THE EVALUATION AND DEVELOPMENT OF TECHNIQUES FOR THE PRESERVATION OF LIVING FILAMENTOUS FUNGI

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To my wife for all her patience

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Abstract

The objective of this study was to critically compare the variety of techniques available for the preservation of living fungi for the first time, and from the data obtained to increase their effectiveness. The preservation techniques that allow a growth stage, oil, water and soil storage, were compared with freeze drying and liquid nitrogen storage which reduce metabolism. The fungi stored in water and in oil showed marked deterioration when compared with isolates recovered from liquid nitrogen and freeze drying. The liquid nitrogen storage technique was most effective and was improved when mechanical damage was avoided to enable its use for the majority of fungi.

For the first time cryomicroscopic studies were carried out on the hyphae of fungi. These studies showed that hyphae survived intracellular ice formation and death correlated with freeze induced shrinkage when no ice formed and events during thawing when it formed. The addition of glycerol reduced the effects of freezing and gave improved viabilities.

The technique of freeze drying was improved by controlling the cooling stage and modifying the drying stages. It was found that the final residual water content must be between 1 and 2% for examples of fungi to retain viability and the water content of the freeze dried pellet must be below 5% when the temperature rises to $-15\,^{o}C$ for greatest survival. However, there still remain isolates of fungi that can be frozen successfully but do not survive dehydration. This is also shown by the many isolates that could not withstand the dehydration of silica gel storage.

Although the freeze drying process can be improved it does

not give the same success that is achieved by liquid nitrogen storage. Many isolates were tested and even those belonging to the same species responded differently to the preservation techniques.

Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

APPENDICES

CHAPTER 1

INTRODUCTION

Contents

1.01 Introduction to the problem

Fungi are important as plant and animal pathogens, in the biodeterioration of economically important materials or storage products and in biotechnology. Culture collections are important genetic resources (Hawksworth, 1985; Kirsop, 1983) that are becoming increasingly valuable to industry and they must employ techniques which retain both the viability and stability of the organisms. The organisms can then be used with confidence for reference purposes, taxonomic comparison, for future experimental and industrial work and confirmation of results. This study examines some of the techniques used for the preservation of fungi, assesses their suitability to meet these objectives and consequently develop them to increase their effectiveness. Particular attention has been given to determining why some fungi fail to survive, because this is a key to the development of improved techniques.

When fungi are kept for taxonomic purposes they are often preserved as dead dried specimens together with permanent microscope slides, drawings, photographs and descriptions. If this is linked with a living culture then the collection becomes much more valuable. It is essential to maintain living cultures of certain groups of organisms, where biochemical data is used extensively, for example with the yeasts. Biochemical data is being used increasingly as an aid to the identification of fungi (Bridge & Hawksworth, 1984, 1985). A few secondary metabolites of fungi can be detected in dead dried cultures preserved in herbaria, thereby enabling the application of some identification techniques relying on such products to be carried

out (Paterson & Hawksworth, 1985). However, the organisms may not have been dried when the production of the metabolite was at its optimum and may only be present below the detection level of the technique. When living cultures are used the tests can be carried out at all stages of growth increasing the chance of detection. Furthermore the opportunity of observing the whole life cycle with all the developmental stages and examining the physiology of the fungus is only available with living specimens.

Traditionally, continuous growth techniques have been employed to maintain cultures for teaching, taxonomic research or industrial collections. This involves the serial transfer of the fungi from staled media to fresh as the fungus grows and metabolizes. There are, however, many problems associated with keeping cultures by such means over the long term. A suitable growth medium has to be found and a decision made on the interval between transfers from old to new media. During growth the morphology or physiology of the fungus may change. Changes that have been observed at the Commonwealth Mycological Institute (CMI) have been the loss of the ability to sporulate, reproduce sexually, or produce pigments (Onions, 1983). Furthermore some plant pathogens often lose the ability to parasitize their usual host (Onions, 1983). This may be the result of sexual recombination, the segregation of heterokaryons or adaptation to growth on artificial media.

Growing cultures are particularly subject to airborne contamination. In addition, microbial spores carried by mites

may contaminate the cultures and in some instances the contaminant overgrows the stored organism so that it is lost. At the very least, contamination makes necessary the re-isolation of the organism. This is often difficult and if storage is unsuccessful it may be necessary to find a replacement.

A variety of long term storage techniques have been used to eliminate the problems encountered by continuous growth methods. Slow drying in the air or more rapid drying under vacuum, freeze drying and freezing are all methods by which the rate of metabolism may be reduced or stopped. This subsequently eliminates problems such as variation due to long term growth on artificial media and the sealed ampoules prevent contamination during storage. At present no technique is available which will preserve all fungi (Smith & Onions, 1983a).

This study was initiated to examine the techniques available for the preservation of fungi and in particular those that offer a reliable means of keeping the organisms stable. Present technology has made the control and monitoring of preservation by frozen storage and freeze drying more exact. These techniques (Onions & Smith, 1984) can now be investigated more thoroughly.

In the selection of a preservation technique for fungi the criteria upon which the choice is normally made usually depends on the purpose of the collection, the importance of the individual isolates and the funds and facilities available (Smith & Onions, 1983a). When organisms are being kept for teaching purposes the individual isolates are seldom irreplaceable and the requirement is for representatives of particular taxonomic groups. Therefore techniques for long term

stability may not be necessary. However, when an industrial collection is being maintained, the metabolic products and the physiological characteristics of the organisms are usually the most important factors. Each isolate is valuable and in some instances may be irreplaceable. In this instance continuous growth techniques are unsuitable because of the high risk of variation and therefore, storage methods which offer genetic and metabolic stability are essential (Smith & Onions, 1983a).

The search is thus for techniques that will maintain long term genetic stability and enable the storage of a wider range of fungi than are currently maintained. Freeze drying and liquid nitrogen storage promise to fulfil these requirements most completely and it is in these areas that most work has been concentrated.

1.02 Literature review

1.021 Serial transfer of cultures incubated on agar media

Fungi that can grow on artificial media can be kept by transferring from staling to fresh media. The period between transfers is specific in many cases and may differ for strains of particular species. It has been recommended that to avoid complete desiccation, cultures grown at room temperature should be transferred to fresh media every 3-6 months (Onions, 1971a). Saprolegniaceae can be kept for 15 months on hemp seed in water but it is recommended that they should be subcultured every year (Dick, 1965; Clark & Dick, 1974). At the Centralbureau voor Schimmelcultures (CBS) in the Netherlands where 35000 isolates of fungi are maintained most of the stock cultures are transferred

every 6 months. However, the genera including Allomyces, Achlya, Isoachlya, Phytophthora, Pythium, Saprolegnia, Boletus, Coprinus, Corticium, Cortinarius and Mycena are subcultured every 2-3 months or in some cases once per month (von Arx & Schipper, 1978). The incubation of cultures on dilute media (Dade, 1960) has been used to increase the intervals between transfers and can extend these periods from 1-6 months to 2-12 months.

1.022 Serial transfer of cultures in mineral oil storage

Covering cultures growing on agar slopes with a layer of mineral oil has been a successful technique for many groups of fungi and significantly extended storage periods between transfers to fresh media (Smith & Onions, 1983b). Wood inhabiting fungi have been stored for 27 years (Perrin, 1979). Not only do fungi remain viable but it has been found that plant pathogenic fungi can be stored under a layer of mineral oil retaining their pathogenicity. Fusarium and Alternaria were stored for 6 months (Sherf, 1943), Botryosphaeria ribis for 6 years (Smith, Lewis & Fergus, 1970), and Fusarium oxysporum and Verticillium dahliae also can be kept by this means (Brezhneva & Khokhryakov, 1971).

Some fungi have been found likely to die under oil, the Saprolegniaceae and some water moulds only survive 12-30 months (Reischer, 1949). Onions (1977) recommended 6 monthly subculturing of the CMI collection of water moulds and a 2 yearly cycle for other strains (Smith & Onions, 1983b). However, von Arx and Schipper (1978) have reported 10 years survival of 85% of fungi representing all subdivisions, although many isolates showed the reduction, or loss, of sporulation and deterioration of

morphological characteristics when compared with subcultures of the original isolates. Preservation under mineral oil is cheap and easy and is recommended for the laboratory with limited resources, but it is subject to contamination and selection (Onions, 1983). It has been observed at CMI that the first transfer from oil tends to be retarded in growth rate and it is often necessary to reculture until normal growth rate and colony appearence are restored. This not only requires time and labour but also introduces additional transfers that may result in variation of cultures.

1.023 Water storage

Storage in sterile water has proved very successful for storing human pathogenic fungi and retaining both viability and pathogenicity (Castellani, 1939, 1967). Pathogenicity is a characteristic which may be lost during maintenance by serial transfer on agar and therefore the method has proved useful. McGinnis, Padhye & Ajello (1974) evaluated Castellani's methods and were able to store 417 isolates of 147 species belonging to 66 genera of filamentous fungi, yeasts and aerobic Actinomycetes for periods ranging from 12-60 months. Their chosen method was to store suspensions of hyphae or spores in sterile water in sealed tubes to prevent evaporation. However, other workers (Marx & Daniel, 1976; Person, 1961; Ellis, 1979) have shown that the method is equally successful if agar discs or blocks cut from the growing edges of colonies are suspended in sterile water in containers sealed to prevent evaporation.

Water storage has proved successful in the

preservation of a wide range of fungi (Bosewinkel, 1976). Marx & Daniel (1976) were able to store ectomycorrhizal fungi for one year although they lost viability over a three year storage period. The method of Person (1961) proved successful in the maintainance of sporulation of Ascochyta (20-25 months) and Colletotrichum (6 months), some isolates of which were difficult to keep in a sporulating form. Additionally virulence of plant pathogens has been maintained by this technique (Figueiredo & Pimentel 1975; Boeswinkel, 1976). Ellis (1979) reported storage of strains of Entomophthorales, Ascomycetes, Hymenomycetes, Gasteromycetes and Hyphomycetes although he emphasises that all strains of any one species, such as Amylomyces rouxii, do not react in the same way to the storage conditions. When species of Phytophthora and Pythium were stored in water by Marx & Daniel (1976) they gave poor revivals, however, Boeswinkel (1976) was able to preserve them for up to 7 years without loss in pathogenicity. Thus there are differing responses of strains of the same species.

1.024 Soil storage

A sterile soil storage method was developed by Miller (1945) and successfully used by Cormack (1951) to maintain cultures of Fusarium, isolated from cereal seed, in a stable condition. Storage in soil has proved useful in the maintenance of characteristics often lost during maintenance by serial transfer. Fusarium species frequently mutate in culture (Gordon, 1952; Jong & Davis, 1978) and their maintainance on agar, even by reduced growth rates under mineral oil, is largely unsatisfactory (Booth, 1971). Shearer, Zeyen & Ooka (1974) obtained good preservation

in soil of isolates of Septoria for more than 20 months without loss of sporulation or pathogenicity, both characteristics are frequently lost by culturing techniques. The technique has also been used for storage and mass spore production of conidia by Pseudocercosporella herpotrichoides (Reinecke & Fokkema, 1979). Alternaria raphani has been shown to survive and retain pathogenicity (Atkinson, 1953) and the quantitative studies carried out on the survival of 5 year old dried soil cultures show that stability can be maintained (Atkinson, 1954).

1.025 Air and vacuum drying techniques at normal temperatures

Some simple drying techniques have been employed successfully for the storage of fungi (Fennell, 1960). Air dried basidiospores of Schizophyllum commune sealed under a vacuum of less than 0.1mm Hg by A.H.R. Buller in 1910 were germinated after 34 years (Bisby, 1945) and 50 years (Ainsworth, 1962). Cultures of Allomyces arbuscula grown in liquid media can be slowly dried on filter paper strips and will survive 14 and 17 years (Goldie-Smith, 1956). Spores of Glomus and Acaulospora species have been successfully dried under vacuum on a fibre filter pad or in soil when the culture is not frozen but dried from the liquid state without freezing (Tommerup & Kidby, 1979). Although long term storage was not investigated spores remained viable when heated to 80 ^{o}C for 40 min.

1.026 Silica gel storage

Fungi can also be successfully dried by adding spore suspensions to the desiccant silica gel without indicator dye. Neurospora species have been successfully stored for several

years by this means (Perkins, 1962; Ogata, 1962). A wide range of fungi have kept their viability in silica gel at CMI but the technique is not suitable for the preservation of mycelial cultures, such as Pythium or Phytophthora (Onions, 1977; Smith & Onions 1983a, 1983b). Sleesman, Larsen and Safford (1974) found that Helminthosporium maydis retained its pathogenicity for 12 months when kept in a dehydrated state on silica gel crystals and Trollope (1975) found that 77% of the 22 fungi stored survived for one year or more. Fungi have been stored above silica gel in desiccators (Elliott, 1975), on agar discs in aluminium foil or on filter paper above self indicating silica gel in bijou bottles (Seaby, 1977) and Ustilago scitaminea has been stored using silica gel as a desiccant at 5 °C (Mata & Tokeshi, 1976).

1.027 Freeze drying

Freeze drying, or lyophilization, is the dehydration of frozen material by the sublimation of ice (Meryman, 1966). The method has been recognised for a long time for preserving microorganisms. The first reference to the preservation of a microorganism appeared in 1903 when Vansteenberghe reported the desiccation by freeze drying of rabies virus over sulphuric acid under vacuum. Shackell (1909) emphasised that to get high viabilities the organism must be frozen during vacuum drying and by an improved technique again rabies virus was successfully freeze dried (Harris & Shackell, 1911).

From very simple beginnings described by Flosdorf & Mudd (1935) centrifugal freeze drying techniques were developed (Greaves, 1944; Fry & Greaves, 1951; Haskins, 1957). Reviews of

the freeze drying process (Rowe, 1970, 1971; Rowe & Snowman, 1978) show how the methods have improved and describe the equipment now available. If suspensions of cells were not frozen before evacuation the rapidly escaping water vapour and gases caused them to overflow from their containers. The development of a freeze drier which centrifuged the suspensions during evacuation thus keeping them in their containers, enabled cooling and freezing by evaporation that did not necessitate a prefreezing step (Greaves, 1944). The centrifugal technique has been adopted for the freeze drying of microorganisms by many workers (Fennell, 1960; Heckly, 1978).

Freeze drying was first applied on a large scale to fungi belonging to the Hyphomycetes, Zygomycetes and Ascomycotina by Raper & Alexander (1945) and subsequently Ellis & Roberson (1968) reported successful revival of most of Raper & Alexander's cultures. Long storage periods are achieved by freeze drying and some fungi have been preserved for 20 years (Rhoades, 1970) and 30 years (Jong, Levy & Stevenson, 1984).

Many surveys have been made of this technique describing varying degrees of success (Heckley, 1961,1978; Nei, 1964; Jong, 1978; Alexander, Daggett, Ghena, Jong, Simione & Hatt, 1980; Smith, 1983a).

Some fungi are killed by freeze drying; Raper & Alexander (1945) were unable to preserve Entomophthorales, the water moulds Achlya, Pythium or Plectospira and the basidiomycete Stereum. However, the apothecia of Moellerodiscus lentus (Kokko & Elliott, 1977) and non-sporulating isolates of Claviceps (Pertot, Puc &

Kremser, 1977) which are normally difficult to maintain have been successfully preserved by this technique. Isolates that do not sporulate in culture can be freeze dried successfully if damage caused by freezing can be avoided (Last, Price, Dye & Hay, 1969).

The freeze drying technique is used by many large collections of fungi which offer a distribution service for example the CBS (von Arx & Schipper, 1978), the American Type Culture Collection (Alexander, Daggett, Gherna, Jong, Simione & Hatt, 1980) and CMI (Onions, 1983). Many prefer to use freeze drying because the cultures do not have to be grown prior to distribution and the small and light freeze dried culture is suitable to send by post.

Despite reported success with freeze drying, selection of strains and genetic damage, especially to the DNA, has been observed in microorganisms following freeze drying (Heckly, 1978). However, techniques of freeze drying have been dependant on the equipment available and methods have not been developed with a particular type of microorganism in mind. As equipment now available enables the variation and monitoring of the cooling and warming applied during the process the effect of these parameters can be determined and the possibility of improved techniques now exists.

1.028 Storage at low temperatures and freezing

(i) Cold hardening and storage at temperatures above freezing

The incubation of cultures at low temperatures (Taterenko, Igolkina & Man'ko, 1976; Ruppell, 1971) have been used to increase the intervals between transfers. The storage of Alternaria brassicola at 5 °C (Kilpatrick, 1976), some forest

pathogens at 5°C (Chu, 1970) and Oomycetes at 4°C (Dick, 1965; Clark & Dick, 1974) has proved successful. A disadvantage of storage at temperatures between 5–8°C is that condensation forms on the outside of the cold bottles. This allows microbial growth and may result in contamination (Dade, 1960; Onions, 1971).

Cold hardening, or acclimation, occurs in plants by several mechanisms some of which are described below. Techniques have been used to induce hardiness to freezing in microorganisms. The vegetative cells of algae can become resistant to freezing following a period of cold acclimatization (Leeson, Cann, & Morris, 1984). The cold hardening of fungi is achieved at the CMI by incubation of cultures prior to freezing at temperatures between 4 and 7°C. Pythium aphanidermatum (2 isolates) and P. arrhenomanes (1 isolate) required incubation for 1–2 weeks at temperatures between 4 and 7°C before they survived freezing and storage in liquid nitrogen (Smith, 1982a).

(ii) Storage at temperatures between -17 and -24°C

By reducing storage temperature to between -17 to -24°C, isolates of the Zygomycotina, Ascomycotina and Deuteromycotina can be stored successfully on agar slopes (Carmichael, 1956, 1962). Albugo occidentalis and Peronospora effusa have been stored successfully for 5–6 months at -23°C on spinach leaves (O'Brien & Webb, 1958). In initial trials the cultures removed from the refrigerator were discarded after use but it was soon found that if the cultures were not allowed to thaw subcultures could be taken from frozen material and the main culture returned to the freezer without loss in viability (Kramer & Mix, 1957).

Storage by this means relies on the reliability of the equipment and power supply or breakdown could result in the loss of all stored cultures if thawing occurs (Onions, 1971). Storage of microorganisms at this temperature may give poor results (Snell, 1984) and it is now generally accepted that, for long-term maintenance, temperatures below $-139^{o}C$ are essential (Morris, 1981). Such temperatures and lower are obtained by storage in liquid nitrogen as discussed below.

(iii) Liquid nitrogen storage

The availability of liquid nitrogen has made the use of ultra low temperatures for the preservation of cultures more attainable (Clark & Loegering, 1967). Living cells were first stored at ultra low temperatures by Polge, Smith & Parkes (1949) with the storage of spermatozoa protected by glycerol (see 1.029 v). The successful storage of some fungi that were previously difficult to preserve by the techniques then available, soon followed (Hwang, 1960). Some modifications were made to the method for its use with fungi. Precooling to $+7^{o}C$ was shown to improve viabilities (Hwang, 1966) and a cooling rate of $1^{o}C\ min^{-1}$ has proved to be most successful with fungi (Hwang, 1966, 1968; Butterfield, Jong & Alexander, 1974; Onions, 1971a; Smith, 1982a). After storage cultures are usually thawed rapidily and normally this is achieved by immersion of ampoules in a heated water bath (Hwang, 1966; Onions, 1971a; Smith, 1982a). If cultures are cooled slowly before preservation the rate of thawing seems to have little effect but if rapid cooling is employed then rapid thawing seems to be essential if viability is to be retained (Mazur, 1968; Heckly,

1978). The viability of spores of Aspergillus niger kept between the temperatures of -70 and -75 °C in distilled water was found to be increased by using rapid rather than slow heating (Mazur, 1956).

A wide range of fungi and fungal structures have been preserved successfully by liquid nitrogen storage (Smith, 1982a). Mycelial fragments and hyphal tips of Neurospora crassa and Sordaria fimicola (Wellman & Walden, 1964) and many plant pathogens have been frozen and stored successfully in liquid nitrogen. These include Peronospora tabacina (Bromfield & Schmitt, 1967), Peronospora phaseoli (Antonio & Blount, 1973), Sclerospora species (Gale, Schmitt & Bromfield, 1975), Septoria passerinii (Anderson & Skormand, 1971), Macrophomina phaseolina (Wyllie & Fry, 1973) and some rust and smut spores (Loegering, 1965; Loegering, Harrison & Clark, 1966; Kilpatrick, Harmon, Loergering & Clark, 1971; Prescott & Kernkamp, 1971).

