	1	0	1	0	0	1	0	1	1	1
LN1	1	0	1	0	0	1	0	1	1	1
LN2	1	0	1	0	0	1	0	1	1	1
LN3	1	0	1	0	0	1	0	1	1	1
LN4	1	0	1	0	0	1	0	0	1	1
LN5	1	0	1	0	0	1	0	1	0	1

viii) *Fusarium oxysporum* isolate F3 : After 16 weeks of storage

Enzyme	17	20	21	19	13	16	14	18	22	2
Preservation treatment										
CS1	1	0	1	0	0	1	0	1	1	1
CS2	1	0	1	0	0	1	0	1	1	1
CS3	1	0	1	0	0	1	0	1	1	1
CS4	1	0	1	0	0	1	0	1	1	1
CS5	1	0	1	0	0	1	0	1	1	1
FD1	1	0	1	0	0	1	0	1	1	1
FD2	1	0	1	0	0	1	0	1	1	1
FD3	1	0	1	0	0	1	0	1	1	1
FD4	1	0	1	0	0	1	0	1	1	1
FD5	1	0	1	0	0	1	0	1	1	1
MP1	1	0	1	0	0	1	0	1	1	1
MP2	1	0	1	0	0	1	0	1	1	1
MP3	1	0	1	0	0	1	0	0	1	1
MP4	1	0	1	0	0	1	0	1	1	1
MP5	1	0	1	0	0	1	0	1	1	1
FZ1	1	0	1	0	0	1	0	1	1	1

FZ2	1	0	1	0	0	1	0	1	1	1
FZ3	1	0	1	0	0	1	0	1	1	1
FZ4	1	0	1	0	0	1	0	1	1	1
FZ5	1	0	1	0	0	1	0	1	1	1
LN1	1	0	1	0	0	1	0	1	1	1
LN2	1	0	1	0	0	1	0	1	1	1
LN3	1	0	1	0	0	1	0	0	1	1
LN4	1	0	1	0	0	1	0	1	1	1
LN5	1	0	1	0	0	1	0	1	1	1

viii)*Fusarium oxysporum* isolate F3 : After 1year of storage

	17	20	21	19	13	16	14	18	22	2
Preservation treatment										
CS1	1	0	1	0	0	1	0	1	1	1
CS2	1	0	1	0	0	1	0	1	1	1
CS3	1	0	1	0	0	1	0	1	1	1
CS4	0	0	0	0	0	1	0	1	1	1
CS5	1	0	1	0	0	1	0	1	1	1
FD1	1	0	1	0	0	1	0	1	1	1
FD2	1	0	1	0	0	1	0	1	1	1
FD3	1	0	1	0	0	1	0	1	1	1
FD4	1	0	1	0	0	1	0	1	1	1
FD5	1	0	1	0	0	1	0	1	1	0
MP1	1	0	1	0	0	1	0	1	1	1
MP2	1	0	1	0	0	1	0	1	1	1
MP3	1	0	1	0	0	1	0	1	1	1
MP4	1	0	1	0	0	1	0	1	1	1
MP5	1	0	1	0	0	1	0	1	1	1
FZ1	1	0	1	0	0	1	0	1	1	1
FZ2	1	0	1	0	0	1	0	1	1	1
FZ3	1	0	1	0	0	1	0	1	1	1
FZ4	1	0	1	0	0	1	0	1	1	1
FZ5	1	0	1	0	0	1	0	1	1	1
LN1	1	0	1	0	0	1	0	1	1	1
LN2	1	0	1	0	0	1	0	1	1	1
LN3	1	0	1	0	0	1	0	1	1	1
LN4	1	0	1	0	0	1	0	1	1	1
LN5	1	0	1	0	0	1	0	1	1	1

ix) *Fusarium oxysporum* isolate F3 : After 1 year of storage and a recovery period

ENZYME	17	20	21	19	13	16	14	18	22	2
Preservation treatment										
CS1	1	0	1	0	0	1	0	1	1	1
CS2	1	0	1	0	0	1	0	1	1	1
CS3	1	0	1	0	0	1	0	1	1	1
CS4	0	0	1	0	0	1	0	1	1	1
CS5	1	0	1	0	0	1	0	1	1	1
FD1	1	0	1	0	0	1	0	1	1	1
FD2	1	0	1	0	0	1	0	1	1	1
FD3	1	0	1	0	0	1	0	1	1	1
FD4	1	0	1	0	0	1	0	1	1	1
FD5	1	0	1	0	0	1	0	1	1	1
MP1	1	0	1	0	0	1	0	1	1	1
MP2	1	0	1	0	0	1	0	1	1	1
MP3	1	0	1	0	0	1	0	1	1	1
MP4	1	0	1	0	0	1	0	1	1	1
MP5	1	0	1	0	0	1	0	1	1	1
FZ1	1	0	1	0	0	1	0	1	1	1
FZ2	1	0	1	0	0	1	0	1	1	1
FZ3	1	0	1	0	0	1	0	1	1	1
FZ4	1	0	1	0	0	1	0	1	1	1
FZ5	1	0	1	0	0	1	0	1	1	1
LN1	1	0	1	0	0	1	0	1	1	1
LN2	1	0	1	0	0	1	0	1	1	1
LN3	1	0	1	0	0	1	0	1	1	1
LN4	1	0	1	0	0	1	0	1	1	1
LN5	1	0	1	0	0	1	0	1	1	1