Some other liquid gases can provide temperatures low enough to preserve fungi successfully. For example, liquid air has been used to preserve Puccinia uredinospores and the chlamydospores of Ustilago (Joshi, Wilcoxson, Gera & Chatterjee, 1974). There are risks of explosion with liquid air and therefore liquid nitrogen is preferred (Anon, 1965).

1.029 The nature of low temperatures, mechanisms of survival, cryoinjury and cryoprotection

(i) Low temperature

Low temperature is a relative term. In life sciences it is

usually identified with subzero temperatures with a lower limit of -70^{o}C below which no life processes persist (Franks, 1981). This is not so with many tropical plants and thermophilic microorganisms which show signs of chill injury well above 0^{o}C. The total physiological temperature range can usually be divided into those temperatures where the aqueous substrate is liquid and those temperatures where it is partly frozen. In living organisms the temperature where these two ranges meet, the freezing temperature, is normally in the region of -20^{o}C but this depends on many factors. Some of these factors may be the species, the environment, the degree of cold acclimatization and the state of development of the organism. Cooling to low temperatures reduces the rate at which chemical reactions occur, increases the viscosity of water, denatures protein, increases the dielectric permittivity of water, which enables it to reduce the attraction between ions of opposite charge, and acidic and basic residues of proteins are markedly affected. The manifestation of such changes contribute to chill injury to which only a few fungi are apparently susceptible as most survive cooling and storage at low temperature (see 1.028i above).

(ii) Freezing

In the terminology of thermodynamics, freezing is referred to as a first order phase change. This phase change in water requires low temperatures but will not occur without a nucleation event (Franks, 1981). A critical group of water molecules adopts a configuration that can be recognised by other molecules which condense on it forming an ice crystal, such events become more likely as the temperature decreases below 0^{o}C. The configuration

of water molecules may arise due to density and energy fluctuations at low temperature or nucleation may be aided by a solid particle around which the water crystalizes. The initial ice crystals that form are normally unstable and recrystalize which in turn can be injurious to a living organism. Without a nucleation event water supercools (undercools).

It is at a cellular level that an understanding of the freezing process must begin. Steponkus (1984) has presented a clear picture of events occurring during the freezing of plant cells and protoplasts. Initially supercooling of both the cell and suspending medium occurs during cooling. Subsequently, ice nucleation occurs in the suspending medium at a temperature depending on the freezing point of the solution and the presence of effective ice nucleating agents. Freezing occurs until the eutectic is reached where the chemical potential of water in the unfrozen portion is in equilibrium with ice. In turn the chemical potential of the intracellular solution must also come into equilibrium with the extracellular ice. Equilibrium is achieved either by intracellular ice nucleation or cell dehydration, the plasma membrane acting as a barrier to extracellular ice and as a semipermeable membrane allowing dehydration the consequence of which depends upon the stability of the plasma membrane.

The rate of cooling affects the cells response. At rates where the efflux of water allows the cell contents to concentrate and to come into equilibrium with the extracellular solution the cell shrinks. At rates where the efflux is not sufficiently

rapid extensive supercooling occurs and if the cell cools to -39^{o} C or below homogeneous nucleation of ice occurs. If a suitable particle is present to act as a nucleus heterogenous nucleation will occur before this temperature is attained. Seeding by extracellular ice requires perturbation or penetration of the plasma membrane.

After extracellular ice nucleation both the intracellular and extracellular solutes are concentrated and in some instances their solubility limits may be exceeded. Precipitation of buffers may result in pH changes. Gas bubbles are formed during freezing as gases are excluded from ice. These bubbles are often seen as a blackening or granulation of the specimen and it is this event that is usually observed on intracellular ice nucleation in plant cell protoplasts. A rapid blackening of the cell interior occurs and this is often termed 'flashing'. Electrical potentials are also created during freezing by charge separation thereby giving rise to potential differences between the liquid and the solid phases.

(iii) Mechanisms of cryoinjury

The combined effect of low temperature and freezing gives rise to changes in positions of chemical and biochemical equilibria, reduction in the rates of motion of molecules, increases the concentration of components in the mixture resulting from phase separation and the possibility of irreversible aggregation or dissociation events involving macromolecules. These events will inevitably affect the structure and metabolism of cells.

Mazur (1970) proposed a two factor hypothesis of injury: at

relatively fast cooling rates injury is caused by intracellular ice formation, whereas at slow cooling rates injury is caused by prolonged exposure to 'solution effects' resulting from the concentration of the extracellular solution or cell dehydration. However, Steponkus (1984) states that there is no evidence to support the theory that intracellular ice causes injury. An alternative view is that ice forms as a consequence of injury to the plasma membrane. Many hypotheses have been put forward for freezing injury caused by cellular dehydration and catagorised under 'solution effects' (Mazur, 1969, 1970, 1977; Meryman, Williams & Douglas, 1977). These include volumetric and area contraction, concentration of intra- and extracellular solutes, possible pH changes because of different solubilities of buffering compounds, cystalization and possible removal of water of hydration from macromolecules (Meryman, 1974; Steponkus et al, 1982, 1983).

Cells shrink during cooling and are said to have a minimum critical volume. However, Steponkus (1984) observed that this hypothesis is not applicable to isolated protoplasts. The demise of many hypotheses based upon a single stress occurs when the proposed mechanism does not apply under all conceivable conditions. Given that freezing results in a multitude of stresses, it is more reasonable to suppose that injury is the result of the interaction of several stresses. Cryomicroscopic studies have been used to discover what constitutes cellular injury in plant cell protoplasts during freeze-thaw cycles. Very little information is available on these mechanisms in fungi but

they may be similar to those of plant cells.

Steponkus (1984) carried out extensive studies of rye leaf cell protoplasts during cooling and observed many manifestations of freezing injury.

(a) The expansion-induced lysis during warming as decreasing osmolality of the suspending medium allowed the expansion of the protoplasts. The duration of time in the plasmolized state influenced the incidence of injury. Large surface area contractions were found to be irreversible and survival was directly correlated. When plasma membranes reduce in area a loss of material into the cell interior occurs and numerous vesicles become apparent in the cytoplasm. The contractions become irreversible when the material for incorporation becomes less readily available as the rate of reincorporation necessary increases. However, others state that membrane lesions are the result of other lethal stresses and that this is a pathological symptom (Grout & Fuller, 1982; McLellan, Morris & Kalininia, 1982).

(b) Loss of osmotic responsiveness during slow cooling and warming. Electrolyte concentration, changes in pH, removal of water from the membrane due to crystalization, electrical perturbations and thermotropic phase transitions in membrane lipids destabilize the plasma membrane causing a loss of osmotic responsiveness.

A membrane is a heterogeneous system of lipids and proteins which exist in dynamic equilibrium with each other. Lowering of temperature reduces the fluidity within the membrane with subsequent reaching of the phase transition temperature where

the phospholipids change from the liquid to the gel phase. This inevitably affects the permeability and transport system of membranes (Pringle & Chapman, 1981).

(c) Alterations in osmotic behaviour during warming which is suggestive of a prior transient loss of intracellular solutes or leakage through the plasma membrane. The failure of the cell to attain its original volume on return to ambient temperature occurs at low frequency both in cold acclimated and non-acclimated rye cell protoplasts and is thought to be a sublethal injury.

(d) Intracellular ice formation during rapid cooling ($>3\ ^{o}C\ min^{-1}$). There are two prerequisites for intracellular ice formation, supercooling and seeding of the intracellular solution. It is commonly assumed that intracellular nucleators are not responsible for intracellular ice formation but it is a consequence of seeding by extracellular ice when the plasma membrane is damaged.

Cryoinjury is dependent upon the rate of cooling, cell type (whether it is sensitive or resistant to cold) and the composition of the suspending medium. The types of injury described above for plant cell protoplasts have also been observed in whole cells (Steponkus, Evans & Singh, 1982).

(iv) Some natural mechanisms of cold resistance

Very little has been published on the natural mechanisms of freezing resistance of fungi. However, extensive studies have been carried out on the mechanisms of plant and plant cell acclimation leading to cold and frost resistance. In some plants

survival may depend on their ability to survive or prevent an excessive loss of water when translocation is impossible from other parts of the plant which are frozen. A plant may develop mechanisms to avoid ice formation in its tissue (Levitt, 1966). One way of achieving this is to avoid low temperatures by developing thick tissues that protect more delicate structures such as the scales on a bud or bark of a tree. A second way is to avoid freezing by the undercooling of cell solutions although this is usually only for a short period or over a few degrees and would not protect the plant throughout the winter. A third way is to accumulate solutes and other compounds that offer the cell some degree of cryoprotection (see 1.029 v below). However, in plants the cell sap rarely has a freezing point below -4^{o}C. It has been observed that in hardened cortical cells of Catalpa less ice formed at -6^{o}C than in non-hardened cells therefore some tolerance to ice is necessary but smaller amounts of ice may mean that less damage occurs.

It is more relevant here to discuss how cold acclimation has affected the individual cells and some of the observations may be useful in determining the mechanisms of cold resistance in fungi. Steponkus, (1984) has reported how plant protoplasts from acclimated and non-acclimated plants differ in their properties. Cold acclimation may be a progressive cold hardiness not by becoming more resistant to one stress but overcoming each stress barrier in turn. In non-acclimated protoplasts membrane deletions occur to a much greater extent and the tension necessary to effect area increase is considerably smaller than in acclimated protoplasts. When reductions in membrane area occur endocytotic

vesiculation of membranes is observed in non-acclimated rye cell protoplasts which necessitates membrane breakage and predisposes the protoplast to other events such as intracellular ice nucleation by the extracellular ice. However, in cold acclimated protoplasts which are more resistant to cooling exocytotic extrusions form without membrane breakage.

A second difference is that the freezing point of the cytoplasm within the protoplast of an acclimated plant is considerably lower than that of a non-acclimated plant. In rye the intracellular ice nucleation temperature is $-15^{o}C$ for non-acclimated and $-45^{o}C$ for acclimated. This was invariably due to a more concentrated cell solution. However, this second difference is less important as intracellular ice nucleation usually occurs after a breakdown of the membrane. Therefore it is more likely that cold acclimation is affected by an increase in membrane stability. The plasma membrane stability is increased by the inclusion of unsaturated fatty acids and sterols. In bacteria there is an increase in the proportion of unsaturated phospholipids in the walls of cold tolerant cells (Herbert, 1981). However, only small changes in content have been seen in cold acclimated protoplasts.

(v) Cryoprotection

Despite other mechanisms of natural resistance to freezing it is the compounds produced within the cell that impart protection to low temperature and freezing that have received most attention and use in the laboratory. The lowering of the freezing point by the production of "antifreeze" compounds such

as glycoprotein, which is also seen in Artic and Antarctic fish, and the formation of water binding molecules in microorganisms enables the resistance to dehydration not only caused by the cold but also by heat and salinity (Gould & Measures, 1977).

Methods of osmoregulation in fungi have been studied by Luard (1982a, 1982b, 1982c). This is an important aspect of resistance to cryoinjury. As the extracellular solution freezes the concentration of suspending medium around the fungus increases. The ability of the fungus to reduce the effect will increase survival. In studies carried out on Penicillium chrysogenum and Chrysosporium fastidium, Luard (1982a) found that as water potentials fell the fungi accumulated potassium ions, and the concentration of magnesium and calcium ions in the hyphae decreased. Carbohydrate analysis suggested that malic acid had also been accumulated and additionally both fungi accumulated glycerol. It appears that glycerol and solutes absorbed from the medium act as osmoregulators. Other osmotica such as fatty acids may also be involved in the increased osmotic potential of the more tolerant strains. Fungi are able to accumulate organic acids together with the negatively charged amino acids aspartate and glutamate and these will balance the preferential accumulation of the positively charged potassium ions. A change in the potassium/sodium ion ratio which occurs in the contents of the hyphae is associated with tolerance to low water potential and may serve as an indicator of tolerance.

In studies of Phytophthora cinnamomi, which is not tolerant to low water potential, the accumulation of potassium ions, sucrose and proline was noted and the only polyol detected was

arabitol at extremely low levels. Mucor hiemalis, Pythium debaryanum and a water mould also accumulated proline as osmotic potential decreased. Glycerol was not detected in any of the strains. Glycine and alanine were found to be the predominant components of the amino acid pool. M. hiemalis and the water mould accumulated sucrose whereas P. debaryanum hydrolysed the sucrose to its constituent components. Twenty three species of Mucor accumulated large amounts of glycerol when stimulated by the addition of sodium hydrogen sulphite and sodium carbonate (Takahashi & Asai, 1933). The lower fungi differ from other fungi in the mode of osmoregulation. Osmotic shock experiments confirmed that glycerol was involved in osmoregulation in P. chrysogenum and C. fastidium (Luard, 1982c). The differences in permeability to internal osmotica was considered to account for their different range of tolerances. The accumulation of compounds such as glycerol and the general increase in the concentration of the hyphal contents will give a degree of cryoprotection.

Meryman & Williams (1985) discuss how colligative cryoprotectants reduce the amount of ice formed at any temperature and this in turn reduces the water loss from cells. They state that properties of a suitable cryoprotectant are that it is non-toxic, it will penetrate the cell membrane and it must be able to permeate the cell rapidly.

During kinetic freezing too much dehydration causes cell injury due to membrane stress. When the cell freezes internally injury could be due to mechanical distortion of intracellular

structures or physical rupture of the membrane due to the 10% increase in volume of the water when ice is formed.

Cryoprotection is achieved by,

(a) Non-critical volume loss by the reduction of ice formation.

(b) An increase in viscosity which slows down ice crystal growth and formation and solute effects.

(c) Reduction of the rate of diffusion of water caused by the increase of solutes.

Different cryoprotectants give different cooling rate optima. In the case of blood platelets the optimum is 30 $^{o}C\ min^{-1}$ in glycerol and 1 $^{o}C\ min^{-1}$ in dimethylsulphoxide (DMSO).

Extracellular cryoprotectants, for example polyvinyl pyrrolidone (PVP), Dextran and hydroxethyl starch (HES), protect by the rapid increase in viscosity at low temperatures reducing the cooling rate for optimum recovery. PVP reduces the melting and homogeneous nucleation temperatures and increases the glass formation temperature to a greater extent than glycerol. At high concentrations (50% v/v) PVP may prevent heterogeneous nucleation of extracellular ice and therefore stop dehydration allowing supercooling and homogeneous ice nucleation. However, on thawing crystalization of ice occurs as the rapid rates of thawing necessary cannot be achieved in the viscous material. PVP is also thought to increase surface tension sufficently to prevent protein loss through damaged membranes though when restored to normal media, if the membrane is not repaired, lysis will occur. Despite these benificial properties during cooling PVP does not protect all cells for example it does not protect red blood cells (Meryman & Williams, 1985).

The use of cryoprotective additives in suspending media has been used since Polge, Smith & Parkes (1949) used glycerol to protect spermatozoa. Glycerol lowers the concentration of salts in equilibrium with ice at any temperature below freezing whether in the medium or when it has penetrated the cell (Nash, 1966). If enough glycerol is present the salt concentration does not rise to a damaging level until the temperature is so low that the rate of damage is tolerable. This theory of the mechanism of cryoprotection and others have been discussed by Calcott (1978). It is evident that different susbtances protect by different mechanisms. The large molecular weight compounds such as polyvinyl pyrrolidone are non-penetrating but are said to change the cells permeability to ions and allow influx and efflux of sodium and potassium ions during freezing and thawing as well as the alteration to the viscosity of the extracellular solution discussed above. Thus they avoid excessive osmotic stress. Another theory is that they alter the structure of water both in and around cells so that on freezing a glass structure is formed and no crystalization occurs. There are many compounds that impart protection during freezing and thawing and the mechanisms by which they all protect is not fully understood (Calcott, 1978).

The suspending medium in which the fungi are frozen is often very important and usually a cryoprotectant such as dimethyl sulphoxide (DMSO) or glycerol is employed (Calcott, 1978). The latter additive has been used successfully by many workers (Hwang, 1966, 1968; Butterfield, Jong & Alexander, 1974; Smith,

1982a). Dimethyl sulphoxide has been shown to be an effective cryoprotectant where glycerol has failed (Hwang & Howells, 1968; Barnhart & Terry, 1971). It has also been used in the successful cryogenic storage of conidia of Sclerospora sorghi (Gale, Schmitt & Bromfield, 1975; Long, Woods & Schmitt, 1978).

Many other compounds have proved to be successful cryoprotectants for example, polyvinyl-pyrrolidone and dextran (Ashwood-Smith & Warby, 1971), ethylene glycol, propylene glycol (Lovelock, 1954), acetamide and urea (Keane, 1953). Mixtures of cryoprotectants have also been used successfully for example, DMSO and glucose has been employed for the protection of fungi (Smith, 1983b), glycerol with any one of the sugars lactose, maltose or raffinose for some bacteria and algae (Daily & Higgens, 1973) and dimethyl sulphoxide with glucose for the storage of sugar cane cells (Finkle & Ulrich, 1979).

1.03 Analysis of preservation records

1.031 Introduction

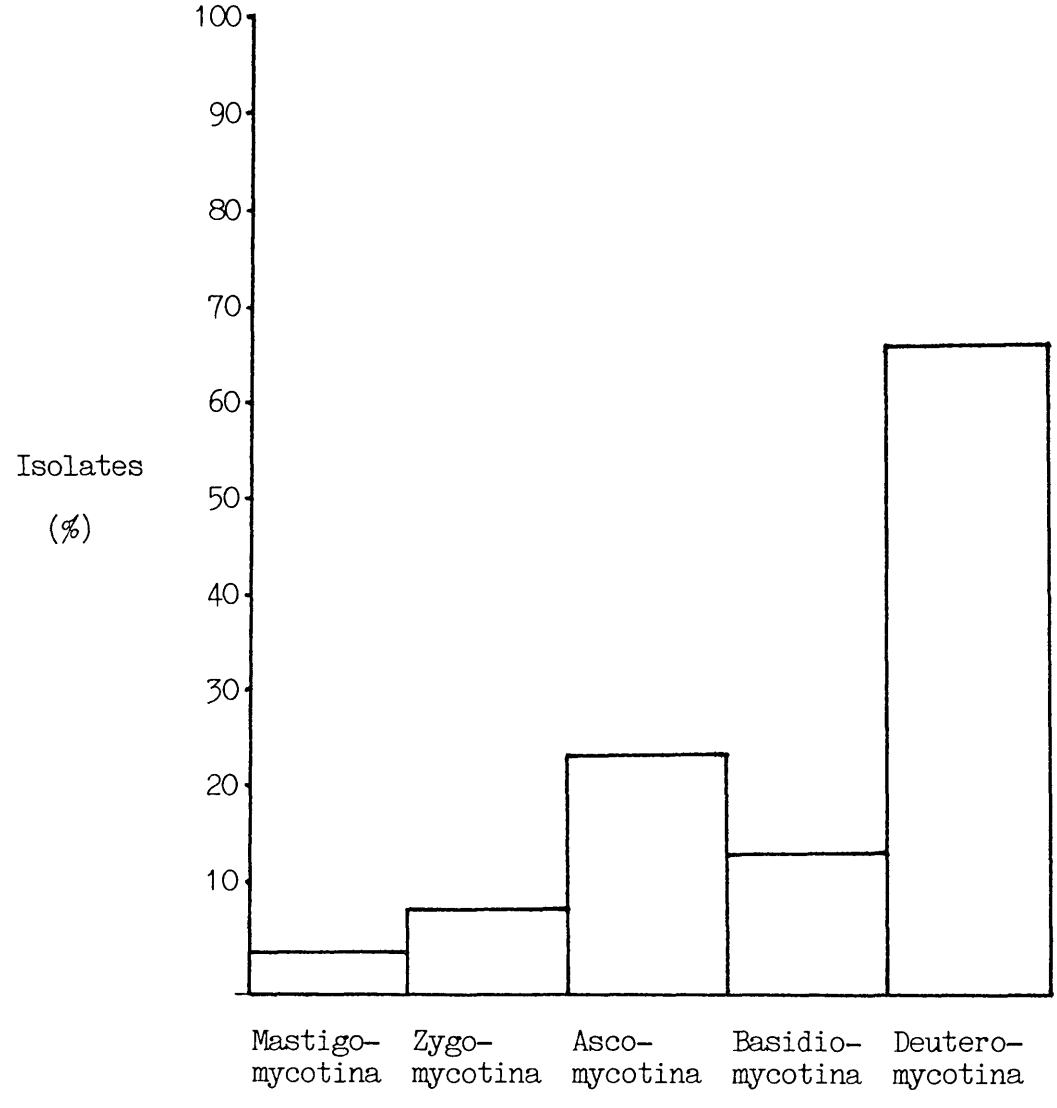
There are over 200 culture collections of fungi throughout the world (McGowan & Skerman, 1982) and it is here that the wealth of information on preservation lies. Many collections list the organisms they maintain and the methods used for their preservation but the detailed information is rarely made available in the literature. The main problems encountered by collections are the number and range of organisms they maintain and the limited number of staff available to carry out their maintenance (Simmons, 1984). Research is rarely carried out into the preservation technique and it is only when organisms die during routine maintenance that other methods are sought. There

are few reports on why organisms died and it is therefore necessary to determine this before improved techniques can be developed.