2) *Metarhizium anisopliae*

i) *Metarhizium anisopliae* isolate M1: After 1 year of storage

ENZYME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Preservation treatment																						
CS1	0	1	1	0	1	1	1	0	0	1	1	1	0	0	0	1	1	0	1	0	1	0
CS2	0	1	1	0	0	0	0	0	0	1	1	0	1	0	0	1	1	0	1	0	1	1
CS3	1	1	1	0	1	1	1	0	0	1	1	0	0	0	1	1	1	0	1	0	1	0
FD1	1	1	1	0	1	1	0	0	0	1	1	1	1	0	0	1	1	0	1	0	1	0
FD2	0	1	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	0	1	0	1	1
FD3	0	1	1	0	0	0	0	0	0	1	1	0	1	0	0	1	1	0	1	0	1	1
MP1	0	1	0	0	0	0	0	0	0	1	1	1	1	0	0	1	1	0	1	0	1	0
MP2	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	1	1	0	1	0	1	0
MP3	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	1	1	0	1	0	1	0
LN1	0	1	1	0	1	0	1	0	0	1	1	1	1	0	1	1	1	0	1	0	1	0
LN2	0	1	1	0	0	0	0	0	0	1	1	0	1	0	0	1	1	0	1	0	1	1
LN3	0	1	1	0	0	0	0	0	0	1	1	0	1	0	0	1	1	0	1	0	1	1

ii) *Metarhizium anisopliae* isolate M1: After 2 years of storage

ENZYME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Preservation treatment																						
FD1	1	1	1	0	1	1	1	1	0	1	1	1	1	0	1	1	1	0	1	0	1	0
FD2	0	1	1	0	1	0	0	0	0	1	1	1	1	0	1	1	1	0	1	0	1	0
FD3	0	1	1	0	0	0	0	0	0	1	1	0	1	0	0	1	1	0	1	0	1	1
FD4	0	1	1	0	1	1	0	1	0	1	1	1	1	0	1	1	1	0	1	0	1	0
FD5	0	1	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	0	1	0	1	0
MP1	0	1	1	0	1	1	0	1	0	1	1	1	1	0	1	1	1	0	1	0	1	0
MP2	0	1	1	0	1	1	0	1	0	1	1	1	1	0	1	1	1	0	1	0	1	0
MP3	0	1	1	0	1	0	0	0	0	1	1	1	1	0	1	1	1	0	1	0	1	0
MP4	0	1	1	0	1	1	0	1	0	1	1	1	1	0	1	1	1	0	1	0	1	0
MP5	1	1	1	0	1	1	0	0	0	1	1	1	1	0	1	1	1	0	1	0	1	0
LN1	0	1	0	0	1	0	0	0	0	1	1	1	1	0	1	1	1	0	1	0	1	0
LN2	1	1	1	0	1	0	0	0	0	1	1	1	1	0	0	1	1	0	0	0	1	1
LN3	0	1	1	0	0	0	0	0	0	1	1	0	1	0	0	1	1	0	0	1	0	0
LN4	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	1	1	0	1	1	0	1
LN5	0	1	1	0	0	0	0	0	0	1	1	0	1	0	1	1	1	0	1	1	0	0

iii) *Metarhizium anisopliae* isolate M2: After 1 day of storage

ENZYME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Preservation treatment																			
CS1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1
CS2	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1
CS3	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1
FD1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1
FD2	1	1	0	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1
FD3	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1
MP1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1
MP2	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1
MP3	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1
FZ1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1
FZ2	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0	1	1	0	0
FZ3	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1
LN1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1
LN2	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1
LN3	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1

iv) *Metarhizium anisopliae* isolate M2: After 1 year of storage

ENZYME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Preservation treatment																						
CS1	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0
CS2	1	1	1	0	0	0	0	0	0	1	1	0	1	1	0	1	1	0	1	0	1	0
CS3	1	1	1	0	0	0	0	0	0	1	1	0	1	1	0	1	1	0	1	0	1	0
CS4	1	1	1	0	0	0	0	0	0	1	1	0	1	1	0	1	1	0	1	0	1	0
CS5	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
FD1	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
FD2	1	1	1	0	0	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0
FD3	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
MP1	1	1	1	0	0	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0
MP2	1	1	1	0	0	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0
MP3	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
MP4	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
MP5	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
FZ1	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
FZ2	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
FZ3	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
LN1	1	1	1	0	0	0	0	0	0	1	1	1	0	0	0	1	1	0	0	0	0	0
LN2	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
LN3	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0

v) *Metarhizium anisopliae* isolate M2: After 2 years of storage

ENZYME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Preservation treatment																						
FD1	1	1	1	0	1	1	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0
FD2	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
FD3	1	1	1	0	1	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0
FD4	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
FD5	0	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
MP1	1	1	1	0	1	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0
MP2	1	1	1	0	1	1	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0
MP3	1	1	1	0	1	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0
MP4	1	1	1	0	1	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0
MP5	1	1	1	0	1	1	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0
FZ1	1	1	1	0	1	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0
FZ2	1	1	1	0	1	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0
FZ3	0	1	1	0	1	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0
FZ4	1	1	1	0	1	1	0	1	0	1	1	1	0	1	0	1	1	0	1	0	1	0
FZ5	1	1	1	0	1	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0
LN1	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
LN2	1	1	1	0	1	1	0	1	0	1	1	1	0	1	0	1	1	0	1	0	1	0
LN3	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
LN4	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	0	1	0
LN5	0	1	1	0	0	0	0	0	0	1	1	0	1	0	0	1	1	0	1	0	1	0