After reviewing the available literature (1.02) it can be seen that researchers have been more concerned with preserving their own particular isolates than with any systematic evaluation of the preservation techniques. Their isolates were usually single strains, strains of one species or several related species. The methods used tended to be the simplest available which would retain the viability and characteristics of the organisms. They were not necessarily the best available, or indeed applicable to other fungi. Many reviews have been written summarising the results of these preservation attempts (Westerdijk, 1947; Van Beverwijk, 1959; Fennell, 1960; Heckley, 1978; Onions, 1983). The methods employed fall into the main categories, subculturing, storage in oil and water, drying in soil and silica gel, freeze drying and freezing, though the details of technique described in some cases differed. No significant additions to the methods have been made since the introduction of liquid nitrogen storage for the preservation of fungi (Hwang, 1960).

Information on most of these preservation techniques is available in the CMI records. The analysis of the records of the 11,000 isolates held enables experiments to be designed to improve the techniques used. Over 60% of the isolates in the collection belong to the Deuteromycotina, 12% are Basidiomycetes and less than 4% to the Mastigomycotina the remainder represent the Ascomycotina and the Zygomycotina (Fig. 1.031a).

Fig. 1.031a Percentage of isolates in the subdivisions in the CMI culture collection



1.032 Serial transfer

At CMI serial transfer was employed as the only method of maintenance until it was discontinued in 1955, when oil storage was introduced as the main preservation technique. Onions stated that serial transfer every 3-6 months was labour intensive and therefore the longer intervals between transfer offered by storage under mineral oil was advantageous and adopted the technique. A subsequent report on the results of the oil storage method showed that 88% had remained viable for 21 years (Onions, 1977). Furthermore, some cultures grew in a better condition after recovery from oil than they did after maintenance by more frequent transfer techniques (Table. 1.0321). Approximately 150 strains of Phytophthora and Pythium species were maintained satisfactorily between 1954 and 1957. However, these isolates were fairly resistant to oil storage and other fungi were found to deteriorate rapidly, for example Penicillium baarnense and other ascosporic strains of penicillia. Many fungi in the CMI survived 32 years under oil without transfer (Smith & Onions, 1983b). Forty six (79%) isolates of the 58 stored belonging to 23 genera survived for the whole period (Table, 1.0322). Two isolates of Aspergillus, one of Corticium, one of Mucor and three of Penicillium died after 20 years or more storage without transfer. Sporendonema casei survived 17 years, Mortierella alpina and Thielaviopsis basicola 12 years and an isolate of Podospora fimbriata 10 years. Although most isolates that survived were eventually recovered without morphological change some needed further transfers before a healthy culture was obtained.

The general opinion is that the use of synthetic media and the change of environmental conditions can allow variation and change of an organism (Snell, 1984; Onions, 1983). Therefore continued growth or metabolism should be avoided.

1.033 Water storage

Water storage was introduced at CMI in 1978 because reports suggested that this technique would be valuable for the preservation of Oomycetes. The conflicting reports of results with these fungi discussed above (1.023) and the need for alternative methods made it necessary for further work and therefore experiments were designed and carried out as part of this study (Chapter 3). During the course of the experiments some of the isolates recovered from water storage were sent to customers of the CMI who carried out virulence tests. In order that the technique could be evaluated they were provided with samples from liquid nitrogen which were tested simultaneously. The results of these tests are given in tables 1.0331 and 1.0332.

Of 16 isolates of Phytophthora tested for virulence after storage in water or liquid nitrogen for the period preceding the last sucessful viability test, 15 recovered from liquid nitrogen were more virulent and 4 recovered from water were non-infective (Table 1.0331).

Of the 5 isolates of Pythium for which virulence tests were performed all had deteriorated after storage in water. Two isolates recovered from liquid nitrogen showed no deterioration (Table 1.0332).

Table 1.0321 Strains retaining characteristics after recovery from oil storage after 20 years which were lost during more frequent serial transfer

Name	IMI Number	Retained characteristic
Aspergillus avenaceus	16140	Production of abundant sclerotia
Botryosphaeria obtusa	38560	Production of ascospores
Penicillium adametzii	39751	Profuse sporulation
P. asperum	39739	Production of sclerotia
P. lapidosum	39743	Sclerotia and sporulation
P. oxalicum	39750	Profuse sporulation
P. waksmanii	39746	Profuse sporulation

Data from CMI records

Table 1.0322 Isolates of fungi surviving 32 years in mineral oil storage without transfer

Name	IMI number	Taxonomic group
Aspergillus avenaceus	16140	Hyphomycete
A. citrisporus	25285*	Hyphomycete
Botryosphaeria obtusa	38560	Dothideales
B. ribis	36476	Dothideales
Ceratocystis paradoxa	37270	Ophiostomatales
C. paradoxa	39075	Ophiostomatales
C. radicicola	36479*	Ophiostomatales
Chlamydomyces palmarum	39639*	Hyphomycete
Corticium praticola	34886	Aphyllophorales
C. rolfsii	33912	Aphyllophorales
Drecslera portulacae	37710*	Hyphomycete
Eleutherascus terrestris	25845*	Pezizales
Helicodendron trilitziense	38968*	Hyphomycete
Humicola sp	38777*	Hyphomycete
Nectria pityrodes	37228a*	Hypocreales
Penicillium adametzii	39751	Hyphomycete
P. aurantio-violaceum	39740	Hyphomycete
P. ehrlichii	39737*	Hyphomycete
P. fellutanum	39734	Hyphomycete
P. fuscum	39747	Hyphomycete
P. javanicum	39733*	Hyphomycete
P. lapidosum	39743	Hyphomycete

*Showed inconsistency on retrieval and required several transfers to achieve good growth

Data from CMI records

Table 1.0322 (continued) Isolates of fungi surviving 32 years in mineral oil storage without transfer

Name	IMI number	Taxonomic group
Penicillium levitum	39735	Hyphomycete
P. lividum	39736	Hyphomycete
P. oxalicum	39750	Hyphomycete
P. sclerotiorum	39742	Hyphomycete
P. shearii	39739	Hyphomycete
P. striatum	39741	Hyphomycete
P. trzebinskii	39749	Hyphomycete
P. turbatum	39738	Hyphomycete
Petriella sordida	38601	Microascales
Phaeoscopulariopsis sp.	16401*	Hyphomycete
Phaeoscopulariopsis sp.	16404*	Hyphomycete
Phytophthora citricola	21173	Oomycete
P. nicotianae	22176	Oomycete
Podospora fimbriata	38111	Sordariales
Rhizoctonia oryzae-sativae	31287	Agonomycete
R. solani	20697	Agonomycete
Sclerotium coffeicolum	37953*	Agonomycete
Setosphaeria rostrata	22971	Dothideales
Stephanosporium cereale	38105	Hyphomycete
Torula herbarum	31291	Hyphomycete
T. ligniperda	36123	Hyphomycete

*Showed inconsistency on retrieval and required several transfers to achieve good growth

Data from CMI records

Table 1.0322 (continued) Isolates of fungi surviving 32 years in mineral oil storage without transfer

Name	IMI number	Taxonomic group
Ustilago scitaminea	36859	Ustilaginales
Verticillium theobromae	31432a	Hyphomycete
Volutella ciliata	38780	Hyphomycete

*Showed inconsistency on retrieval and required several transfers to achieve good growth

Data from CMI records

Table 1.0331 Virulence of isolates of *Phytophthora* after storage in water and liquid nitrogen (Data from CMI records)

Name	IMI Number	Host	Part Infected	Virulence after storage (years)	
				Water	Liquid nitrogen
P. cactorum	21168	*Malus pumila*	fruit	+(2)	++(2)
P. cactorum	49562	*Dispyros virginiana*	root	-(2)	++(12)
P. cactorum	62471	*Malus pumila*	fruit	+(3)	++(3)
P. capsici	45528	*Lycopersicon esculentum*	fruit	+(2)	++(2)
P. cinnamomi	22938	*Cinnamonmum burmanni*	root	+(3)	++(3)
P. cinnamomi	158786	*Tristania conferta*	root	-(3)	++(3)
P. cinnamomi	211105	*Eugenia caryophyllata*	root	+(3)	+(1)
P. drechsleri	40499	*Chrysanthemus cinerariaefolium*	root	-(3)	++(11)
P. erythroseptica	34684	*Solanum tuberosum*	tuber	+(2)	++(12)
P. erythroseptica	181716	*Solanum tuberosum*	tuber	+(1)	++(9)
P. megasperma	131375	*Malus pumila*	fruit	+(2)	++(2)
P. megasperma	131555	*Malus pumila*	fruit	+(2)	++(2)
P. nicotianae	77972	*Citrus sinensis*	fruit	-(2)	++(6)
P. nicotianae	158733	*Citrus sinensis*	fruit	+(2)	++(12)
P. palmivora	46333	*Annoma squamosa*	fruit	+(4)	++(4)
P. palmivora	80298	*Theobroma cacoa*	pod	+(4)	++(8)
P. syringae	131190	*Malus pumila*	fruit	+(4)	NT

+, weak low infectivity; ++, good infectivity; -, failed to infect host; NT, not tested;

Table 1.0332 Virulence of isolates of *Pythium* after storage in water and liquid nitrogen (Data from CMI records)

Name	IMI Number	Host	Part Infected	Virulence after storage (years)	
				Water	Liquid nitrogen
P. aphanidermatum	104926	*Agave sisalana*	root	+(2)	++(12)
P. debaryanum	48558	*Fragaria* spp.	root	+(4)	NT
P. middletoni	42098	*Malus pumila*	fruit	+(4)	++(13)
P. oligandrum	78731	*Hordeum vulgare*	Seedling cotyledons	+(2)	NT
P. oligandrum	133857	*Nasturtium officinale*	Seedling cotyledons	+(2)	NT

+, weak low infectivity; ++, good infectivity; -, failed to infect host; NT, not tested;

1.034 Soil storage

Booth (1971) introduced a soil storage technique to CMI and reported that this technique was preferable to oil storage for the retention of characteristics of species of Fusarium. Seven hundred and sixty four isolates of 8 genera are held in the soil collection (Table 1.0341). Of these there are 652 isolates of Fusarium belonging to 55 species. These cultures have been kept in this way for up to 20 years and most are still viable. Those that died had remained viable for 10 years in storage (Smith, 1984). However, there are no reports on the stability of characteristics of these fungi after long term storage.

1.035 Silica gel storage

The silica gel storage technique was introduced to the CMI in 1971 (Onions, 1977). Since then 420 isolates of fungi have been processed by the technique (Table, 1.0351) 73% of which survived the initial dehydration. A further 55 isolates died between 1 and 5 years storage the remainder have survived between 8 and 14 years. The cultures preserved were sporulating; those that did not sporulate in culture and all the representatives of the Mastigomycotina failed to survive. A rather high percentage of the sporulating Zygomycotina died. The majority of these belonged to the genera Coemansia and Martensiomyces of the Kickxellales and Conidiobolus, Entomophthora and Piptocephalis of the Entomophthorales. The only member of the Mucorales that failed the initial dehydration was Syzigites megalocarpus.

Table 1.0341 Isolates in the soil collection surviving for 10 to 20 years , (All isolates tested survived).

Genus	Number of species	Number of isolates
Calonectria	6	8
Cylindrocarpon	12	32
Cylindrocladium	5	9
Fusarium	55	652
Gibberella	4	6
Melanospora	2	9
Nectria	17	47
Thielavia	1	1
Totals	102	764

Data from CMI records

Table 1.0351 The viability of fungi after storage with silica gel for 8 to 11 years at the CMI. Arranged taxonomically after Hawksworth, Sutton & Ainsworth, 1983.

	No. of genera viable	No. of species viable	No. of isolates		
			Tested	Viable	%
MASTIGOMYCOTINA					
Chytridiomycetes	0	0	5	0	0
Oomycetes	0	0	5	0	0
ZYGOMYCOTINA					
Zygomycetes	9	17	34	20	59
ASCOMYCOTINA					
Clavicipitales	1	1	1	1	100
Diaporthales	2	2	5	3	60
Dothideales	4	4	10	4	40
Endomycetales	1	1	2	2	100
Eurotiales	2	3	4	4	100
Helotiales	0	0	1	0	0
Hypocreales	3	3	4	3	75
Ophiostomatales	1	1	4	1	25
Pezizales	3	3	4	3	75
Sordariales	8	15	38	30	79
Sphaeriales	3	3	6	3	50
BASIDIOMYCOTINA					
Hymenomycetes	8	10	19	10	52
Gasteromycetes	0	0	1	0	0
DEUTEROMYCOTINA					
Coelomycetes	7	18	28	21	75
Hyphomycetes	38	136	250	202	81

1.036 Centrifugal freeze drying

This technique was first used at CMI for the preservation of fungi in 1966 and by 1980 over 7000 isolates were successfully stored by this method (Table, 1.0361). The data on these isolates have been summarized (Smith, 1982c). Most sporulating fungi survived the initial process, over 90% of the Zygomycotina (754 isolates), and Deuteromycotina (5348) survived. However, the Basidiomycotina survived less well, namely 42% of the Hymenomycetes and 79% of the Ustilaginomycetes. Forty four isolates belonging to the Mastigomycotina failed. The remaining isolate, Trachysphaera fructigena, a representative of the Peronosporales died after 3 years storage. The survival of the isolates belonging to the Ascomycotina showed some variation between orders. In most orders survival of isolates was over 89% but only 62% of the isolates belonging to the Clavicipitales and 67% of those of the Sphaeriales survived. One of the 2 isolates belonging to the Diatrypales failed. As the isolates of all groups were processed over a period of 14 years their storage lives range between 4-14 years.

Table 1.0362 lists the 62 genera of fungi that failed the process of centrifugal freeze drying. Not all genera were tested extensively, for example only one representative was tested for 37 of those listed. However, 28 isolates of Phytophthora and 16 of Pythium were attempted. The minimum storage periods for the 134 isolates that died in storage are given in Appendix VII and summarized in table 1.0363.

Table 1.0361 The viability of fungi after centrifugal freeze drying and storage for 4-14 years at CMI. Arranged taxonomically after Hawksworth, Sutton & Ainsworth, 1983.

	No. of genera viable	No. of species viable	No. of isolates		
			Tested	Viable	%
MASTIGOMYCOTINA					
Chytridiomycetes	0	0	6	0	0
Oomycetes	0	0	45	0	0
ZYGOMYCOTINA					
Zygomycetes	48	222	821	754	92
ASCOMYCOTINA					
Ascosphaerales	2	6	7	7	100
Clavicipitales	3	5	13	8	62
Diaporthales	8	18	36	32	89
Diatrypales	1	1	2	1	50
Dothideales	35	118	236	225	95
Elaphomycetales	1	1	1	1	100
Endomycetales	12	22	36	36	100
Eurotiales	16	33	85	85	100
Gymnoascales	19	50	88	86	98
Helotiales	15	23	44	42	95
Hypocreales	11	57	132	123	93
Microascales	5	12	49	49	100
Ophiostomatales	1	22	45	44	98
Pezizales	14	24	56	51	91
Polystigmatales	2	4	13	13	100
Rhytismatales	2	4	6	6	100
Sordariales	21	187	351	341	97
Sphaeriales	17	31	66	44	67
Taphrinales	3	5	6	6	100
BASIDIOMYCOTINA					
Hymenomycetes	19	31	92	41	42
Gasteromycetes	2	2	5	2	40
Urediniomycetes	1	2	3	0	0
Ustilaginomycetes	7	14	24	19	79
DEUTEROMYCOTINA					
Coelomycetes	106	302	576	526	91
Hyphomycetes	320	1565	5091	4822	95

Table 1.0362 Genera with no representatives surviving centrifugal freeze drying

Genus	Number of isolates tested	Genus	Number of isolates tested
Achlya	3	Lacellinopsis	1
Allomyces	3	Lasiobolidium	1
Areolospora	2	Lentinus	1
Armillariella	3	Lenzites	1
Arthrocladium	1	Leptoporus	1
Ascocalvatia	1	Lomachashaka	1
Balansia	2	Marasmius	1
Battaraea	1	Melanconis	2
Biscogniauxia	1	Monotosporella	1
Blastocladiella	1	Nummularia	3
Calospora	1	Panus	1
Camposporium	5	Penicillifer	2
Chytridium	1	Phaeoisariopsis	2
Cladobotryum	1	Phyllosticta	1
Coriolus	1	Physarum	2
Dactuliophora	1	Phytophthora	28
Eleutherascus	1	Piedraia	1
Entomophthora	2	Platysomum	1
Eremomyces	1	Puccinia	3
Fomes	2	Pythium	16
Ganoderma	2	Quaternaria	1
Herpotricha	1	Saprolegnia	2
Kretzschmaria	1	Searchomyces	2

Data from CMI records

Table 1.0362 Genera with no representatives surviving centrifugal freeze drying (continued)

Genus	Number of isolates tested	Genus	Number of isolates tested
Selenosporella	1	Syzigites	1
Selinia	1	Tetracladium	1
Sigmoidea	1	Tetranacrium	1
Sphaerobolus	2	Umbelopsis	1
Sphaerostilbe	2	Urohendersonia	1
Spondylocladiopsis	1	Ustilaginoidea	1
Stereum	2	Ustilina	6
Sympodiella	2	Volvariella	2

Data from CMI records

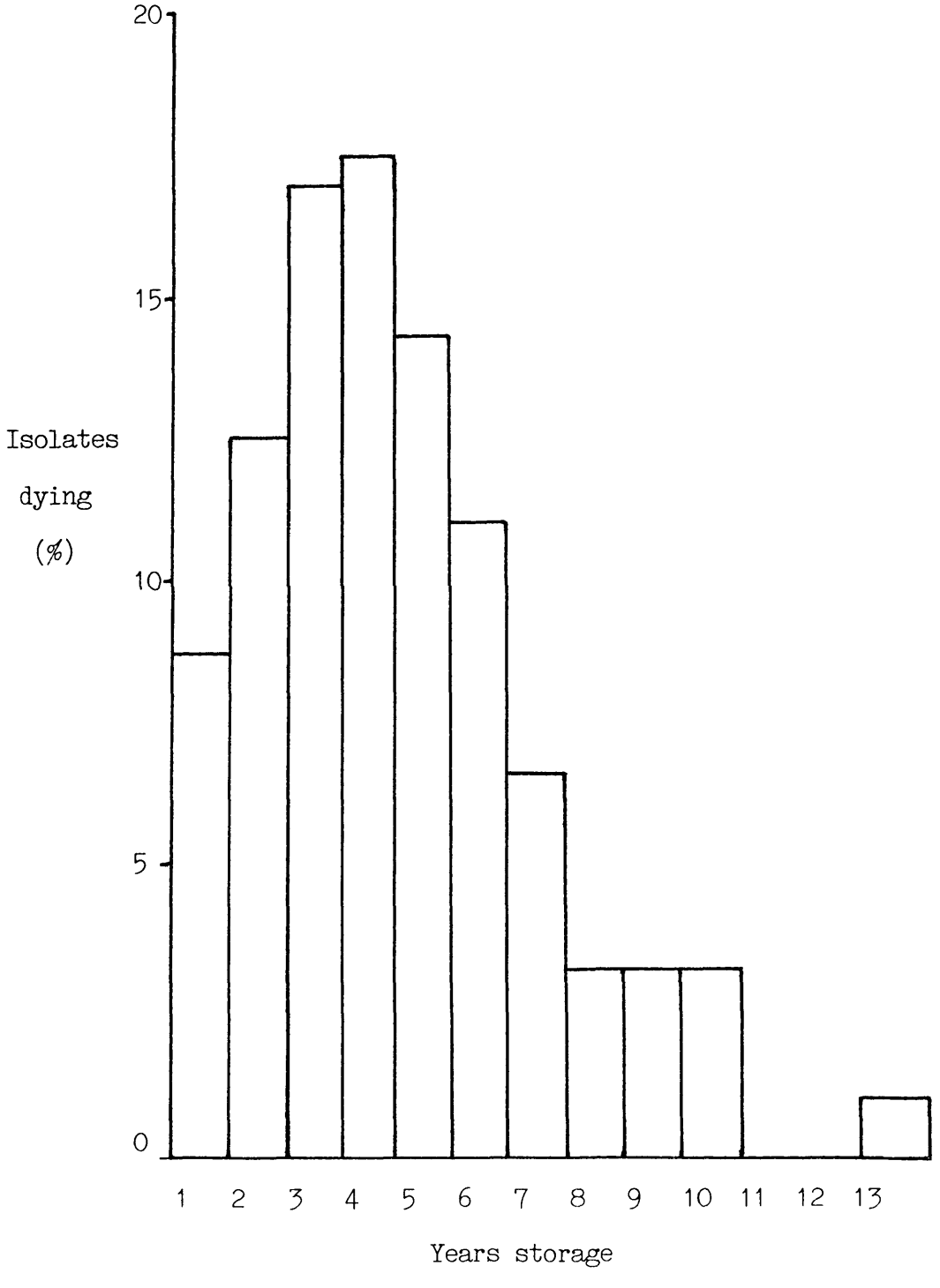
Of the organisms that survived the centrifugal freeze drying technique 134 died during storage, 12 of them in the first year (Appendix VII). Most of these isolates died in the first 6 years of storage (Fig. 1.036a). Of those that died in the first year of storage Ascochyta fimbriata IMI 87300, Caloscypha fulgens IMI 144877 and Monacrosporium oxysporum IMI 78728 are not represented by any other surviving isolates. Among the other ten, all have at least 1 isolate that remains viable in storage. Not all taxonomic groups had representatives that died in storage and in most of those that did 5% or less died (Table 1.0363). Only two orders of the Ascomycotina had higher percentages of death in storage, the Clavicipitales (13%), though only one isolate died from the 8 initially preserved successfully, and the Pezizales where 6 (12%) died.