vi) *Metarhizium* spp. isolate M4 : After 1 week of storage

ENZYME	17	20	21	19	13	16	14	18	22	2
Preservation treatment										
CS1	1	0	1	1	1	1	1	0	0	1
CS2	1	0	1	1	1	1	1	0	0	1
CS3	1	0	1	1	1	1	1	0	0	1
CS4	1	0	1	1	1	1	1	0	0	1
CS5	1	0	1	1	1	1	1	0	0	1
FD1	1	0	1	1	1	1	1	0	0	1
FD2	1	0	1	1	1	1	1	0	0	1
FD3	1	0	1	1	1	1	1	0	0	1
FD4	1	0	1	1	1	1	1	0	0	1
FD5	1	0	1	1	1	1	1	0	0	1
MP1	1	0	1	1	1	1	1	0	0	1
MP2	1	0	1	1	1	1	1	0	0	1
MP3	1	0	1	1	1	1	1	0	0	1
MP4	1	0	1	1	1	1	1	0	0	1
MP5	1	0	1	1	1	1	1	0	0	1
FZ1	1	0	1	1	1	1	1	0	0	1
FZ2	1	0	1	0	0	1	0	0	0	1
FZ3	1	0	1	1	1	1	1	0	0	1
FZ4	1	0	1	1	1	1	1	0	0	1
FZ5	1	0	1	1	1	1	1	0	0	1
LN1	1	0	1	1	1	1	1	0	0	1
LN2	1	0	1	1	1	1	1	0	0	1
LN3	1	0	1	1	1	1	1	0	0	1
LN4	1	0	1	1	1	1	1	0	0	1
LN5	1	0	1	1	1	1	1	0	0	1

vii) *Metarhizium* spp. isolate M4 : After 16 weeks of storage

ENZYME	17	20	21	19	13	16	14	18	22	2
Preservation treatment										
CS1	1	0	1	1	1	1	1	0	0	1
CS2	1	0	1	1	1	1	1	0	0	1
CS3	1	0	1	0	1	1	0	0	0	1
CS4	1	0	1	1	1	1	1	0	0	1
CS5	1	0	1	1	1	1	1	0	0	1
FD1	1	0	1	1	1	1	1	0	0	1
FD2	1	0	1	1	1	1	1	0	0	1
FD3	1	0	1	1	1	1	1	0	0	1
FD4	1	0	1	1	1	1	1	0	0	1
FD5	1	0	1	1	1	1	1	0	0	1
MP1	1	0	1	1	1	1	1	0	0	1
MP2	1	0	1	1	1	1	1	0	0	1
MP3	1	0	1	1	1	1	1	0	0	1
MP4	1	0	1	0	1	1	0	0	0	1
MP5	1	0	1	0	1	1	0	0	0	1
FZ1	1	0	1	1	1	1	1	0	0	1

FZ2	1	0	1	1	0	1	0	0	0	1
FZ3	1	0	1	1	1	1	1	0	0	1
FZ4	1	0	1	1	1	1	1	0	0	1
FZ5	1	0	1	1	1	1	1	0	0	1
LN1	1	0	1	1	1	1	1	0	0	1
LN2	1	0	1	1	1	1	1	0	0	1
LN3	1	0	1	1	1	1	1	0	0	1
LN4	1	0	1	1	1	1	1	0	0	1
LN5	1	0	1	1	1	1	1	0	0	1

viii) *Metarhizium* spp. isolate M4 : After 1 year of storage

ENZYME	17	20	21	19	13	16	14	18	22	2
Preservation treatment										
CS1	1	0	1	1	0	1	1	0	0	1
CS2	1	0	1	1	1	1	1	0	0	1
CS3	1	0	1	1	1	1	1	0	0	1
CS4	1	0	1	1	1	1	1	0	0	1
CS5	1	0	1	1	0	1	1	0	0	1
FD1	1	0	1	1	1	1	1	0	0	1
FD2	1	0	1	1	0	1	1	0	0	1
FD3	1	0	1	1	1	1	1	0	0	1
FD4	1	0	0	1	1	1	1	0	0	1
FD5	1	0	1	1	0	1	1	0	0	1
MP1	1	0	1	1	0	1	1	0	0	1
MP2	1	0	1	1	1	1	1	0	0	1
MP3	1	0	1	1	1	1	1	0	0	1
MP4	1	0	1	1	1	1	1	0	0	1
MP5	1	0	1	1	1	1	1	0	0	1
FZ1	1	0	1	1	1	1	1	0	0	1
FZ2	1	0	1	1	0	1	1	0	0	1
FZ3	1	0	0	1	1	1	1	0	0	1
FZ4	1	0	0	1	1	1	1	0	0	1
FZ5	1	0	0	1	0	1	1	0	0	1
LN1	1	0	1	1	1	1	1	0	0	1
LN2	1	0	0	1	1	1	1	0	0	1
LN3	1	0	0	1	1	1	1	0	0	1
LN4	1	0	1	1	1	1	1	0	0	1
LN5	1	0	0	1	1	1	1	0	0	1

ix) *Metarhizium* spp. isolate M4 : After 1 year of storage and a recovery period

ENZYME	17	20	21	19	13	16	14	18	22	2
Preservation treatment										
CS1	1	0	1	1	1	1	1	0	0	1
CS2	1	0	1	1	1	1	1	0	0	1
CS3	1	0	1	1	1	1	1	0	0	1
CS4	1	0	1	1	1	1	1	0	0	1
CS5	1	0	1	1	1	1	1	0	0	1
FD1	1	0	1	1	1	1	1	0	0	1
FD2	1	0	1	1	1	1	1	0	0	1
FD3	1	0	1	1	1	1	1	0	0	1
FD4	1	0	0	1	1	1	1	0	0	1
FD5	1	0	1	1	1	1	1	0	0	1
MP1	1	0	1	1	1	1	1	0	0	1
MP2	1	0	1	1	1	1	1	0	0	1
MP3	1	0	1	1	1	1	1	0	0	1
MP4	1	0	1	1	1	1	1	0	0	1
MP5	1	0	1	1	1	1	1	0	0	1
FZ1	1	0	1	1	1	1	1	0	0	1
FZ2	1	0	1	1	1	1	1	0	0	1
FZ3	1	0	0	1	1	1	1	0	0	1
FZ4	1	0	0	1	1	1	1	0	0	1
FZ5	1	0	0	1	1	1	1	0	0	1
LN1	1	0	1	1	1	1	1	0	0	1
LN2	1	0	0	1	1	1	1	0	0	1
LN3	1	0	0	1	1	1	1	0	0	1
LN4	1	0	1	1	1	1	1	0	0	1
LN5	1	0	1	1	1	1	1	0	0	1

APPENDIX E

Appendix to chapter 7.