Different isolates of the same species respond differently to storage by centrifugal freeze drying. For example one isolate of Actinomucor elegans IMI 56159 survived only 6 months, while another survived 10 years by the same technique. Apiocrea chrysosperma IMI 109891 died after just 3 months storage whereas another isolate of the same species remains viable and has been stored for 6 years. For other examples see Appendix VII. As isolates of the same species of fungi behave differently selecting suitable test isolates to assess preservation techniques is difficult.

Table 1.0363 The periods of successful storage of fungi that died during storage after centrifugal freeze drying at CMI with percentage of the total that remain viable. Arranged taxonomically after Hawksworth, Sutton & Ainsworth, 1983.

	Number of isolates									
	Survived process		Period (years) which the number died						Total that died in storage	
	Number	%	0–2	3–4	5–6	7–8	9–10	13	Number	%
MASTIGOMYCOTINA										
Oomycetes	1	2	0	1	0	0	0	0	1	100
ZYGOMYCOTINA										
Zygomycetes	754	92	6	6	3	3	0	0	18	2
ASCOMYCOTINA										
Ascosphaerales	7	100	0	0	0	0	0	0	0	0
Clavicipitales	8	62	1	0	0	0	0	0	1	13
Diaporthales	32	89	1	1	0	0	0	0	2	6
Diatrypales	1	50	0	0	0	0	0	0	0	0
Dothideales	225	95	2	2	1	0	0	0	5	2
Elaphomycetales	1	100	0	0	0	0	0	0	0	0
Endomycetales	36	100	0	0	0	1	0	0	1	3
Eurotiales	85	100	1	0	0	0	0	0	1	1
Gymnoascales	86	98	0	0	0	0	0	0	0	0
Helotiales	42	95	2	0	0	0	0	0	2	5
Hypocreales	123	93	1	0	0	0	0	0	1	1
Microascales	49	100	0	0	0	0	0	0	0	0
Ophiostomatales	44	98	2	0	0	0	0	0	2	5
Pezizales	51	91	4	2	0	0	0	0	6	12
Polystigmatales	13	100	0	0	0	0	0	0	0	0
Rhytismatales	6	100	0	0	0	0	0	0	0	0
Sordariales	341	97	2	1	2	0	0	0	5	1
Sphaeriales	44	67	1	0	1	0	0	0	2	5
Taphrinales	6	100	0	0	0	0	0	0	0	0
BASIDIOMYCOTINA										
Hymenomycetes	41	42	1	0	0	0	0	0	1	2
Gasteromycetes	2	40	0	0	0	0	0	0	0	0
Ustilaginomycetes	19	79	0	0	1	0	0	0	1	5
DEUTEROMYCOTINA										
Coelomycetes	526	91	5	2	0	0	1	0	8	2
Hyphomycetes	4822	95	21	28	16	6	5	1	77	2

Fig. 1.036a The isolates that died during storage after centrifugal freeze drying. The proportion dying in each year of storage is given.



Data from CMI records

The freeze drying data, with the exception of the Mastigomycotina tested and some of the orders of the Ascomycotina, did not show any particular taxonomic grouping to the failures. It was apparent that other factors may be involved and experiments were designed to determine if the fungi were dying as a result of a particular treatment during the preservation procedures (Chapter, 7).

1.037 Liquid nitrogen storage

A taxonomic list of the isolates tested based on Hawksworth, Sutton & Ainsworth, (1983) is given (Table 1.0371). This comprises 3304 different isolates belonging to 2922 species and 582 genera (Smith, 1982b). The isolates were preserved using 10% (v/v) glycerol as cryoprotective agent over a period of 15 years and therefore have storage periods of 1 - 15 years. The Deuteromycotina and Zygomycotina, excluding the Trichomycetes were stored successfully as over 90% of the isolates tested survived. Survival of isolates in various orders of the Ascomycotina ranged from 75% (6/8) of the Polystigmatales to 100% of the Diaporthales (17/17), Endomycetales (10/10), Helotiales (33/33), Microascales (13/13), Ophiostomatales (22/22), Rhytismatales (7/7) and the Taphrinales (5/5). The isolates of Basidiomycotina tested survived the technique with a success rate of 56-92%.

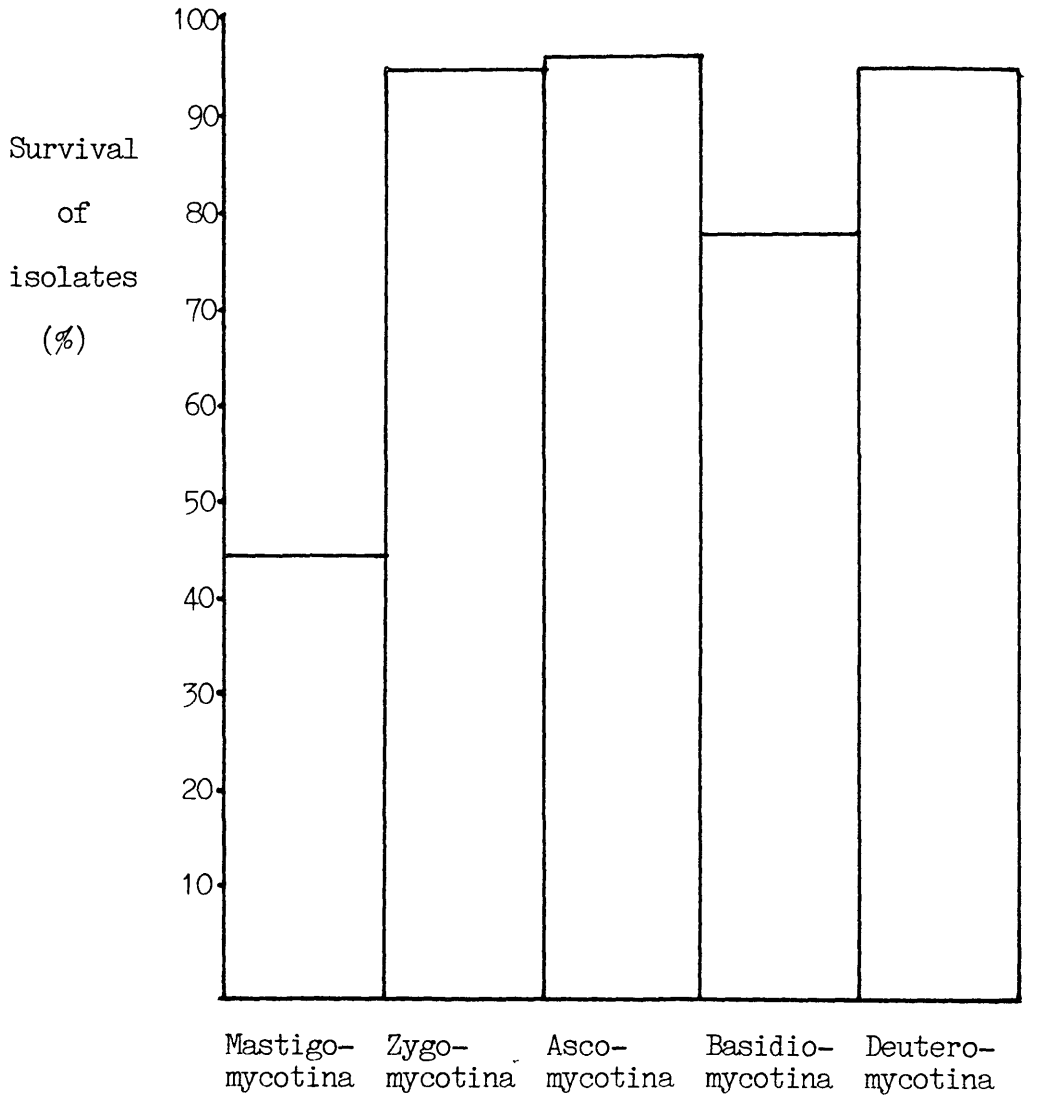
Of the total processed 379 isolates failed, of which 258 were of species with other surviving representatives. Many of the cultures that failed either sporulated weakly or not at all prior to preservation and this may have contributed to their failure.

The highest failure was seen in the Mastigomycotina (Fig. 1.037a). Over 40% of those isolates that had no other representatives of the species surviving (Appendix IV; Fig. 1.037b) belonged to the subdivision Mastigomycotina.

Table 1.0371 The viability of fungi after liquid nitrogen storage for 1-15 years at CMI. Arranged taxonomically after Hawksworth, Sutton & Ainsworth, 1983.

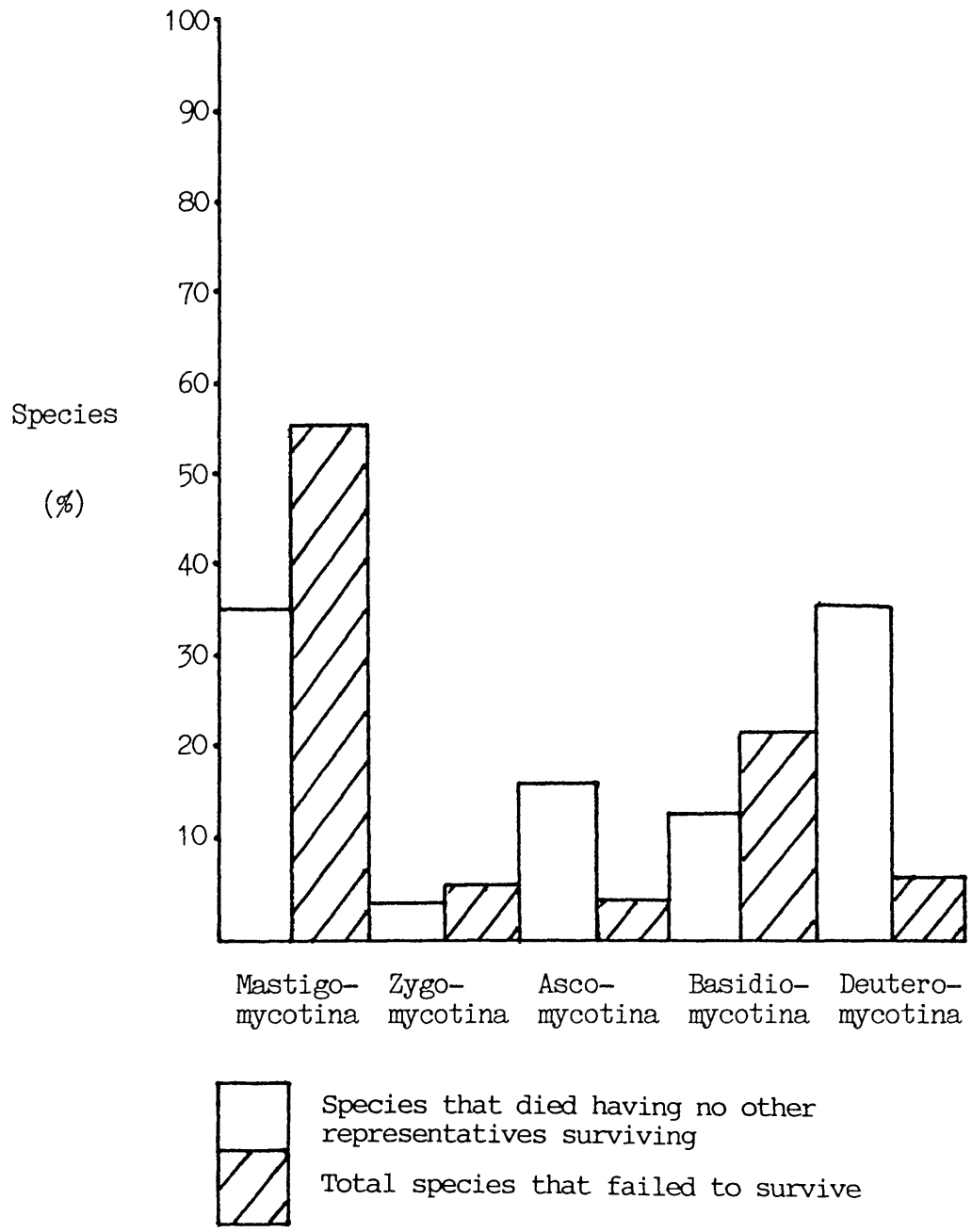
	Number of genera viable	Number of species viable	Number of isolates		
			Tested	Viable	% viability
MASTIGOMYCOTINA					
Chytridiomycetes	4	7	56	9	16
Hyphochytriomycetes	1	1	5	3	60
Oomycetes	9	54	348	172	50
ZYGOMYCOTINA					
Zygomycetes	43	176	267	254	95
ASCOMYCOTINA					
Ascosphaerales	2	6	7	6	86
Clavicipitales	3	11	15	13	87
Diaporthales	7	16	17	17	100
Dothideales	23	65	109	97	89
Elaphomycetales	0	0	1	0	0
Endomycetales	5	9	10	10	100
Eurotiales	16	38	56	55	98
Gymnoascales	14	28	42	41	98
Helotiales	9	18	33	33	100
Hypocreales	7	34	39	38	97
Microascales	3	9	13	13	100
Ophiostomatales	1	15	22	22	100
Pezizales	15	20	41	39	95
Polystigmatales	2	2	8	6	75
Rhytismatales	2	5	7	7	100
Sordariales	17	119	178	170	96
Sphaeriales	22	37	59	51	86
Taphrinales	3	4	5	5	100
BASIDIOMYCOTINA					
Hymenomycetes	38	61	149	143	96
Gasteromycetes	3	3	8	8	100
Uredinomycetes	2	5	18	10	56
Ustilaginomycetes	5	5	6	6	100
DEUTEROMYCOTINA					
Hyphomycetes	259	1212	1543	1465	95
Coelomycetes	67	163	238	224	94

Fig. 1.037a The percentage survival of isolates in the subdivisions of fungi stored in liquid nitrogen



Data from CMI records

Fig. 1.037b Species having no isolates surviving liquid nitrogen storage in their subdivisions compared with the percentage of the total number of species in each group that were inviable after treatment



Data from CMI records

The Zygomycotina made up less than 3% of those failures that had no representatives of their species surviving and 5% of the total number of isolates that failed. Less than 16% of the failures within the Ascomycotina had no other representatives of the species surviving although they made up only 4% of the total number of failures. Eleven percent of the failures with no other representatives surviving and 21% of the total number of failures belonged to the Basidiomycotina. Although 35% of those species with no representatives belonged to the Deuteromycotina only 6% of the total number of isolates tested failed (Fig. 1.037b). Again 35% of the species without representatives surviving the technique belonged to the Mastigomycotina but in this case 55% of the total number of isolates tested failed. It is apparent from this analysis that only particular strains of fungi fail in the majority of groups but all strains of the Mastigomycotina have a high probability of failing and failure is not restricted to particular species.

The Mastigomycotina make up less than 4% of the CMI culture collection (Fig. 1.031a) but among the isolates are important plant pathogens and as discussed earlier members of this subdivision are difficult to maintain in culture or by other methods of preservation. It is therefore necessary to improve techniques of preservation for this group of fungi. The taxonomic analysis of this group is examined below.

Few representatives of the Hyphochytridiomycetes were tested (5 isolates of 1 species). However 56 isolates of the Chytridiomycetes and 348 isolates of Oomycetes were frozen and stored in nitrogen (Table, 1.0372). Two hundred and twenty five

isolates belonging to these three classes failed which makes up 59% of the total failures. The majority of isolates tested belonged to the genera Phytophthora, of which 50% survived, and Pythium, of which 48% survived (Table 1.0373).

Seven genera belonging to the Saprolegniales were processed of which only 12 isolates (15%) survived from the 79 tested. It is evident that the method was not entirely suitable for members of the Mastigomycotina and an improved technique should be sought.

Table 1.0372 The viability and longevity of the Mastigomycotina stored in liquid nitrogen

	Number of isolates								
	Surviving the process			Surviving 1 year		Surviving 6 years		Surviving 12 years	
	Tested	Viable	%	Tested	Viable	Tested	Viable	Tested	Viable
Chytridiomycetes	56	9	16	9	9	7	7	2	2
Hyphochytridiomycetes	5	3	60	3	3	3	3	3	3
Oomycetes	347	146	42	146	146	127	127	43	43

Data from CMI records

Table 1.0373 The viability and longevity of Oomycetes stored in liquid nitrogen for 1 year or more

	Number of isolates								
	Surviving the process			Surviving 1 year		Surviving 6 years		Surviving 12 years	
	Tested	Viable	%	Tested	Viable	Tested	Viable	Tested	Viable
Peronosporales									
Phytophthora	210	104	50	104	104	92	92	21	21
Pythium	58	28	48	28	28	23	23	17	17
Saprolegniales									
Achlya	42	6	14	6	6	6	6	2	2
Aphanomyces	10	2	20	2	2	2	2	1	1
Dictyuchus	2	0	0	0	0	0	0	0	0
Isoachlya	1	0	0	0	0	0	0	0	0
Protoachlya	3	0	0	0	0	0	0	0	0
Saprolegnia	16	3	19	3	3	3	3	1	1
Thraustotheca	5	1	20	1	1	1	1	1	1

Data from CMI records

Table 1.0374 Viability of *Phytophthora* species stored in liquid nitrogen

Name	Number isolates viable after storage			Survival period (years)
	Tested	Viable	%	
P. arecae	1	1	100	4
P. boehmeriae	2	2	100	5
P. botryosa	4	2	50	4,8
P. cactorum	4	3	75	8–10
P. cambivora	6	5	83	6–9
P. capsici	6	0	0	<4days
P. cinnamomi	12	7	58	10
P. citricola	4	3	75	5–9
P. citrophthora	2	0	0	<4days
P. cryptogea	6	3	50	8–10
P. drechsleri	3	2	66	9–10
P. erythroseptica	12	7	58	6–11
P. fragariae	5	0	0	<4days
P. hevae	7	2	29	4,8
P. infestans	8	3	38	8–9
P. iranica	1	0	0	<4days
P. lateralis	1	0	0	<4days
P. meadii	1	0	0	<4days
P. megasperma	19	8	42	6–10
P. megasperma var. *sojae*	1	0	0	<4days
P. mexicana	1	0	0	<4days

Data from CMI records

Table 1.0374 Viability of *Phytophthora* species stored in liquid nitrogen

Name	Number isolates viable after storage			Survival period (years)
	Tested	Viable	%	
P. nicotinae	16	16	100	5-12
P. palmivora	80	39	49	5-12
P. porri	5	1	20	9
P. syringe	1	0	0	<4days

Data from CMI records

Table 1.0374 gives details of the Phytophthora species stored in liquid nitrogen. All 16 isolates of P. nicotianae tested survived and 30 of 80 (49%) of P. palmivora were preserved. The survival periods given are the range in years the isolates have remained viable to date (1985) and are not limiting as all isolates that survived the initial treatment have remained viable during storage.