1) Comparison experiment

i) Radial growth data for cultures of *Metarhizium anisopliae* line M5
Regression analysis and analysis of variance

REGRESSION EQUATIONS

M5A CBS y=1.55x -0.62
M5B IMI y=1.40x -1.79
M5D MUCL y=1.17x -1.06

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	⇒	254.755	84.918	2160.72	0.04
Error	2	0.079	0.039		
Total	5	254.833			

All other data is presented within the chapter.

2) Sectorisation experiment

i) Binary matrix used to construct dendrogram (on page 202), compiled from the extracellular enzyme profiles of the lines of *Metarhizium* spp. isolate M4 derived from sectors.

	ENZYME ASSAYED FOR									
Line (replicate)	β-glucosamidase	α-arabinofuranosidase	β-chitobiosidase	α-fucosidase	β-glucosidase	β-galactosidase	β-glucuronidsidase	α-mannosidase	β-xylosidase	esterase
Bia 3	1	0	1	1	1	1	1	0	0	1
Bib 2	1	0	1	1	1	1	1	0	0	1
Biia 3	1	0	1	1	1	1	0	0	0	1
Ciii 4	1	0	1	1	0	1	0	0	0	1
Biib 2	1	0	1	1	1	1	1	0	0	1
Di 2	1	0	1	0	1	1	0	0	0	1
Dii 2	1	0	1	1	1	1	1	0	0	1
Diii 2	1	0	1	1	1	1	0	0	0	1
Ei 3	0	0	1	0	1	1	0	0	0	1
Eiii 3	1	0	1	0	1	1	0	0	0	1
Control	1	0	1	1	1	1	1	0	0	1

1= metabolite detected, 0= metabolite not detected. See chapter 7 (page 198) for line and replicate details. Control= original extracellular enzyme profile of isolate M4

ii) Binary matrix used to construct dendrogram (on page 203), compiled from the extracellular secondary metabolite profiles of the lines of *Metarhizium* spp. isolate M4 derived from sectors.

	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RFA	RFB	RF8
Line/rep										
Biia 3	1	1	1	1	1	1	0	1	1	1
Bia 3	1	1	1	1	1	1	0	1	1	1
Ciii 4	1	1	1	1	1	1	0	1	1	1
Ei 3	1	1	1	0	0	1	1	0	0	1
Bib 2	1	1	1	0	1	1	0	0	0	1
Biib 2	1	1	1	1	1	1	0	0	0	1
Di 2	1	1	0	1	0	1	1	0	0	1
Dii 2	1	1	1	1	0	1	1	0	0	1
Diii 2	1	1	1	1	0	1	1	0	0	1
Eiiib 2	1	1	1	1	1	1	1	0	0	1
Cont	1	1	1	1	1	1	1	0	0	1
Cont	1	1	1	1	1	1	1	0	0	1

RF's 1-8 refer to the original extracellular secondary metabolite profile of *Metarhizium* spp. isolate M4. Rf A and Rf B are additional secondary metabolites to the original profile (see chapter 7). 1= metabolite detected, 0= metabolite not detected. Cont= control replicate of isolate M4. See chapter 7 (page 198) for line and replicate details.

APPENDIX F

A Decision - based Key to Determine the Most Appropriate Protocol for the Preservation of Fungi

Running title: Decision-based key for fungal preservation

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Key words: Cryopreservation, Culture collection, Lyophilisation, Preservation, Sub-culture, Storage

Summary

The long-term preservation of valuable fungal cultures can be achieved in several ways, and the choice of methodology can be problematical. Firstly, there is the decision whether to use a public service culture collection or "in-house" facilities. Secondly, the wide variety of preservation methods available often leads to confusion about which protocol(s) are best suited for specific fungi. No method can be universally applied to all fungi. Some species are notoriously difficult to preserve, whilst other fungi can be preserved by almost any method. A decision-based key has been devised which uses questions related to fungal characters and user facilities and economics to determine the most appropriate method for long-term preservation of cultures. This key should facilitate the decisions of microbiologists when considering preservation of important fungal cultures.

Introduction

The development of fungal biotechnology has coincided with increased concern for the need to provide reliable and safe preservation and storage protocols for yeasts and filamentous fungi. Physiological or genetic damage to economically important strains, induced by stresses that occur during preservation and storage, could potentially result in considerable loss of income to the biotechnology industry or investment in a research programme. To ensure good practice during preservation and storage, guidelines for the establishment and operation of culture collections were produced by the World Federation For Culture Collections (WFCC), a federation of the International Union of Microbiological Societies (IUMS) (Hawksworth 1990). However, there is a need to help the biotechnologist assimilate the vast amount of information available in the literature, in order to assist them in the decision about how and where to preserve their fungi.

Several factors, such as economics, often have to be considered when fungi are to be preserved. Continual sub-culture, storage under oil (Kobayashi 1984, Smith and Onions 1994) or water (Burdsall 1994, Smith and Onions 1994), in soil (Smith and Onions 1994) or on silica gel (Elliot 1975, Smith and Onions 1994) are cost-effective and not very labour intensive. However, their suitability as protocols for longer-term storage is doubted and stability of characteristics may not be guaranteed. Lyophilisation (Tan 1997, Kolkowski and Smith 1995) and cryopreservation in liquid nitrogen (Hwang 1966, Smith 1998) are provided by all the major national culture collections and many industrial and university collections have the appropriate facilities. However, the equipment often requires substantial initial outlay and the methods are quite labour intensive. Cryopreservation demands a constant supply of liquid nitrogen which may be expensive and there is potential for disaster if the supply

is disrupted. It may be economically feasible for mycologists to get their strains preserved by a recognised collection, lyophilised cultures for example are easy to transport once preserved. However, where large numbers have to be preserved, it may be more cost effective and beneficial for institutions to purchase hardware and establish their own "in-house" collections. The security offered by major culture collections such as that at CABI Bioscience (formerly the International Mycological Institute (IMI)) and the back-up services it and the UK National Culture Collection (http//:ukncc.co.uk) provide are pre-eminent. However, longer-term financial and practical considerations may promote scientists to consider their own alternatives.

The advantages and disadvantages of different preservation techniques for fungi were summarised by Smith and Waller (1992) but did not include any species-specific information. No preservation method can be universally applied to all fungi and intraspecific variability makes it impossible to apply standard protocols, even at the species level. Cryopreservation, at temperatures below –196°C, is considered to be the best preservation method (Smith and Allsopp 1993). However, changes in the morphology of hyphae during freezing and thawing, and variable viability following thawing, necessitate the application of different freezing and thawing rates (Morris et al. 1988, Smith and Thomas 1998); this requires species-specific information to be considered. Attempts have been made to establish the optimal protocols for a wide range of fungi using cryomicroscopy (Smith and Thomas 1998). However, there are estimated to be 1.5 million fungi of which less than 0.1 million have been characterised (Hawksworth *1991), and of these only* a very small percentage have had cryopreservation protocols established. In order to facilitate the selection of an appropriate preservation and storage regime, the following key has been developed:

Decision-based key

NOTE TO THE USER- This key incorporates two sections. Consider section A and then proceed to section B. Section A consists of questions designed to allow the user to consider preservation methods that may be applicable to the particular fungus using species-specific information. Section B asks the user a series of questions, taking into account more general aspects of preservation dynamics and resources available.

Section A: Species-specific criteria

1. Does the fungus readily sporulate asexually in culture?

YES ---------- **9**

NO ---------- **2**

2. Can the fungus be readily maintained by continual sub-culture?

YES---------- **3**

NO ---------- **4**

3. The following preservation methods may be suitable

-- Cryopreservation in liquid nitrogen at –196°C[1] [1]

-- Cryopreservation at –70°C or below in a mechanical freezer [2]

-- Storage under oil [3]

-- Storage in water [4]

-- Storage in a freezer at –20°C [5]

4. Is the fungus endomycorrhizal ?

YES ---------- **5**

NO ---------- **6**

[1] For footnotes see Table 1

5. Store as viable inoculum in growth substrate in association with plant material.

6. Are sexual structures formed by the fungus?

YES ---------- 7

NO ---------- 8

7.Induce sexual reproduction and consider preservation of propagule by cryopreservation or lyophilisation. (Note- The inducement of meiotic crossing over may result in any future cultures being characteristically different from the parent fungus)

8. Consider preservation with substrate from which the fungus was isolated.

9. Does the fungus have motile spores?

YES (Oomycota)---------- 10

NO ----------11

10. The following preservation methods may be suitable

-- Cryopreservation in liquid nitrogen at -196°C [1]

-- Cryopreservation at –70°C or below in a mechanical deep freeze [2]

-- Storage under oil [3]

-- Storage in water [4]

-- Continual sub-culture [6]

11. The following preservation protocols may be suitable-

-- Cryopreservation in liquid nitrogen at –196°C [1]

-- Cryopreservation at –70°C or below in a mechanical deep freeze [2]

-- Storage under oil [3]

-- Storage in water [4]

-- Storage in a domestic freezer at –20°C [5]

-- Continual sub culture [6]

-- Lyophilisation [7]

Section B: Practical and economic criteria

1. How long is it necessary to store your isolate for?

Short-term (<2years) ---------- **2**

Long-term (>2 years) ---------- **3**

2. Consider one of the following

---------- Storage in water [4]

---------- Storage in a freezer at – 20°C [5]

---------- Continual Sub-Culture [6]

---------- Storage on silica gel [8]

3. Is your isolate of economical or scientific importance?

YES ---------- **5**

NO ---------- **4**

4. Consider

---------- Storage under oil [3]

---------- Storage on silica gel [8]

---------- Storage in sand/soil [9]

5. Are resources freely available?

NO ---------- 6

YES ---------- 7

6. Consider sending isolate to WFCC-recognised culture collection for preservation and storage if costs permit or send your isolate to a recognised centre for lyophilisation and arrange for your culture to be returned by post.

7. Consider either or both

Lyophilisation---------- 8

Cryopreservation---------- 11

8. Do you have, or have the resources to obtain lyophilisation facilities?

No---------- 9

Yes, but I don't need to preserve many cultures-----9

Yes ---------- 10

9. Send your isolate to a WFCC recognised collection for lyophilisation and storage.

10. Freeze dry your culture, store at reduced temperature and away from direct light, and check for viability every 3 to 5 years.

11. Consider

-------- Cryopreservation in a mechanical deep freeze at -70°C or below -12

or

-------- Cryopreservation in liquid nitrogen at -196°C [1]

12. Are electrical supplies guaranteed or liquid nitrogen/cold gas back up available to keep temperature stable or low whilst electricity is disrupted?

NO ---------- 13

YES---------- 14

13. Consider alternative or send your isolate for storage in a WFCC-recognised culture collection.

14 --- Cryopreservation at -70°C or below in a mechanical deep freeze [2]

Table 1: Suggested preservation protocols

[1] Cryopreservation in liquid nitrogen at –196°C – Prepare a conidial suspension or hyphal plug of your isolate, submerge in a suitable cryoprotectant and cool at a controlled rate (see Smith and Thomas 1998). Store in the liquid or vapour phase of liquid nitrogen for as long as required (Smith and Onions 1994).
[2] Cryopreservation in a mechanical deep freeze at -70°C - Store your isolate on a slope or as a conidial suspension (preferably with a cryoprotectant) in a mechanical deep freeze at the lowest temperature possible.