The species of Phytophthora that have been stored without failure using liquid nitrogen are, P. arecae (1 isolate), P. boehmeriae (2 isolates) and P. nicotianae (16 isolates). Some isolates of the remaining 21 species died and this may have been due to poor growth in the preliminary culture. These were isolates that only gave poor restricted growth on Lima bean agar, Oat agar or Corn meal agar.

Table 1.0375 lists the 27 species of Pythium tested and indicates the species which were successfully processed. Of the 58 isolates that were tested, 30 (52%) failed to survive. The number of isolates tested of each species was quite low. All the isolates of some species failed, P. aristosporum (0/4) and P. iwayamae (0/4) and all isolates of some other species survived P. flevoense (4/4).

Table 1.0375 Viability of *Pythium* species stored in liquid nitrogen

Name	Number isolates viable after storage			Survival period (years)
	Tested	Viable	%	
P. acanthicum	1	0	0	<4days
P. aphanidermatum	2	2	100	8
P. aristosporum	4	0	0	<4days
P. arrhenomanes	1	1	100	7
P. artotrogus	1	0	0	<4days
P. butleri	3	2	66	7,9
P. coloratum	1	1	100	5
P. debaryanum	3	3	100	6–8
P. deliense	2	1	50	9
P. flevoense	4	4	100	5
P. fluminum	4	0	0	<4days
P. graminicola	2	0	0	<4days
P. helicoides	3	1	33	5
P. hydnosporum	1	0	0	<4days
P. irregulare	3	1	33	7
P. iwayamai	4	0	0	<4days
P. middletonii	2	2	100	4,8
P. myriotylum	3	3	100	4–8
P. oligandrum	2	0	0	<4days
P. periplocum	1	0	0	<4days
P. polymorphum	1	1	100	7
P. spinosum	1	1	100	8

Data from CMI records

Table 1.0375 Viability of *Pythium* species stored in liquid nitrogen (continued)

Name	Number isolates viable after storage			Survival period (years)
	Tested	Viable	%	
P. splendens	1	1	100	9
P. sulcatum	2	0	0	<4days
P. sylvaticum	2	1	50	4
P. tracheiphilum	1	1	100	2
P. undulatum	1	1	100	8
P. vexans	2	1	50	5

Data from CMI records

Table 1.0376 Viability of other isolates belonging to the Mastigomycotina excluding *Phytophthora* and *Pythium* stored in liquid nitrogen

Species	Number isolates tested	Number isolates viable	Survival period (years)
Achlya ambisexualis	13	3	1-5
A. americana	2	1	4
A. bisexualis	10	0	<4days
A. debaryana	3	0	<4days
A. racemosa	7	3	4-8
A. radiosa	4	0	<4days
Allomyces anomalus	2	2	3,8
A. cystogenus	1	1	4
A. javanicus	3	2	4,5
Aphanomyces brassicae	2	1	4
A. euteiches	12	5	4-7
Chytridium olla	2	0	<4days
C. ottariense	1	0	<4days
Dictyuchus sterilis	2	0	<4days
Entophlyctis confervae-glomeratae	2	2	4,8
Hyphochytrium catenoides	7	5	4-10
Phlyctochytrium acuminatum	4	2	4,10
P. arcticum	2	1	4
P. californicum	3	1	3
P. plurigibbosum	2	0	<4days
P. reinboldtae	1	0	<4days
Protachlya paradoxa	3	1	<4days

Data from CMI records

Table 1.0376 (continued) Viability of other isolates belonging to the Mastigomycotina excluding *Phytophthora* and *Pythium* stored in liquid nitrogen

Species	Number isolates tested	Number isolates viable	Survival period (years)
Rhizoclosmatium globosum	3	0	<4days
Rhizophydium biporosum	2	1	4
R. capillaceum	3	0	<4days
R. chlorogonii	1	0	<4days
R. granulosporum	3	0	<4days
R. haynaldii	3	0	<4days
R. karlingii	1	0	<4days
R. patellarium	3	0	<4days
R. sphaerocarpum	2	0	<4days
R. sphaerotheca	7	0	<4days
Saprolegnia ferax	4	1	4
S. glomerata	1	0	<4days
S. litoralis	3	0	<4days
S. megasperma	2	2	4,6
S. parasitica	6	1	1
Thraustotheca clavata	5	1	4

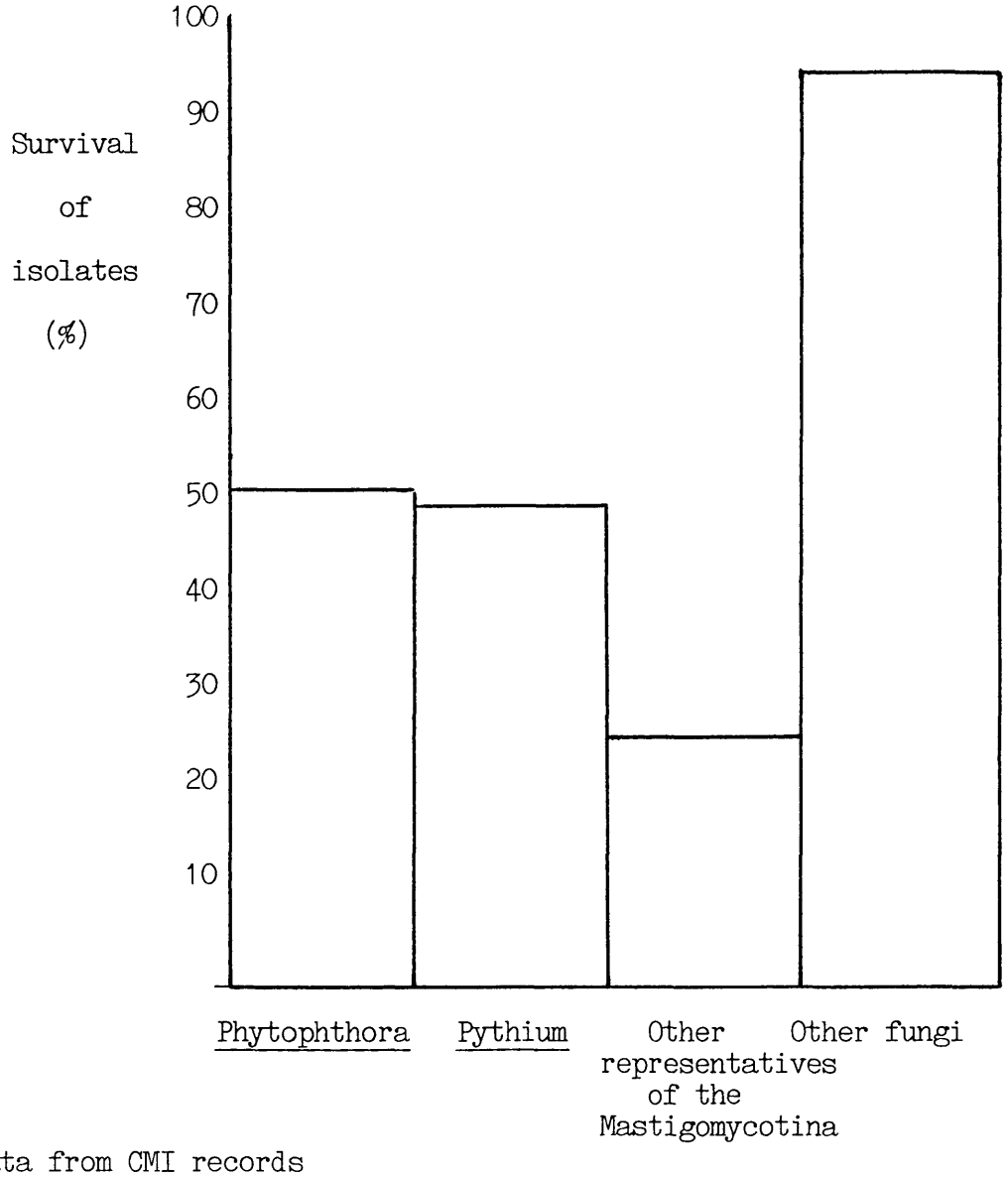
Data from CMI records

The Mastigomycotina excluding Phytophthora and Pythium show poor results (Table 1.0376). All 10 isolates of Achlya bisexualis tested failed and only 3 isolates of the 13 tested of A. ambisexualis remained viable for perids of 1-5 years.

The other representatives of the Mastigomycotina belonging to the Blastocladiales, Chytridiales, Hyphochytridiales and Saprolegniales which were frozen and stored in liquid nitrogen numbered some 38 species totalling 137 isolates of which only 36 survived more than 4 days. One hundred and one isolates failed to survive freezing, 27% of the total failures of the liquid nitrogen storage method to date (1985). However, the isolates that were tested had been in the CMI culture collection for up to 30 years before attempts were made to freeze them. This may have adversely affected their ability to survive.

Unfortunately the viabilities of these fungi were not carried out quantitatively at CMI. However, 14 ampoules of each isolate were frozen and stored and, although a positive result was scored by the regrowth of a healthy culture from 2 ampoules, all 14 were openned to record a failure.

Fig. 1.037c The comparison of percentage survival of the genera Phytophthora and Pythium, the other Mastigomycotina including representatives of the Blastocladiales, Chytridiales, Hyphochytridiales and Saprolegniales with other fungi



Data from CMI records

As can be seen from the data assembled from the CMI records the Mastigomycotina proved to be more susceptible to failure in liquid nitrogen storage than fungi belonging to other subdivisions (Fig. 1.037d). Almost 94% of the fungi tested, excluding the Mastigomycotina, survived freezing and recovery from liquid nitrogen whereas only 50% of the isolates of Phytophthora, 48% of Pythium and 26% of the remaining representatives of the Mastigomycotina survived. The survival periods were similar. As the isolates were preserved over a period of 1-14 years longevities could not be compared directly with the groups as a whole.

It was observed that many isolates that failed to survive had grown poorly on agar prior to preservation. Many of the isolates had been stored for several years under mineral oil and had shown deterioration before freezing and storage in or above liquid nitrogen. The preservation of fresh isolates and avoiding excessive manipulation may therefore improve results.

1.038 Comparsion of the efficiency of silca gel, oil, centrifugal freeze drying and liquid nitrogen storage.

As discussed above oil storage was introduced as a preservation technique at CMI in 1955, and over 10,000 isolates have been stored by this means. Most isolates of fungi can be kept under mineral oil but some show variation and others survive only short periods of time and require regular transfer. However, many isolates cannot be stored by the techniques of silica gel storage, centrifugal freeze drying and liquid nitrogen storage. Taxonomic groupings have been made of the fungi tested by these latter techniques and presented in tables 1.0351, 1.0361

and 1.0371. The fungi tested were processed on different occasions and consequently longevities cannot be compared directly for the isolates that are still viable. The methods of preservation were introduced at CMI over a period of years and therefore the maximum storage period for oil is 32 years whereas it is 15 years for centrifugal freeze drying, 14 years for liquid nitrogen storage and 11 years for silica gel. A comparison of these results show that silica gel storage is inferior to centrifugal freeze drying and liquid nitrogen storage. Only 59% (20 from 34 isolates) of the Zygomycotina survived silica gel storage whereas 92% (754/821) survived centrifugal freeze drying and 95% (254/267) survived liquid nitrogen storage. Of the Basidiomycotina tested 52% (10/19) of the Hymenomycetes survived silica gel storage, only 42% (41/91) survived centrifugal freeze drying but 96% (143/149) survived in liquid nitrogen storage. The only Gasteromycete tested in silica gel died, 2 of the 5 isolates centrifugally freeze dried survived whereas all 8 tested survived liquid nitrogen storage. When attempts were made to preserve the Coelomycetes in silica gel 75% (21/28) survived but both centrifugal freeze drying, 91% (526/576), and liquid nitrogen storage, 94% (224/238) were more successful. The Hyphomycetes were easily preserved although only 81% (202/250) survived silica gel storage whereas 95% (4822/5091) survived centrifugal freeze drying and also 95% (1465/1543) survived liquid nitrogen storage. The Ascomycotina also survived silica gel storage less well. Forty percent (4/10) of the Dothideales survived whereas 95% (225/236) and 89% (97/109) survived

centrifugal freeze drying and liquid nitrogen storage respectively. A similar pattern of survival was seen with the Diaporthales where 60% (3/5) survived in silica gel, 89% (32/36) survived by freeze drying and all 17 isolates survived liquid nitrogen storage. This analysis shows that centrifugal freeze drying and liquid nitrogen storage are equally effective preservation methods for these fungi. However liquid nitrogen and mineral oil storage are the only ones of the four that allow the long term preservation of isolates of the Mastigomycotina.

1.04 A summary of the preservation techniques

When a method of preservation is chosen there must be a balance between requirements, cost in material and labour, longevity and genetic stability. In this study the methods available were evaluated and their effectiveness in the retention of viability and stability compared. The methods used for the preservation of filamentous fungi range from the very simple and inexpensive to the complex techniques that require expensive equipment. It is important that culture collections choose their methods carefully. By far the most important criterion is that no morphological or physiological changes of the fungi are allowed to occur during storage. All culture collections must ensure that they use methods that are cost effective and in many cases choose the inexpensive technique. The cheaper technique may not retain the desired stability. In this study data will be gathered to show which techniques are most suitable.

1.05 Objectives

The methods available for the preservation of filamentous fungi will be investigated and compared. The simple and

inexpensive techniques of water, soil, silica gel and oil storage will be examined and compared with the more sophisticated techniques of freeze drying and liquid nitrogen storage. Their usefulness, particularly to large service collections, will be assessed and discussed.

To evaluate the water storage technique isolates of Phytophthora and Pythium will be recovered from liquid nitrogen or oil storage and stored in water and their viabilities determined during storage. The isolates will be stored in liquid nitrogen if not already preserved by this technique and their viabilities compared on recovery.

As there are no reports on the stability of characteristics of Fusarium after long term storage in soil the effectiveness of this technique will be assessed by retrieving some of the isolates stored by Booth and examining their morphological stability by comparison with the descriptions and the microscope slides of the original material preserved.

To evaluate the oil, silica gel, liquid nitrogen and centrifugal freeze drying techniques the work of Onions (1977) will be followed up. Storage of fungi in oil had been compared with their short term storage in silica gel and longer storage in liquid nitrogen and centrifugal freeze drying. This would show if the continuous growth technique of oil storage allowed more variation than the other techniques. Such experiments would also evaluate the suitability of silica gel storage and give valuable comparison with what are believed to be the best available preservation techniques, freeze drying and

liquid nitrogen.

The reasons why some filamentous fungi fail to survive the technique of liquid nitrogen storage have been discussed (1.03) and will be considered in the development of the technique. A technique will be developed to reduce mechanical damage and cultures will be grown at optimum conditions prior to preservation for examples of fungi normally difficult to maintain.

Many cryoprotectants have been used to preserve other microorganisms but not all have been used to protect fungi during cooling. Experiments will therefore be initiated to determine if examples of fungi could be better protected by compounds other than glycerol during freezing.

The cooling rate of 1 $^{o}C\ min^{-1}$ has been used since the first experiments with limited numbers of test organisms. A cooling rate that allows survival may not be the optimum as has been shown by cooling rate/viability curves of other cell types (Leeson, Cann & Morris, 1984). Use of a cryomicroscope can show the physical changes that occur during cooling (McGrath, 1985). Experiments will be designed to observe examples of fungi at different cooling rates both with and without cryoprotectant and viability tests will be carried out in parallel. It is hoped that these experiments will give the optimum cooling rate and elucidate the mechanisms of cryoinjury and cryoprotection.

A review of the available literature on the freeze drying (1.027) of fungi revealed that many isolates failed to survive this method of preservation but the stage of this technique where death occurred had not been determined. If the point of death

were known it may be possible to discover why the isolate failed to survive and then the necessary changes to the technique made to avoid these conditions. The effect of cooling rate, warming procedure and final residual water content will be investigated using the Minifast 3400 shelf freeze drier (Edwards High Vacuum Ltd.) which is more versatile than the Edwards EF6 centrifugal freeze drier.

The information gathered and the developed techniques may be of valuable assistance to the many collections of fungi in the world. In the incomplete list of 566 collections of microorganisms given in the world directory of collections (McGowan & Skerman, 1982) over 200 hold strains of fungi. There are numerous smaller collections maintained for private use and housed by university departments, research institutes and industry which are not included in this directory (Hawksworth, 1985). All collections are looking for improved techniques that will retain the characteristics of their isolates. It is therefore hoped that this study will go some way to meet these ends.

CHAPTER 2.

MATERIALS AND METHODS

2.01 The organisms tested

The 1120 isolates of fungi selected for the investigation of the preservation techniques are listed in Appendix III. The methods they were tested by, their growth medium, temperature and light requirement are given. They were grown on media that gave optimum sporulation and were illuminated with near ultraviolet light to stimulate sporulation (2.18) if necessary. The fungi represent the subdivisions, Mastigomycotina, Zygomycotina, Ascomycotina, Basidiomycotina and the Deuteromycotina. The specific reasons why some of the isolates were chosen to assess a particular method are given in the introduction or in the brief introduction to the relevant chapter.

2.02 Culture media

The formulae of the media used for the cultivation and preparation of fungi prior to preservation tests and for viability tests are given below. Agar slopes were prepared in 30 ml universal bottles. The media were prepared in medical flats and either poured immediately after cooling into Petri dishes (15 ml per dish) or stored. Solid media were melted prior to pouring from the stored medical flats by heating them in an autoclave for 5 min at 121 °C.

Cornmeal (Maize) Agar (CMA)

Cornmeal (T.R. Suterwalla & Sons Ltd.)..... 30g

Agar (Oxoid, No. 3) 20g

Water ..... 1 l

The cornmeal and water were heated in a beaker in a boiling water bath and stirred at 5 min intervals for 1 h. The mixture was filtered through muslin, the agar added, and heated until it dissolved. The medium was autoclaved for 15 min at 121 °C.

Czapek (Dox) Agar (CZ)

Stock Czapek solutions (A and B) were made up as follows:-

A

$NaNO_3$ 40g

KCl 10g

$MgSO_4 7H_2O$ 10g

$FeSO_4 7H_2O$ 0.2g

The salts were dissolved in 1 l of distilled water and stored at 4 °C.

B

K_2HPO_4 .....20g

This was dissolved in 1 l of distilled water and stored at 4 °C.

The trace element solutions a and b were prepared as follows.

(a) $ZnSO_4 7H_2O$ .....1.0g in 100ml water

(b) $CuSO_4 5H_2O$ .....0.5g in 100ml water

Stock solution A (50 ml) and B (50 ml) were added to 900 ml of distilled water. Twenty grams of agar were dispersed in the solution mixture, 30 g of sucrose and 1 ml each of solutions a and b were added. The medium was autoclaved for 20 min at 120 °C.

Czapek (dox) broth

This medium was prepared as above (Czapek dox agar) omitting the agar.

Dimethyl sulphoxide cryoprotectant medium (DMSO)

Dimethyl sulphoxide was prepared as 5 or 10% (v/v) solutions in distilled water, dispensed into glass universal bottles in 15 ml amounts and autoclaved for 15 min at 121 °C.

Dimethyl sulphoxide/glucose cryoprotectant medium (DMSO + Glu)

DMSO-Glucose mixtures were prepared in aqueous solution in the following concentrations (v/w/v), 10%/5%, 10%/8%, 10%/10%, 5%/10%, 8%/10% and 15%/10%. The mixtures were dispensed into glass universal bottles in 15 ml amounts and autoclaved for 15 min at 121 °C.

Egg Yolk Medium (EGG)

A fresh hen's egg was soaked in 90% alcohol, with 1 ml of

acetone for 2h. The alcohol mixture was flamed off and a 5 mm hole punctured into each end. The egg white was discarded and the yolk membrane punctured. The yolk was poured into glass universal bottles or Petri dishes and sterilized in steam at 80 °C for 30 to 45 min.

Glucose cryoprotectant medium

Glucose was prepared as 5 or 10% w/v solutions in distilled water, dispensed in 15 ml amounts into glass universal bottles and autoclaved for 10 min at 114 °C.

Glycerol cryoprotectant medium

Glycerol was prepared as a 10% v/v solution in distilled water, dispensed in 15 ml amounts into glass universal bottles and autoclaved for 15 min at 121 °C.

Hemp seed medium (HEMP)

Twenty hemp seeds were placed in 15 ml of distilled water in a glass universal bottle and heated in an autoclave for 20 min at 121 °C.

Malt Czapek Agar (MCZ)

The Czapek Dox solutions A and B were prepared as described above and additions made as follows:-

Stock Czapek solution A 50ml

Stock Czapek solution B 50ml

Sucrose (B.D.H. Ltd.).....30g

Malt extract (Edme Ltd.).....40g

Agar (Oxoid. No.3).....20g

Distilled water 900ml

The malt extract and agar were dispensed in the water, the

two stock Czapek solutions and sucrose were added and heated in a beaker in a boiling water bath until all constituents were dissolved. The medium was dispensed into glass universal bottles and autoclaved for 20 min at 121 °C. The pH was then adjusted to between 4 and 5 with 10% (w/v) sodium hydroxide or 10% (v/v) hydrochloric acid in distilled water as required.

Malt Extract Agar (MA)

Malt extract (Edme Ltd.).....20g

Agar (Oxoid No.3).....20g

Water .....1 l

The malt extract was heated in the water until dissolved, the agar was then added and the mixture boiled until the agar dissolved. The medium was then dispensed into glass universal bottles and autoclaved for 20 min at 121 °C. The pH was between 3 and 4 and was adjusted to 6.5 with 10% w/v sodium hydroxide in distilled water.

Malt Extract-Sucrose(M)

Malt extract agar was prepared as above and supplemented by addition of sucrose just before boiling to reduce caramelization. The concentrations of sucrose, expressed as % (w/v) of the water included were 20 (M/20), 40 (M/40) and 60 (M/60).

Oat agar (OA)

Powdered oatmeal (30 g) was added to 1 l of water in a beaker, heated in a boiling water bath and simmered for 1 h with occasional stirring. The mixture was filtered through muslin, made up to 1 l and 20 g of Japanese Kobe agar (Oxoid) was added. The mixture was heated until dissolved, dispensed into glass

universal bottles or 250 ml medical flats and autoclaved for 20 min at 121°C.

Oat Agar plus Rice (OA+R)

Rice grains in a glass universal bottle were autoclaved for 20 min at 121°C. When cold 20 grains were added to each Petri dish or universal bottle of oat agar medium prepared as above.

Onion seed medium (ONION)

Twenty onion seeds were placed in 15 ml of distilled water in a glass universal bottle autoclaved for 20 min at 121°C.

Polyvinyl pyrrolidone cryoprotectant medium (PVP)

A 10% v/v solution of PVP was prepared. PVP was filter sterilized using a millipore syringe filter (0.5u pore) and added to water which had been sterilized by autoclaving for 15 min at 121°C.

Potato Carrot Agar (PCA)

The potatoes and carrots were washed, peeled and grated. Grated potato (20 g) and carrot (2g) were boiled for 1 h in 1 l of tap water. The mixture was passed through a fine sieve, 20g agar added and heated in a beaker in a boiling water bath until the agar dissolved. This was then dispensed into glass universal bottles or 250 ml medical flats and heated in an autoclave for 20 min at 121°C.

Note

Unripe or immature potatoes are usuitable for this medium PDA and PSA below.

Potato carrot agar plus filter paper (PCA+FP)

Strips of filter paper 10 mm x 30 mm were sterilized in the

vapour from 0.1 ml of propylene oxide in a 100 ml screw cap jar for 24 h. One strip was added to a Petri dish or universal bottle slope of potato carrot agar.

Potato Dextrose Agar (PDA)

Potatoes were scrubbed and cut, without peeling, into 12mm cubes. Two hundred gram quantities were rinsed rapidly under a running tap and boiled in 1 l of tap water for 1 h. The potatoes were blended, 20g agar added and the mixture heated until the agar dissolved when 15 g of dextrose was added. The volume was made up to 1 l with distilled water and the mixture was dispensed into glass universal bottles after agitating the stock to ensure that the solid matter was evenly distributed. The medium was autoclaved for 20 min at 121 °C. See note under PCA above.

Potato Sucrose Agar (PSA)

Potatoes (1.8 kg) were peeled and diced into 10 mm cubes, suspended in a double cheesecloth in 4.5 l of water and boiled until almost cooked (about 8 min). Sucrose (20 g), agar (20 g) and distilled water (500 ml) were added to 500 ml of potato water. The mixture was heated in a beaker in a boiling water bath until the agar dissolved and autoclaved for 15 min at 121 °C. The pH was adjusted to 6.5 with calcium carbonate. See note under PCA above.

Rabbit Dung Agar (RDA)

Five pellets of dung from wild rabbits were placed in a glass universal bottle and 20 ml of tap water/agar (TWA below) added. This was then heated in an autoclave for 20 min at 121 °C.

Skimmed milk medium

The skimmed milk (50 g) was dissolved in 1 l distilled water

and dispensed into glass universal bottles in 15 ml amounts which were heated in an autoclave for 10 min at 114 °C.

Skimmed milk and inositol medium

Skimmed milk powder (100 g) was dissolved in 1 l of distilled water and 50 g inositol added. The mixture was dispensed into glass universal bottles and heated in an autoclave for 10 min at 114 °C.

Skimmed milk and glycerol cryoprotectant medium

Skimmed milk (85 g) was dissolved in 900 ml of distilled water and 100 ml of glycerol added. The medium was mixed and dispensed in 15 ml amounts into glass universal bottles and heated in an autoclave for 10 min at 121 °C.

Tap water agar (TWA)

Agar (15 g) was dissolved in 1 l of distilled water and dispensed into glass universal bottles or 250 ml medical flats. The medium was heated in an autoclave for 20 min at 121 °C.

Tap water agar plus wheat straw (TWA+W)

Wheat straw was cut into 30 mm lengths and heated in an autoclave in glass universal bottles for 20 min at 121 °C. Two pieces were added to each Petri dish or universal bottle of TWA medium.

V8 agar

Agar (35 g) was dissolved in 140 ml of distilled water and 355 ml vegetable juice (Campbells, V8 canned) added. The pH was adjusted to 6.0 with 10% w/v sodium hydroxide in distilled water. The medium was heated in an autoclave for 20 min at 121 °C. The pH was checked after autoclaving and found to be 5.8 (Diener,

1955).

Yeast Phosphate Soluble Starch (YPSS)

Yeast Extract (Difco) 4g

Soluble starch (B.D.H. Ltd.).....15g

K_2HPO_4 .....1g

$MgSO_4 7H_2O$ 0.5g

Agar (Oxoid).....20g

Water 1l

The ingredients were mixed, dissolved, and dispensed into glass universal bottles or 250 ml medical flats and heated in an autoclave for 15 min at 121 °C.

All chemicals, unless stated otherwise, were obtained from B.D.H. Ltd., (British Drug Houses Ltd., see Appendix II).

2.03 Temperature measurement

All incubation temperatures were measured using thermometers calibrated against a British standard certificated thermometer (Baird & Tatlock (London) Ltd.).

(i) Temperature on the shelves of the Minifast 3400 freeze drier (Edwards High Vacuum Ltd.) were measured using the integral thermocouple and recorded on a chart recorder.

(ii) Temperatures in the vapour phase of the liquid nitrogen refrigerator (Union Carbide LR 320) were measured using the Minifast 3400 thermocouples and recorder (Edwards High Vacuum Ltd.) or a Digitron 1754 resistance thermometer (Digitron Instrumentation Ltd.).

(iii) Determination of the cooling rate with the Minifast 3400 prior to liquid nitrogen storage

Aliquots (0.5 ml) of glycerol (10% v/v) were placed in a

series of borosilicate glass ampoules which were about 50 mm apart on each shelf of the Minifast freeze drier. The temperature of the glycerol was monitored (2.03i) and the ampoules observed for signs of ice formation as the temperature approached 0 °C to determine the freezing point.

(iv) Cooling rate for cryomicroscopy

The temperatures to determine cooling rates for the viability tests carried out in parallel to the cryomicroscope observations were measured using copper constantan thermocouples connected to a BA5 channel selector (Kipp & Zonen) and recorded on a BD5 Micrograph recorder (Kipp & Zonen).

2.04 Preservation by continuous growth

The fungi were inoculated on to agar slants of selected growth media (Appendix III and section 2.02) in plastic (Sterilin) or glass (Adelphi [Tubes] Manufacturing Ltd.) universal bottles. All bottles were sealed by attaching sterile cigarette paper to the necks with copper sulphate gelatine adhesive. Pieces of cigarette paper had been sterilized in glass Petri dishes by adding 2-3 drops of propylene oxide and leaving overnight in the vapour. The adhesive was prepared from 20 g of gelatine dissolved in 100 ml of distilled water with 2g of copper sulphate. After heating in a boiling water bath to dissolve all the ingredients, a 15 ml quantity was poured into a Petri dish. The culture bottle neck was warmed and rotated in the glue and the sterile cigarette paper was attached. The excess paper was burnt away in a bunsen flame and the screw cap replaced and left loose (Snyder & Hansen, 1947).

When growth had been established the cultures were transferred to a refrigerator between 4 and 7 °C. Fungi sensitive to chilling effects were incubated at 15 °C (for example Piptocephalis species and Rhizopus species.

After prescribed periods of storage, transfers were made by cutting agar plugs from the aging culture and placing them, mycelium downwards, on to fresh media. At least two bottles of each strain was grown and stored. One was used in subsequent transfers and the other was a reserve.

2.05 Mineral oil storage

The method of Buell & Weston (1947) was adapted. Mature healthy cultures on agar slants in 30 ml universal bottles were covered with sterile liquid paraffin (medicinal grade, specific gravity 0.830–0.890). The paraffin had been sterilized by autoclaving twice for 15 min at 121 °C in volumes not exceeding 1 l for the first autoclaving. Approximately 20 ml quantities were dispensed in to universal bottles for the second heating. Cultures were covered to the depth of 10mm above the highest point of the slant with the sterile oil and the bottle caps left slack to allow diffusion of gases. The culture under the layer of oil was stored in an air conditioned room at 15 °C.

Viability was tested by the removal of a small amount of the fungal colony on a sterile needle. The oil was drained from the inoculum on the neck of the bottle and the fungus streaked on to the surface of a suitable growth medium and incubated at a favourable temperature (Appendix III).

2.06 Water storage

The method used was an adaptation of that used by Boeswinkel

(1976). Cubes ($6mm^3$) of agar were cut from the growing edge of a young fungal colony on a suitable growth medium (Appendix III) in a Petri dish. The blocks were transferred into sterile distilled water in 15 ml McCartney bottles (Payne Scientific Ltd.) and the rubber lined caps tightened. The bottles were stored at 15°C in an air conditioned room.

Viability was tested by removing an agar block from the bottle and placing it with the mycelium face down onto a Petri dish of agar medium (Appendix III).

2.07 Soil storage

The fungi were grown on agar slopes in universal bottles, sterile distilled water added and the mycelium and spores brought into suspension by gently scraping the colony with a sterile wire loop. Twice autoclaved soil (15 min at 121°C) in a universal bottle was inoculated with 1 ml of the suspension. The inoculated bottle was then incubated at room temperature with the cap loose for 5-10 days, according to the growth rate of the fungus being preserved, when drying will be complete. The cap of each bottle was tightened before storage at 4°C.

Viability was tested by sprinkling a few grains of the inoculated soil on to a suitable growth medium and incubating at a suitable temperature (Appendix III).

2.08 Silica gel storage

The method used was similar to those used by Ogata (1962) and Perkins (1962). Glass universal bottles were quarter filled with non-indicating silica gel (6-20 mesh) and heat sterilized at 180°C for 3 h. They were then placed in a bath of

water, to a depth level with the height of the silica gel, which was placed in a deep freeze at -20°C over night. Precooled (+4 to 7°C) 5% skimmed milk medium was added to a mature sporulating culture on an agar plate or in a universal bottle which was also precooled to between 4 and 7°C. The spores were released and suspended by gently scraping with a Pasteur pipette or a sterile loop. The frozen bath of ice containing the silica gel filled bottles was removed from the deep freeze 20-30 min before it was required to allow the ice to melt slightly around the bottles and facilitate their removal. An aliquot (approximately 2ml) of suspension was added at the side of the silica gel crystals until three quarters of the crystals were moist. The bottles were agitated to disperse the suspension evenly, incubated at 25°C for 10-14 days and when the crystals readily separated, their caps were screwed down tightly. The bottles were placed in airtight containers with an open bottle of indicator silica gel to absorb any condensing water and stored at 4°C.

Viability was tested by sprinkling small numbers of crystals on to a suitable agar medium (Appendix III).

2.09 Cryomicroscopy

(i) Cells and cell culture

Conidia of *Penicillium expansum* IMI 174158 or agar blocks cut from the edge of colonies of *Phytophthora nicotianae* IMI 158733 or *P. citrophthora* IMI 129906 growing on OA medium were inoculated into liquid media. The *Penicillium* was inoculated into CZ broth and the *Phytophthora* species into onion or hemp seed medium. The cultures were incubated for 48 to 72 h and

agitated occasionally to break up the colonies. The colonies produced were non-sporulating and were in the form of thin mycelial mats. Small intact colonies were selected for direct observation with the cryomicroscope.

(ii) Direct observation with a cryomicroscope

Direct observation of cells during freezing and thawing was carried out on a cryomicroscope conduction stage similar to that described by McGrath (1985), except that a microcomputer (Apple IIe) was used as controller. The Leitz Dialux 22 microscope used was fitted with a hollow brass stage through which nitrogen gas at temperatures lower than 100 ^{o}C was passed. The centre of the stage was fitted with a small heater which controlled the temperature. The different cooling rates were obtained by raising the stage temperature to 20 ^{o}C, placing the fungus colony on the stage and then allowing the nitrogen to cool the stage at rates controlled by the heater.

In all experiments hyphae were cooled on the cryomicroscope stage from 20 ^{o}C to 5 ^{o}C at a rate of 10 ^{o}C min^{-1}, were held at 5 ^{o}C for 0.5 min and then cooled at various uniform rates to -30 ^{o}C or -50 ^{o}C. Cells were maintained at that temperature for 0.5 min and rewarmed at 50 ^{o}C min^{-1} to 20 ^{o}C unless otherwise stated. There were at least three replicates for each cooling rate.

Observations using the temperature controlled stage were made on a Leitz Dialux 22 microscope with a 40/0.7 objective combined with an intermediate 2x magnification changer. Data were recorded with a video camera (Hitachi HV-65), Sony U-matic recorder, model VO-5630 and a video time generator (Panasonic

model WJ-810). The recorded data were analysed with a video monitor and selected frames photographed using a Polaroid Land camera (Model CU-5, film-type 665).

(iii) Hyphal shrinkage

The time recorded on the video (2.09ii) enabled the temperature to be calculated at any time during the recording. Measurements, at any given temperature, were taken of the hyphae directly from the video monitor screen using callipers.

(iv) Viability at different cooling rates

Colonies produced as above (2.09i) were placed in 2 ml polypropylene ampoules (NUNC) in 0.5 ml aliquots of either growth medium or 10% (v/v) glycerol and after equilibration, with the medium for 1 h in the latter case, they were cooled at rates similar to those achieved on the cryomicroscope stage. The number of colonies giving rise to fresh growth were counted and the proportion viable was calculated. Samples were taken from each culture which was used to provide colonies for cooling and the viability tested to give a pre-cooling viability.

The method of varying rates of cooling was similar to that of Morris & Farrant (1972). The tubes were held in a metal basket in an alcohol bath (CAM LAB) maintained at $-6\ ^{o}C$. A thermocouple leading to the BA5 channel selector (see 2.03iv) was placed in one of the ampoules which was used as the control. After 5 min each sample was seeded by touching the surface of the suspension with the tip of a Pasteur pipette which had been cooled to $-196\ ^{o}C$ in liquid nitrogen. Five minutes later the tubes were transferred either to liquid nitrogen and cooled at approximately $200\ ^{o}C\ min^{-1}$ or to a circular aluminium holder

suspended in an inner vessel held in liquid nitrogen in an evacuated silvered dewar (Jencons [Scientific] Ltd.) until its temperature reached $-6\ ^{o}C$. Different cooling rates were obtained by using inner vessels made of different materials, suspending the ampoules in air or in alcohol (Methylated spirit 740P) inside the vessel and by stirring (see Table 2.0941).

After cooling to $-55\ ^{o}C$ the sample tubes were plunged directly into liquid nitrogen. Subsequently the cooling rates in the control ampoule were determined. After storage for 5 min in the liquid nitrogen thawing was carried out by agitating the tubes in a water bath at $+37\ ^{o}C$ until all the ice had melted.

Table 2.0941 Composition of the inner vessel and suspending medium used to achieve various cooling rates prior to viability counts

Inner vessel structure and material	Alcohol		Stirring	Cooling rate achieved ($^{o}C\ min^{-1}$)		
	Presence (+) or absence (-)	Volume (ml)		Nominal	Actual 1	Actual 2
Unevacuated, unsilvered dewar	+	1250	+	1	1.14	1.25
Unevacuated, unsilvered dewar	+	750	+	2	2.11	2.15
Unevacuated, unsilvered dewar	+	250	+	3	2.86	3.05
Plastic beaker (2000 ml)	-	-	-	5	4.72	5.09
Plastic beaker (2000 ml)	-	-	+	7	7.85	7.05
Plastic beaker (2000 ml)	+	1000	-	10	9.1	11.0
Stainless steel beaker	+	1000	-	15	15.27	16.4
Stainless steel beaker	+*	1000	+	20	20.9	21.1
Stainless steel beaker	+	500	+	25	25.0	22.91
Stainless steel beaker	+*	1000	+	100	114.5	148.64

Alcohol temperature was $-6\ ^{o}C$ except where asterisked where it was $-60\ ^{o}C$.

2.10 Centrifugal freeze drying

All fungi tested were grown in universal bottles on selected agar media (Appendix III) to give maximum sporulation. Some fungi required stimulation by near ultraviolet light (2.16).

(i) Centrifugal freeze drying technique

Spore suspensions were prepared by adding 10ml of skimmed milk-inositol medium to the mature sporulating cultures, and gently scraping the fungal colony with a sterile wire loop. Aliquots (0.2 to 0.5 ml) of the suspension were dispensed with a Pasteur pipette into dry heat sterilized 0.5ml neutral glass ampoules (Adelphi [Tubes] Manufacturing Ltd.). Fifteen ampoules of each suspension were prepared. Caps, prepared by folding a 100 mm x 100 mm piece of lint and stapling together the sides and dry heat sterilized with the ampoules, were placed over each set of ampoules to prevent aerial contamination. Batches of ampoules were transferred to the centrifuge carriage of an Edwards EF6 primary freeze drying machine (Edwards High Vacuum Ltd.) and centrifuged at 1450 rpm while the chamber was evacuated. After 15 min, when the suspensions had frozen as indicated by a deflection on the Pirani vacuum guage, the centrifuge was switched off. After drying for 3 h, at a pressure of between 5×10^{-2} and 8×10^{-2} mbar, the chamber was brought to atmospheric pressure and the ampoules removed and plugged with sterile cotton wool which had been sterilized in propylene oxide vapour overnight. The plugs were compressed to 10 mm in depth with a metal or glass rod and pushed down to just above the tip of the slope of the freeze dried specimen. The ampoules were constricted using an air-gas torch (Buck & Hickman) about 10 mm above the cotton wool plug,

attached to the manifold of an Edwards 30S2 secondary freeze drying machine (Edwards High Vacuum) and evacuated over phosphorus pentoxide desiccant. The second stage drying was carried out overnight for a period of 17 h at a pressure of between 2 x 10 and 7 x 10^{-2} mbar, leaving a residual water content of the dried suspension of between 1 and 2%. The ampoules were then sealed using a twin jet crossfire burner (T.W. Wingent) and stored at 15 ^{o}C. After 3-4 days storage sample ampoules were opened by scoring the tube midway down the length of the cotton wool plug and cracking the ampoule by touching it on the score with the tip of a hot glass rod. The dried suspension was reconstituted by adding 3 or 4 drops of sterile distilled water from a Pasteur pipette and allowing 15-20 min for absorption of the moisture by the spores. The contents of the ampoule were streaked on to a suitable agar medium and incubated at an appropriate growth temperature (Appendix III).