[3] Storage in oil – Grow healthy cultures on agar slants, submerge with a maximum of 10mm of mineral oil (Smith and Onions 1994).
[4] Storage in water - Mycelial plugs should be cut from actively growing mycelium, submersed in at least 10ml of sterile deionised water in glass universals or other suitable container and tightly sealed to prevent dehydration (Smith and Onions 1994, Burdsall 1994).
[5] Freezing at -20^{0}C – Maintain cultures to achieve optimal growth on agar slants. Transfer to cold storage in a freezer at -20°C (Smith and Onions 1994)
[6] Continual sub-culture – Cut agar blocks or plugs from the periphery of actively growing cultures and sub-culture onto a fresh nutrient source or slant every two months on agar that does not promote excessive sporulation or meiotic events. Cultures should be sealed, maintained and then stored at reduced temperature (~ 4°C).
[7] Lyophilisation - Prepare spore suspensions in skimmed milk/inositol. Preferably freeze dry in a two-stage centrifugal lyophilisation process. (Smith and Onions 1994).
[8] Storage on silica gel - Store a conidial suspension of your isolate on non-indicating silica gel (Smith and Onions 1994).
[9] Storage in sand/soil - Prepare conidial suspensions and inoculate into sterile (sandy-loam) soil or sand (Smith and Onions 1994).

Conclusion

It is hoped that the key presented may assist microbiologists during their consideration of how and where to preserve their isolates. The key considers the primary decisions facing mycologists world-wide and is aimed to provide researchers with options that are suited to both their fungus and their budgetary limitations. It should be noted that fungi show immense variability intraspecifically and the

information produced should only be used as a guide. No preservation method should be assumed to guarantee the physiological and genetic stability of an isolate. Scientists may wish to preserve many replicates of their fungus and use more than one technique to reduce the chance of strain deterioration.

Acknowledgements

We would like to thank Glaxo Wellcome Research and Development for their support of M.J. Ryan through a BBSRC Industrial studentship.

References

Burdsall, H.H. & Dorworth, E.B. 1994 Preserving cultures of wood decaying Basidiomycotina using sterile distilled water in cryovials. *Mycologia* **86**, 275-280

Elliot, R.F. 1975 Viability of fungal cultures dried and stored over silica gel. *New Zealand Journal of Sciences* **18**, 577-583

Hawksworth, D.L. 1991 The fungal dimension of biodiversity: magnitude, significance and conservation. *Mycological Research* **95**, 641-655

Hawksworth, D.L., Sastramihardja, K.I., Kokke, R. & Stevenson, R. 1990 *Guidelines for the establishment and operations of collections of cultures of microorganisms*. WFCC Secretariat, Brazil:WFCC

Hwang, S.W. 1966 Long term preservation of fungus cultures with liquid nitrogen refrigeration. *Applied Microbiology* **14**, 784-788

Kobayashi, T. 1984 Maintaining cultures of Basidiomycetes by mineral oil method 1. *Bulletin of Forestry and Forestry Product Research Institute* **325**, 141-147

Kolkowski, J.A. & Smith, D. 1995 Cryopreservation and freeze drying of fungi. In *Methods in Molecular Biology 38, Cryopreservation and freeze drying protocols,* ed. Day J.G. & McLellan M.R. pp 49-61, Humana Press NJ U.S.A

Morris, G.J., Smith, D. & Coulson, G.E. 1988 A comparative study of the morphology of hyphae during freezing with the viability upon thawing of 20 species of fungi. *Journal of General Microbiology* **134**, 2897-2906

Smith, D. 1998 The use of cryopreservation in the ex-situ conservation of fungi *Cryo-Letters* **19**, 79-90

Smith, D. & Allsopp, D. 1993 Preservation and maintenance of biotechnological important microorganisms. In *Industrial Biotechnology International,* ed. Greenshields, R.N. pp 103-107 Stirling Press, U.K. ISSN 0967-2044

Smith, D. & Onions, A.H.S. 1994 *The Preservation and Maintenance of Living Fungi*. Second edition, CAB International, Wallingford, U.K.

Smith, D. & Thomas, V.E. 1998 Cryogenic light microscopy and the development of cooling protocols for the cryopreservation of filamentous fungi. *World Journal of Microbiology and Biotechnology* **14**, 49-57

Smith, D. & Waller, J.M. 1992 Culture collections of microorganisms: their importance in tropical plant pathology. *Fitopathologi Brasiliensis*. **17**, 1-8

Tan, C.S. 1997 Preservation of fungi. *Cryptogamic Mycology* **18**, 157-163

APPENDIX G

The Use of Immobilisation for the Preservation of *Serpula lacrymans*

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Abstract

The encapsulation of mycelial formulations of two isolates of the dry rot fungus (*Serpula lacrymans)* in calcium alginate beads was successfully accomplished. Immobilised mycelium was stored in a freezer at –20°C or in water for one month at 20°C and then tested for viability. *Serpula lacrymans* survived the immobilisation process and beads formulated from one of the isolates and stored for one month had greater viability compared to non-immobilised replicates preserved at –20°C or in water. Immobilisation of fungi in calcium alginate beads has potential to complement existing preservation regimes.

Key words:

Basidiomycota, Calcium alginate beads, Culture collections, Immobilisation,

Introduction

Methods of immobilisation have been used to entrap microorganisms for many years. Immobilised fungi have been used by the biotechnology industry to produce enzymes and other novel products (Kwak and Rhee 1992), for formulation of biological control agents (Pereira and Roberts 1991), biodegradation (Lestan *et al.* 1998), carriers of bioherbicides (Walker and Connick, 1983) as a source of inocula for ectomycorrhizal fungi (Mauperin *et al.* 1987) and as a pathogen-free inoculum of *Agaricus bisporus* (Romaine and Schlagnhauffer, 1992). Entrapment of fungi in

calcium alginate beads has been widely reported (Walker and Connick 1983, Mauperin *et al.* 1987, Pereira and Roberts 1991, Kwak and Rhee 1992, Daigle and Cotty 1997). The method is relatively simple and involves the preparation of a spore or mycelial suspension in sodium alginate solution and then the drop-wise addition to calcium chloride solution. Insoluble calcium alginate beads are formed by a displacement reaction between the sodium and calcium. Gilson et al. (1990) noted that to avoid leakage of cells after immobilisation and to achieve optimal gelling strength, beads they should be left in calcium chloride solution. However, long periods in calcium chloride can increase bead shrinkage. Pre-sterilisation of sodium alginate solutions can depolymerise the sodium alginate and cause a decrease in gelling strength, although buffering can reduce depolymerisation during autoclaving (Daigle and Cotty, 1997). Abdullah *et al.* (1995) suggest that immobilisation could be used for the storage of fungal cultures as it is cheap and simple method and does not exert physical damage on hyphae. It could be applied to non-sporulating groups of fungi, such as the Basidiomycota and Oomycota, some of which are difficult to preserve by conventional preservation and storage procedures (Smith and Onions 1994). Abdullah *et al.* (1995) noted that the hyphae of eight immobilised basidiomycetes entrapped on vegatable sponge remained viable for at least one year. Mauperin et al. (1987) reported that immobilised ectomycorrhizal mycelium stored in water retained viability for at least five months at 4°C. However, drying of beads resulted in deterioration of viability after 2 months in storage. Immobilisation technology could be applied to preservation procedures to improve the viability of fungi that are otherwise difficult to preserve and maintain in culture collections. *Serpula lacrymans* is an economically-important basidiomycete which does not easily survive conventional preservation protocols. The aim of this investigation was

to establish whether two isolates of *Serpula lacrymans*, could be successfully immobilised in calcium alginate beads and then stored in water at 20°C or in 10% glycerol in a freezer at –20°C.

Materials and Methods

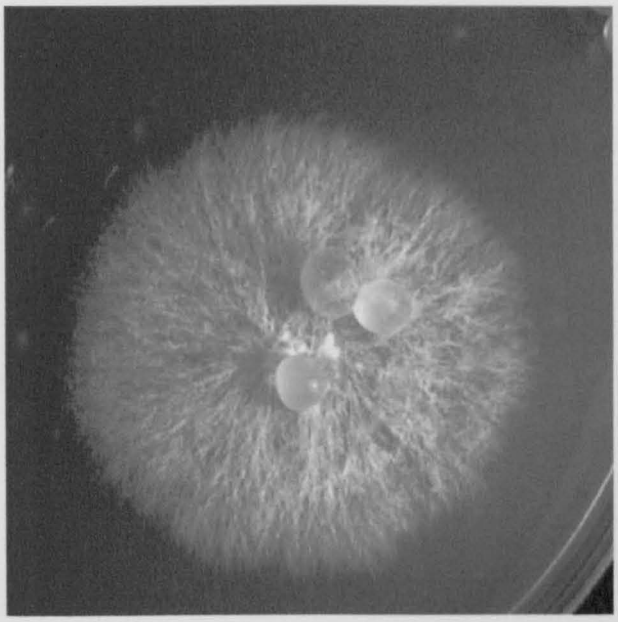
Two isolates of *Serpula lacrymans* were selected. Isolate S1 was isolated in 1994 by Dr P.Jeffries from infected timber in Whistable, Kent. Isolate S3 was isolated in 1998 by Dr P.Bridge from infected timber in Egham, Surrey. Cultures (previously maintained on 1% (w/v) malt extract agar (Oxoid) (MEA)) inoculated with a10mm^3 cube of agar and mycelium, were grown in 10ml 1% (w/v) malt broth (Oxoid) in 90mm diameter Petri dishes. Plates were maintained at 20°C in the dark for 21 days. A 4% (w/v) sodium alginate (BDH) solution was prepared by gradual addition to 100ml sterile distilled water. A 0.2M calcium chloride (Sigma) solution was prepared and both solutions sterilised by autoclaving at (chk) for 10 minutes. The mycelial mats from 2 cultures of *S.lacrymans* in Petri dishes were transferred to the mixing vessel of a vertical homogeniser. 30 ml of alginate solution was added and the solution macerated at high speed for 30 seconds. The macerate was transferred to a sterile 20ml syringe attached with a wide bore pipette tip (1.5mm diam). The syringe was then fastened in a clasp above a conical flask containing the calcium chloride solution. The *Serpula* / alginate macerate was then added drop-wise to the calcium chloride solution at a rate of one drop ever five seconds. The resulting calcium alginate beads were left to harden for 5 minutes in the calcium chloride solution. The excess calcium chloride solution was then decanted off and the beads washed twice in 10ml sterile distilled water. Fifteen beads were immediately taken and placed on 3 plates of MEA to test for viability. The remaining beads were

transferred to either 10ml of 10% (w/v) glycerol solution and stored in a freezer at –20°C or 10ml sterile distilled water and stored at 20°C. Five replicates were set up for each isolate and each preservation treatment. Non-immobilised treatments were set up as mycelial plugs of MEA agar/mycelium in 10ml sterile distilled water (stored at 20°C) and as slopes grown for 21 days on MEA and then stored at –20°C. After one month, replicates were tested for viability. Beads were removed from treatments using blunt end forceps and placed on 1% MEA agar plates. Plates were incubated in the dark for 12 days at 20°C and then viability recorded. Results were statistically compared using the t-test.