(ii) Prefreezing freeze drying technique using the centrifugal freeze drying machine without centrifugation

The fungal suspensions were prepared and dispensed in ampoules as described above (2.10i). These were suspended in the gaseous phase of a liquid nitrogen refrigerator at an angle of 30 degrees to the horizontal in a basket at -35 ^{o}C for 1 h. They were then lowered to just above the surface of the nitrogen in the refrigerator (approximately at -190 ^{o}C) for 5 min. These were then transfered in the basket to the chamber of the EF6 centrifugal freeze drier. The chamber was sealed and evacuated immediately. After a 3 h drying period at a pressure of between

5×10^{-2} and 8×10^{-2} mbar the ampoules were plugged, constricted and transfered to the 30S2 secondary drier for completion of the freeze drying process as above (2.10i).

2.11 Freeze drying using Minifast 3400 freeze drying apparatus (shelf freeze drying)

(i) General technique

Spore suspensions prepared as for centrifugal freeze drying (2.09) were dispensed in 0.5 ml quantities into 5 ml flat bottomed glass vials (Adelphi [Tubes] Manufacturing Ltd.). Sterile, grooved, butyl rubber bungs were inserted aseptically into the necks of the vials which were placed on the precooled shelves of a Minifast 3400 (shelf) freeze drier (Edwards High Vacuum Ltd.). The shelf temperature was maintained at particular temperatures during the series of investigations. When the temperature of the suspension reached -20^{o}C, the chamber was evacuated. This lowered the temperature of the frozen material to below -45^{o}C due to the latent heat of vaporization. The warming rates were varied. When the drying period was complete, vials were automatically sealed with a butyl rubber bung at a vacuum of approximately 4×10^{-2} mbar.

Viabilities were tested by removing the butyl rubber bung and adding 3 to 4 drops of sterile distilled water with a Pasteur pipette. The bungs were replaced to prevent aerial contamination and after 20 to 30 min for absorption of the moisture the bung was again removed and the contents of the vial streaked on to a suitable growth medium (Appendix III).

(ii) The improved shelf technique

The Minifast 3400 shelf freeze drier shelves were cooled to -45^{o} C and the vials containing 1 ml of fungus suspension and a loosely fitted butyl bung were placed 10 mm apart on the two shelves. The integral temperature thermocouples were inserted into 2 similar vials without butyl bungs. The chamber door was closed and when the temperature of the suspension reached -20^{o} C the chamber was evacuated. The temperature of the suspension was held at -45^{o} C for 3 h and then raised to $+10^{o}$ C at 0.08^{o} C min^{-1}. After drying for 24 h, from the time the suspensions reached -45^{o} C, the vials were automatically sealed under vacuum with the butyl rubber bung. The chamber was brought to atmospheric pressure and the vials unloaded. Two vials of each organism were opened, one for a viability test (2.11i) and the other for water content determination.

2.12 Residual water content determinations

(i) Ten vials were labelled and weighed and 0.5 ml of skimmed milk-inositol medium added. The vials were freeze dried with a shelf freeze drier under conditions identical with those used for the fungal suspensions (2.11). Alternatively, 10 preweighed vials containing 1.0 ml of fungal suspension were used for the determinations. When the drying was complete the vials were opened over silica gel in a deep metal container to reduce the risk of moist air entering. The vials were placed in a metal tray with silica gel gel crystals and dried in an oven at 105^{o} C for successive 17 h periods. Contact with the dried vials was avoided by wearing disposable surgical gloves. The vials were weighed after each drying and when two consecutive weights were

within 0.1mg of each other, the loss in weight due to oven drying was calculated. The loss in weight was assumed to be due to the loss of the residual moisture and was calculated as follows:-

Residual water content (%) =

$$\frac{\text{(Weight of vial + pellet) before oven drying} - \text{(Weight of vial)}}{\text{(Weight of vial + pellet) before oven drying} - \text{(Weight of vial)}} \times 100$$

(ii) The recession of the ice front was observed in vials that were marked with a scale of 0.5 mm intervals. The vials were arranged in the shelf freeze drier so that the scale could be seen through the perspex door. The vials were observed at 1 h intervals and the height of the ice interface recorded until no further recession occurred.

2.13 Liquid nitrogen storage

The fungi were grown on slopes of selected agar media at a suitable incubation temperature (Appendix III) in 30 ml universal bottles. When growth was established, the fungi were incubated at between +4 and +7 °C for 5 to 14 days to allow acclimatization prior to cooling and freezing. When the cultures had reached maturity, 10 ml of cryoprotectant was added and the mycelium and/or spores were brought into suspension by gentle agitation and scraping of the fungal colony. An aliquot (0.5ml) of this suspension was added to a 1 ml borsilicate glass ampoule (Adelphi [Tubes] Manufacturing Ltd.) which was then heat sealed using an air-gas torch (flame master, Buck & Hickman). The seals were tested for leakage in an erythrocin B dye bath at +4 to +7 °C which also precooled the fungal suspensions. The fungi were left at this stage to ensure that they were in contact with

the cryoprotectant for at least 1 h. This pretreatment was carried out with every cryoprotectant or suspending medium tested. The ampoules were then clipped to aluminium rods (Jencons [Scientific] Ltd.) and suspended at -35 °C in the vapour phase of a liquid nitrogen refrigerator or placed on the shelf of the Minifast 3400 freeze drying machine set at -35 °C. After 60 min the cooled ampoules were immersed in liquid nitrogen at -196 °C. The ampoules were stored either clipped to aluminium rods in metal boxes in the refrigerator or in a drawer rack inventory control system (Denley Instruments Ltd.) in a liquid nitrogen refrigerator (Union Carbide LR320).

Ampoules were retrieved from the refrigerator and thawed by immersion in a water bath at +37 °C. After surface sterilization by immersion in, or wiping with, 70% alcohol the ampoules were opened by scoring the neck with a glass cutter and snapping it open. The contents were removed with a Pasteur pipette and streaked onto a suitable agar growth medium (Appendix III).

Fungi which are damaged by excessive manipulation, for example some species of Phytophthora, Achlya, Allomyces and other water moulds, were handled by a more elaborate preliminary method. The fungus was grown on a selected agar medium (Appendix I) and either slivers of agar or agar plugs were cut out of the colony and transferred to the ampoules with 0.5 ml of cryoprotectant. When this technique proved unsuccessful, the fungi were grown on small quantities of agar medium (approximately 1ml) in wide necked, polypropylene ampoules with

screw caps (Jencons [Scientific] Ltd.) and 0.5 ml quantities of cryoprotectant added when the colonies had matured. In both cases the subsequent freezing protocol was as described above. Screw capped ampoules were thawed in a 37 °C water bath and surface sterilized by wiping with 70% ethyl alcohol, before the caps were removed.

2.14 Freeze preservation of Sclerospora graminicola and Erysiphe pisi

The infected host plants were provided for this work by Reading University (Sclerospora graminicola) and Imperial College of Science and Technology (Erysiphe pisi).

(i) Tissues infected with S. graminicola

Florets or leaves infected with S. graminicola were removed from the plants and placed in sterile, polypropylene, screw capped ampoules. A cryoprotectant (0.5ml) was added and the ampoules precooled to between 4 and 7 °C and held at this temperature for 1 h for pretreatment to allow the cryoprotectant to peneterate the cells. The ampoules were then placed in the vapour phase of a liquid nitrogen refrigerator (Union Carbide, LR320) at -35 °C. After 1 h they were immersed in liquid nitrogen at -196 °C (for 2 min) and then stored in the vapour phase above it (-150 to -190 °C).

The ampoules were thawed by immersion in a water bath at 37 °C. The florets or leaves infected with S. graminicola were placed on moist filter paper in contact with growing host seedlings. The proportion of seedlings showing growth of the fungus on the cotyledons was recorded.

(ii) Conidia of Erysiphe pisi

Mature conidia were obtained by blowing off the aging spores from an infected pea plant and allowing a 24 h period for maturation of the remaining spore initials. These spores were harvested on aluminium foil strips (5mm x 15mm) by tapping a developed infected leaf over it. To avoid damage that may be caused by harvesting the spores a second method was also tried. The epidermis of the infected pea leaf was removed and placed on similar pieces of aluminium foil. In both cases the pieces of foil were folded into a V-shape and the open edges crimped together with toothed forceps. The packets were cooled rapidly by immersion in Arcton 22 at its melting point (approximately -150^{o} C) which was prepared by passing the gas through a coiled pipe in liquid nitrogen and collecting the liquid in a metal cup floating on liquid nitrogen.

The foil packets were thawed immediately afterwards by immersion in water at 20^{o} C or by holding them between two metal plates at approximately 20^{o} C. The spores were released to the surface of water in small transparent cups, 25mm in diameter.

The spores were examined under the microscope and again after 24 h incubation at approximately 20^{o} C.

2.15 Freeze preservation of Bremia lactucae

Lettuce seedlings were grown and infected with Bremia lactucae at the National Institute of Agricultural Botany. Subsequently infected leaves were detached from the diseased plant and cut into 5 mm x 5 mm pieces. Six pieces were placed in 0.5ml of cryoprotectant in a polypropylene screw capped ampoule. Cooling and thawing was carried out similarly to the method used

for infected tissue of Sclerospora graminicola (2.14i). The thawed leaf portions were removed and placed infected side down on to freshly grown detached lettuce cotyledons on moist filter paper in a Petri dish. The filter paper was kept moist by the addition of sterile distilled water during incubation in daylight at 20°C. The proportion of infected cotyledons were estimated after one week.

2.16 Viability counts

(i) Spore germination on microscope slides

The concentration of spores was determined using a haemocytometer and dilutions made to give a concentration of 1×10^{3} ml^{-1}. Germination tests were carried out by placing a drop of spore suspension on to a microscope slide, immediately examining under the microscope for spores that had already germinated, and incubating them in a moist chamber overnight at 25°C over moist filter paper in a 90 mm petri dish. A bent glass rod acted as a support for the slides. The slide was re-examined and the proportion of germinated spores was counted for at least ten microscope fields at a magnification of x400 to give a total of 100 spores counted where possible.

(ii) Spore germination tests on agar media

A drop of the diluted spore suspension (prepared as above 2.16i) was streaked across a Petri dish (TWA), (MA) or (PCA) and examined under the microscope for spores that had germinated prior to incubation. The plates were incubated at 25°C and examined at intervals up to 7 days. The percentage of germinated spores was calculated for at least ten microscope fields or,

where the spore concentration in the suspension was low, spores were examined along the streaks on the agar at magnification x400 to give a total of approximately 100 or more spores counted where possible.

(iii) Viability of non-sporulating fungi

The viability for non-sporulating fungi was determined by counting the number of agar plugs preserved that gave rise to fresh growth. The proportion of plugs with viable mycelia was calculated.

2.17 Growth tests

Spore germination was followed by a growth test which was assessed by a comparison with the original rate of coverage of a 90 mm Petri dish of agar medium and its original degree of sporulation. This was recorded on a five point scale (Normal (N), reduced (R), further reduced (F), abnormal (AN) to nil

2.18 The use of near ultraviolet light (black light) to induce sporulation

The fungi were grown in plastic Petri dishes or plastic universal bottles (Onions, 1969) for 3-4 days before irradiation and the edges sealed with clear tape to prevent rapid drying. Rich growth media were avoided as they gave rise to excessive growth of mycelium (Smith & Onions, 1983b). Fungi were therefore grown on weak media such as potato carrot agar prior to stimulation under black light.

The illuminators comprised three 1.22 m fluorescent lamps 130 mm apart. A black light tube (Philips TL 40 W/08) was held in the centre and cool white tubes (Philips MCFE 40 W/33) were supported on each side. The lamps were controlled by a time

switch which gave a 12 h on/off cycle. The Petri dishes or bottles were supported on a shelf 320 mm below the light source and were illuminated until sporulation was induced.

2.19 Control of mite infestation

Mites, commonly of the genera Tyrophagus and Tarsonemus which sometimes contaminate fungal cultures, were controlled by several measures. Hygiene, mechanical barriers and protective storage were used to prevent and control mite infestation.

All work surfaces were kept clean and the cultures protected from aerial infestation by storage in cabinets or incubators. The benches were washed with the non-fungicidal acaricides 0.5% (w/w) Tedion V-18 (Mi-dox Ltd.) or 0.2% v/v Actelic (Imperial Chemicals Industries Ltd.).

If mites were detected, the contaminated cultures were destroyed by autoclaving. The work benches were cleaned with one of the above acaricides and the uninfested cultures protected.

Cultures of mite infested fungi which were irreplaceable were stored in a deep freeze at -18 °C for three days before being subcultured. Isolates which could not survive this short term cold storage were covered with a layer of mineral oil and subcultured after 24 h.

The use of cigarette seals described in section 2.04 prevented mites from entering universal bottles containing colonies of fungi on agar slopes.

CHAPTER 3

THE INVESTIGATION OF A WATER STORAGE TECHNIQUE FOR THE PRESERVATION OF FUNGI

Contents

3.01 Introduction

Experiments on water storage (2.06) were initiated with species of *Phytophthora* and *Pythium* because the characteristics of strains of both genera vary greatly during culture and are difficult to preserve (Dahmen, Staub & Schwinn, 1983). The differing results obtained by Boeswinkel (1976) and Marx and Daniel (1976) also stressed the need for further investigation. The viability and condition in culture after recovery from storage were recorded and the results compared with those for liquid nitrogen storage at CMI.

3.02 The comparison of preservation of isolates of Phytophthora by water storage and liquid nitrogen storage

Thirty agar blocks of each of the 56 isolates of the genus *Phytophthora* chosen were stored in water. At yearly intervals two samples were removed for each viability test. When only one sample grew two further samples were taken to confirm the result. If both samples failed to grow the test was repeated until growth was observed or all samples had been used. Thirty one of the isolates had been previously preserved in the CMI liquid nitrogen collection. Viability tests for the two methods are compared (Table, 3.021).

Only one isolate of *Phytophthora* did not grow after one year storage and two further isolates could not be recovered after the second year of storage. The three isolates of *Phytophthora* that failed to survive two years storage were as follows:

P. cambivora IMI 40505; the other isolate tested survived. *P. erythroseptica* IMI 181716; three other isolates survived. *P.*

hevae IMI 147352; two other isolates survived.

Some of the isolates which survived water storage, failed to survive in liquid nitrogen. These were P. erythroseptica IMI 17028 which survived 3 years in water, P. infestans IMI 181530 which survived 2 years in water, P. megasperma IMI 32035 which survived 3 years in water and P. palmivora which survived 4 years in water. One isolate of P. cinnamomi IMI 211105, which survived in water, died between one and two years storage in liquid nitrogen leaving 84% of those tested by both water and liquid nitrogen viable for the rest of the test period. After 3 years storage in water 82% of the isolates still remained viable but at the end of the test period (5 years) only 23% (13/56) were viable. In liquid nitrogen the subsequent deterioration did not occur.

Sporulation of some isolates also declined during storage. After 2 years in water P. hevae IMI 147352 did not sporulate whereas after 11 years in liquid nitrogen it grew and sporulated normally. P. cryptogea IMI 45168 grew normally and sporulated after 5 years in both water and liquid nitrogen but after 13 years in liquid nitrogen it grew only vegetatively. Of the 29% (9/31) of isolates that survived 5 years in water, 56% (5/9) were in poorer condition than the same isolates retrieved from liquid nitrogen.

The CMI records (Table, 1.0331) showed that of 16 isolates tested with their plant hosts, 15 were more virulent after storage in liquid nitrogen than after storage in water. Four of the isolates stored in water had lost the ability to grow on their hosts (Fig. 3.042a).

Table 3.021 Viability and condition of isolates of *Phytophthora* species stored in water and liquid nitrogen for periods of up to 5 years. Where only some samples survived the percentage is given in brackets

Name	IMI Number	Water storage					Liquid nitrogen	
		Samples viable for period given (years)			Condition		Period viable (years)	Condition after final test
		All	Some	None	2 yr	5 yr		
P. arecae	62655	5	-	-	N	V	7	N
P. arecae	62656	2	-	3	N	F	NT	NT
P. botryosa	136915	5	-	-	N	V	7	N
P. botryosa	136916	3	-	4	N	F	12	N
P. cactorum	21168	2	3(50)	5	N	F	13	N
P. cactorum	49562	2	3(50)	4	N	F	13	N
P. cactorum	62471	4	-	5	N	F	4	N
P. cambivora	40505	-	-	1	F	NT	NT	NT
P. cambivora	77374	5	-	-	V	AV	12	V
P. capsici	45528	4	-	5	N	F	8	N
P. cinnamomi	22938	3	-	4	N	F	7	N
P. cinnamomi	158786	3	-	4	N	F	11	N
P. cinnamomi	211105	5	-	-	N	V	2	N
P. cinnamomi	230381	3	-	4	V	F	NT	NT
P. citricola	21173	5	-	-	N	N	NT	NT
P. citricola	202319	3	4(75)	5	N	F	8	N
P. colocasiae	143253	4	-	5	N	F	NT	NT
P. cryptogea	21278	3	4(25)	5	V	F	7	V

N, Normal growth and sporulation; V, Vegetative growth only; AV, Abnormal vegetative growth; NT, Not tested; F, failed to grow.

Table 3.021 (continued) Viability and condition of isolates of *Phytophthora* species stored in water and liquid nitrogen for periods of up to 5 years. Where only some samples survived the percentage is given in brackets

Name	IMI Number	Water storage					Liquid nitrogen	
		Samples viable for period given (years)			Condition		Period viable (years)	Condition after final test
		All	Some	None	2 yr	5 yr		
P. cryptogea	45168	5	-	-	V	F	13	V
P. cryptogea	152646	3	-	4	V	F	NT	NT
P. drechsleri	40499	3	-	4	V	F	12	V
P. drechsleri	77969	2	-	4	N	F	NT	NT
P. drechsleri	136534	3	-	4	V	F	13	V
P. erythroseptica	17028	3	-	5	V	F	F	NT
P. erythroseptica	34684	3	-	4	V	F	13	V
P. erythroseptica	139360	3	-	4	V	F	NT	NT
P. erythroseptica	181716	1	-	2	N	F	10	N
P. heveae	131093	2	-	3	N	F	7	N
P. heveae	131372	4	-	5	N	F	NT	NT
P. heveae	147352	1	-	2	V	F	11	N
P. hibernalis	134760	2	3(75)	4	N	F	NT	NT
P. infestans	181530	2	3(25)	4	V	F	F	NT
P. meadii	36529	3	-	4	N	F	NT	NT
P. meadii	130427	2	-	3	N	F	NT	NT
P. megasperma	32035	3	4(25)	5	V	F	F	NT
P. megasperma	144023	5	-	-	N	V	NT	NT

N, Normal growth and sporulation; V, Vegetative growth only; NT, Not tested; F, failed to grow.

Table 3.021 (continued) Viability and condition of isolates of *Phytophthora* species stored in water and liquid nitrogen for periods of up to 5 years. Where only some samples survived the percentage is given in brackets

Name	IMI Number	Water storage					Liquid nitrogen	
		Samples viable for period given (years)			Condition		Period viable (years)	Condition after final test
		All	Some	None	2 yr	5 yr		
P. megasperma	131375	4	-	5	N	F	8	NT
P. megasperma	131555	2	-	3	N	F	7	N
P. nicotianae	21276	4	-	5	N	F	NT	NT
P. nicotianae	21279	3	5(50)	-	N	AV(50)	8	N
P. nicotianae	130899	4	-	5	N	F	NT	NT
P. nicotianae	77972	5	-	-	N	N	7	N
P. nicotianae	205751	2	-	3	V	F	NT	NT
P. nicotianae	207770	3	5(75)	-	N	N(75)	NT	NT
P. nicotianae	35087	3	-	4	N	F	5	N
P. palmivora	46333	4	5(50)	-	N	N(50)	8	N
P. palmivora	80298	4	5(50)	-	N	N(50)	9	N
P. palmivora	202077	4	-	5	N	F	NT	NT
P. palmivora	206790	4	-	5	V	F	F	NT
P. porri	208979	2	-	3	N	F	NT	NT
P. syringae	38915	2	-	4	N	F	NT	NT
P. syringae	62472	3	4(50)	5	N	F	NT	NT
P. syringae	131190	4	-	5	N	F	NT	NT

N, Normal growth and sporulation; V, Vegetative growth only; AV, Abnormal vegetative growth; NT, Not tested; F, failed to grow.

Table 3.021 (continued) Viability and condition of isolates of *Phytophthora* species stored in water and liquid nitrogen for periods of up to 5 years. Where only some samples survived the percentage is given in brackets

Name	IMI Number	Water storage					Liquid nitrogen	
		Samples viable for period given (years)			Condition		Period viable (years)	Condition after final test
		All	Some	None	2 yr	5 yr		
P. syringae	131191	4	-	5	N	F	NT	NT
P. vesicula	139645	5	-	-	N	N	NT	NT
Species indet.	241683	2	-	3	N	F	NT	NT

N, Normal growth and sporulation; NT, Not tested; F, failed to grow.