Results

Immediately after the immobilisation procedure, 100% of the beads from both isolates produced mycelial growth on MEA. After one month of storage, only 7% of the beads of isolate S1 stored in water produced mycelium compared to zero of non-immobilised samples. None of the beads stored at -20°C produced mycelium. For isolate S3, 97% (+/- 1%) of replicates stored at -20°C produced mycelium compared to 20% of non-immobilised samples and 34% (+/- 5%) stored in water produced mycelium (Fig 1) compared to zero of non-immobilised samples. The improvements in the numbers of immobilised replicates of *Serpula lacrymans* isolate S3 producing mycelium after storage for one month in water ($P<0.005$) and at –20°C ($P<0.005$) was highly significant compared to replicates that were not immobilised. Mycelial growth from beads maintained in a freezer at –20°C was denser than that growing from beads stored in water.

Fig 1: Hyphal growth radiating from calcium alginate bead stored at in water at 20°C for one month and incubated on MEA for 10 days.



Discussion

Both isolates of *Serpula lacrymans*, survived the initial immobilisation process. *Serpula* is usually difficult to preserve, as many hyphae do not survive the initial preparation procedures that are required before the physical process of preservation. Isolate S3 survived immobilisation and storage better than isolate S1, this may be because isolate S3 had been in culture for six months only. Isolate S1 had been in culture for over 4 years and had been sub-cultured at frequent intervals and had lost some of the characteristics, such as pigmentation, which had been described by Donnison (1994). Nevertheless, the 7% viability of replicates stored in water was a substantial improvement over non-immobilised samples, where all samples undergoing prolonged suspension in water lost viability. Mycelial growth

from alginate beads of isolate S3 that had been stored at –20°C was very good, with cultural behaviour identical to the morphology exhibited by the fungus before preservation. However, mycelium radiating from beads stored in water was less dense suggesting that the fungus may have been stressed during storage. The improvement in the number of replicates of isolate S3 producing mycelium after immobilised preservation was highly significant. Preservation regimes seek to reduce the metabolic activity of an organism during culture storage (Smith and Onions 1984). A combination of both immobilisation and preservation could induce further reductions of metabolic activity during storage and ultimately improve the long-term viability and stability of the preserved immobilised organism. Conidia survive preservation well because the spore wall provides a physical barrier during freezing, thawing and drying. Hyphae do not have substantial walls, and are easily damaged during physical preservation processes. Tan (1997) found that hyphae are especially prone to ice damage. Encapsulation of hyphae in the alginate bead matrix could confer physical protection to the hyphae, especially during cooling. The improvement in viability of immobilised replicates compared to non-immobilised replicates stored by the same methods suggests that immobilisation could be applied and developed for fungi that are otherwise notoriously difficult to preserve. The development of species-specific protocols, for example, changing the amount of inoculum and concentration of the chemical constituents could alter the structure of the calcium alginate matrix, and this could be optimised for the immobilisation of different fungal mycelia. Preservation of fungi by immobilised storage could also be improved by the addition of chemicals to the suspending medium that are already known to confer protection on cells during existing preservation processes. For example, the addition of peptides is thought to aid recovery of cells during

resuscitation after freezing (Tan 1997). In conclusion, the use of immobilisation in association with preservation and storage techniques has potential for preservation of non-sporulating fungi such as *Serpula lacrymans*.

Acknowledgements

This work was funded through a BBSRC/GlaxoWellcome Industrial Case Studentship to M.J.Ryan. I would like to thank Ms D. Evans for technical support and Dr T.W. Ford (Royal Holloway, University of London) for initial advice on methodology. Dr P. Jeffries and Dr D.Smith are thanked for commenting on the manuscript.

References

Abdullah, N., Iqbal, M. & Zafar, S.I. (1995) Potential of immobilised fungi as viable inoculum. *Mycologist* **9:** 168-171

Donnison, L. (1994) Molecular biology of *Serpula lacrymans*. *MSc thesis, University of Kent.*

Daigle, D.J. & Cotty, P.J. (1997) The effect of sterilization, pH, filler and spore inoculum concentration on the preparation of alginate pellets. *Biocontrol Science and Technology* **7:** 3-10

Gilson, C.D., Thomas, A. & Hawkes, F.R. (1990) Gelling mechanism of alginate beads with and without immobilised yeast. *Process Biochemistry International* **June:** 104-108

Kwak, M.Y. & Rhee, J.S. (1992) Controlled mycelial growth for kojic acid production using Ca-alginate-immobilized fungal cells. *Applied Microbiology and Biotechnology* **36:** 578-583

Lestan, D., Lestan, M. & Lamar, R.T. (1998) Growth and viability of mycelail fragments of white-rot fungi on hydrogels. *Journal of Industrial Microbiology and Biotechnology* **20:** 244-250

Mauperin, C.H., Mortier, F., Garbaye, F., Le Taycon, F. & Carr, G. (1987) Viability of an ectomycorrhizal inoculum produced in a liquid medium and entrapped in a calcium alginate gel. *Canadian Journal of Botany* **65:** 2326-2329

Pereira, R.M. & Roberts, D.W. (1991) Alginate and cornstarch mycelial formulations of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae. Journal of Economic Entomology* **86**: 1657-1661

Romaine, C.P. & Schlagnhauffer, B. (1992) Characteristics of a hydrated, alginate-based delivery system for cultivation of button mushroom. *Applied and Environmental Microbiology* **58:** 3060-3066

Smith, D. & Onions, A.H.S. (1983) The preservation and maintenance of living fungi. *Commonwealth Mycological Institute. CAB International, Wallingford U.K.*

Tan, C.S. (1997) Preservation of fungi. *Cryptogamic Mycology* **18:**157-163

Walker, H.L. & Connick, Jr. W.J. (1983) Sodium alginate for production and formulation of mycoherbicides. *Weed Science* **31:** 333-338