Contamination of cultures during storage was observed, 10 of the 56 isolates (18%) had contaminants. However, these were recovered contaminant-free from reserve bottles. The contaminants were most often found to be species of Penicillium.

3.03 The comparison of preservation of isolates of Pythium by water storage and liquid nitrogen storage

The results of the recoveries of the twelve isolates of Pythium stored in water and recoveries of the isolates preserved in liquid nitrogen are shown in Table 3.031.

The 5 isolates of Pythium tested in both water and liquid nitrogen were viable after 2 years storage in water but one failed to survive 4 days in liquid nitrogen. There was no difference in the condition of the isolates after retrieval from both techniques. During storage in water one isolate died after 3 years storage and another after 5 years. Of the 3 isolates that remained viable in water at 5 years one had deteriorated and 2 of the 4 samples did not recover. The 2 others were retrieved from both techniques in normal condition.

The CMI records (1.0332) showed that the 2 isolates tested were both less virulent when retrieved from water than when retrieved from liquid nitrogen.

Contamination was also a problem with the storage of Pythium species, 4 of the 12 isolates had contaminants though these were recovered contaminant-free from reserve bottles.

Table 3.031 Viability and condition of isolates of *Pythium* species stored in water and liquid nitrogen for periods of up to 5 years. Where only some samples survived the percentage is given in brackets

Name	IMI Number	Water storage					Liquid nitrogen	
		Samples viable for period given (years)			Condition		Period viable (years)	Condition after final test
		All	Some	None	2 yr	5 yr		
P. aphanidermatum	58847	2	-	3	N	F	NT	NT
P. aphanidermatum	104926	4	-	5	N	F	7	N
P. coloratum	181938	2	-	3	V	F	10	V
P. debaryanum	48558	4	-	5	V	F	NT	NT
P. mamillatum	45622	4	5(25)	-	N	V(25)	NT	NT
P. middletonii	42098	5	-	-	N	N	13	N
P. oligandrum	78731	3	-	4	V	F	NT	NT
P. oligandrum	133857	2	-	3	N	F	NT	NT
P. paroecandrum	92552	4	-	5	N	F	NT	NT
P. sylvaticum	248394	4	5(50)	-	N	V(50)	4	V
P. sylvaticum	248395	5	2(50)	-	N	N	F	NT
P. ultimum	82514	5	-	-	N	N	NT	NT

N, Normal growth and sporulation; V, Vegetative growth only; NT, Not tested; F, failed to grow.

3.04 General conclusions from the water storage technique

1. Most isolates of both genera survived 3 years storage in water. All the isolates of Pythium and 95% of Phytophthora preserved in water survived 2 years. Some of the isolates of both genera survived storage periods of 4, and a few 5, years (Figs. 3.041a & 3.041b). Some isolates have failed to survive the technique whereas others of the same species remained viable.

2. More isolates survived the initial two years in water than in the liquid nitrogen storage method. Where liquid nitrogen storage failed the loss of viability was usually immediate.

3. Between 3 and 5 years storage in water led to a rapid loss of viability but little change occurred in liquid nitrogen.

4. The CMI records show that most isolates have a lower degree of virulence after storage in water than after storage in liquid nitrogen (Fig. 3.042a).

5. Over half the isolates of Phytophthora that survived 5 years sporulated and/or grew abnormally when retrieved from water. Fewer were affected by liquid nitrogen storage.

Fig. 3.041a The viability of species of Phytophthora (31 isolates) and Pythium (5 isolates) during storage in water and liquid nitrogen. Data from Tables 3.021 and 3.031 for species where full data was available

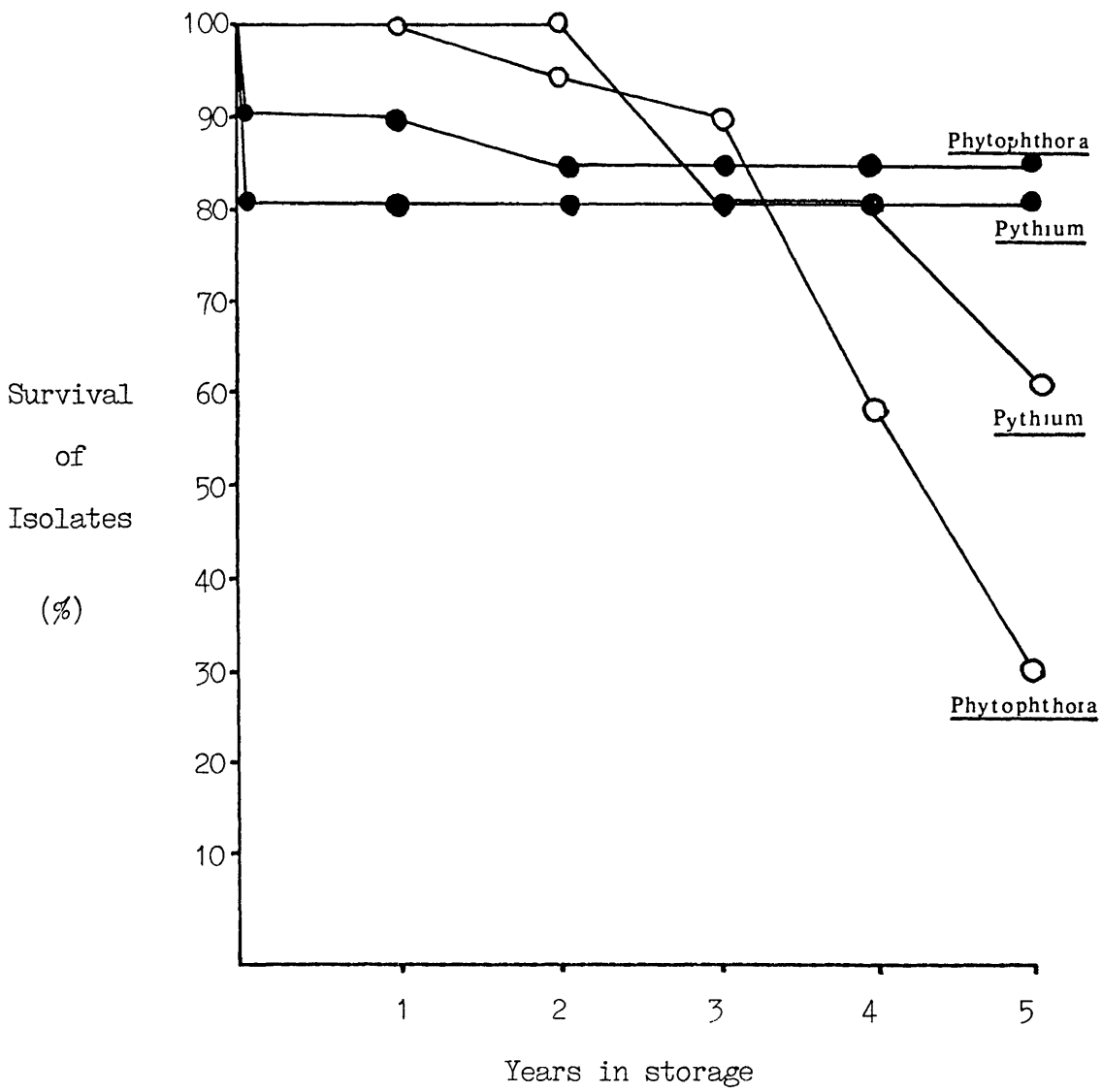


Fig. 3.041b Comparison of the viability after water storage of species of Phytophthora and Pythium in Fig. 3.041a (solid line) with other isolates (Phytophthora 25, Pythium 7) (broken line) which were not tested in liquid nitrogen

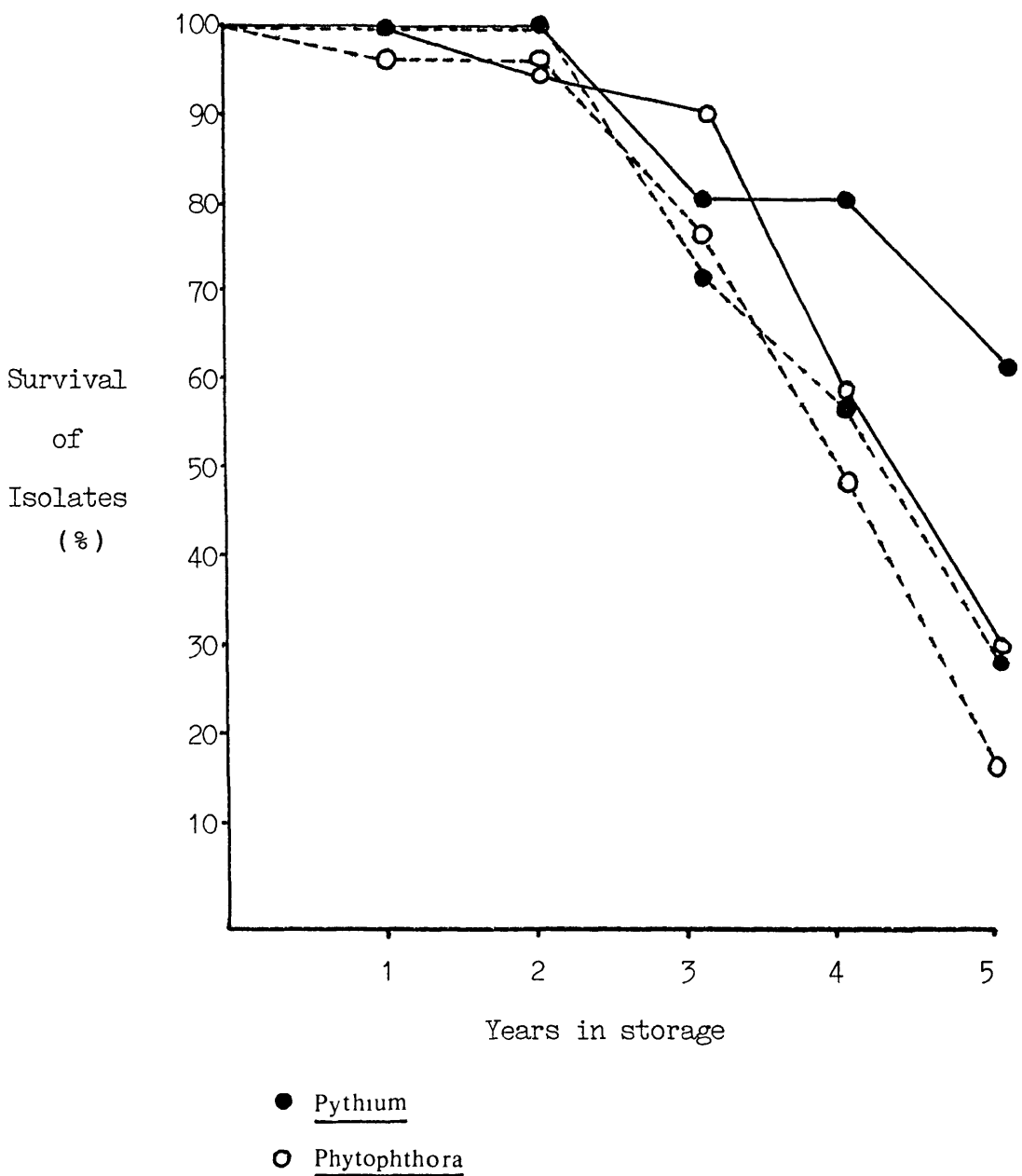
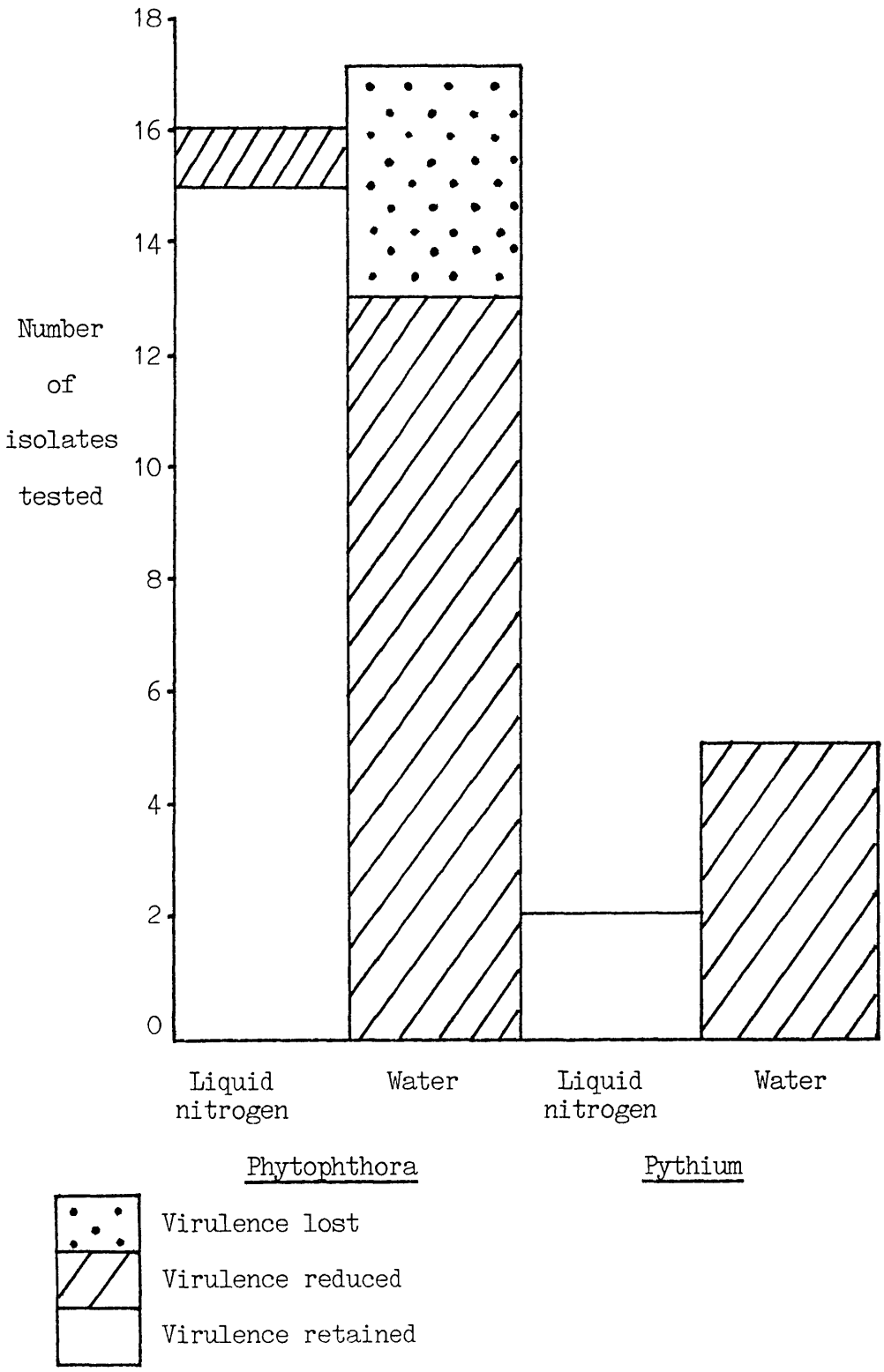


Fig. 3.042a The virulence of the isolates of Phytophthora and Pythium stored in water and liquid nitrogen from the CMI records



The investigation and development of water storage for the preservation of Phytophthora and Pythium showed that although viability of the organisms was maintained they deteriorated in storage (Fig. 3.042a). The virulence of the organism decreased despite the retention of viability and 5 of the 9 isolates of Phytophthora that survived 5 years storage lose the ability to sporulate after 2 years.

A point that should also be borne in mind is that 18% (10/56) isolates of Phytophthora and 33% (4/12) isolates were contaminated on retrieval from the first sample bottle. The reserve storage proved to be contaminant-free. It is therefore recommended that a reserve set of cultures should be stored to obtain cultures from in the event of contamination.

From this study it can be seen that the liquid nitrogen storage technique is more suitable for the long term storage and stability of the species under test. However, this technique can not be used successfully for the preservation of all the isolates and water storage can therefore be used to preserve those that fail. It is recommended that cultures stored in water should be subcultured every 2 years.

CHAPTER 4

THE INVESTIGATION OF SOME DRYING TECHNIQUES USED FOR THE PRESERVATION OF FUNGI

4.01 Introduction

Several drying techniques have been used to preserve fungi, with varying degrees of success and some of which have been discussed above (1.024, 1.025 and 1.026). Here the efficiency of the simple methods of soil and silica gel storage are explored. The soil storage technique (2.07) was first used at CMI for the preservation of fungi in 1965 by Booth (unpublished). The viability of some isolates of Fusarium recovered from samples stored by Booth (2.07) is examined. Secondly, storage in anhydrous silica gel (2.08) is assessed by continuing the work of Onions (1977). The results of the recoveries of isolates previously stored in silica gel are compared with the recoveries of the same isolates from oil storage, centrifugal freeze drying and liquid nitrogen storage. The condition of the organisms in culture was assessed by comparing the growth and sporulation of the isolate after retrieval from storage with CMI records made before preservation,

4.02 The viability and morphological stability of isolates of Fusarium after recovery from the CMI soil collection

Forty five isolates of Fusarium (Table, 4.021) were selected from Booth's soil collection and inoculated on to PSA medium. After 10 to 14 days incubation at approximately 23 °C the cultures were examined both macroscopically and microscopically. Comparison was made of the characteristics of the cultures retrieved with their taxonomic descriptions (Booth, 1971) and the identity of each isolate was confirmed by Booth.

Table 4.021 The period of survival (years) of isolates of the genus *Fusarium* from Booth's soil collection.

Name	Authority	IMI Number	Maximum recorded survival
F. acuminatum	Ellis & Everhart	106901	15
F. acuminatum	Ellis & Everhart	129863	15
F. acuminatum	Ellis & Everhart	136675	13
F. aquaeductuum (anomorph of *Nectria episphaeria*)	Lagerheim	86977	10
F. arthrosporioides	Sherbakoff	163864	6
F. avenaceum	(Corda ex Fries) Saccardo	85564	14
F. avenaceum	(Corda ex Fries) Saccardo	103230b	14
F. avenaceum	(Corda ex Fries) Saccardo	103227	14
F. avenaceum	(Corda ex Fries) Saccardo	175486	9
F. scirpi var. *compactum*	Lambert & Fautrey Wollenweber	52129	10
F. concolor	Reinking	136902	10
F. culmorum	(W.G. Smith) Saccardo	135794	14
F. culmorum	(W.G. Smith) Saccardo	135667	14
F. culmorum	(W.G. Smith) Saccardo	149131	11
F. culmorum	(W.G. Smith) Saccardo	164746	10
F. dimerum [*Microdochium dimerum* (Penzig) von Arx]	Penzig	109832	15
F. equiseti	(Corda) Saccardo	111911	14
F. equiseti	(Corda) Saccardo	127561	14
F. flocciferum	Corda	131515	8
F. graminearum	Schwabe	105494	14
F. graminearum	Schwabe	140790b	13
F. graminearum	Schwabe	155426	11
F. heterosporum	Nees ex Fries	100469a	14

Table 4.021 (continued) The period of survival (years) of isolates of the genus *Fusarium* from Booth's soil collection.

Name	Authority	IMI Number	Maximum recorded survival (yrs)
F. heterosporum	Nees ex Fries	124108	14
F. heterosporum	Nees ex Fries	169066	10
F. lateritium	Nees ex Fries	129623	14
F. lateritium	Nees ex Fries	134593	14
F. merismoides	Corda	101143	10
F. merismoides	Corda	105043	9
F. moniliforme	Sheldon	113173	13
F. moniliforme	Sheldon	151906	12
F. moniliforme	Sheldon	152300	12
F. moniliforme	Sheldon	158047	10
F. oxysporum	Schlechtendahl	136160	13
F. oxysporum	Schlechtendahl	138619	13
F. oxysporum	Schlechtendahl	159029	10
F. poae	(Peck) Wollenweber	128054	14
F. sambucinum	Fuckel	111826	14
F. sambucinum	Fuckel	135683	14
F. sambucinum	Fuckel	136929	13
F. sambucinum	Fuckel	155388	11
F. sambucinum	Fuckel	155389	11
F. sambucinum	Fuckel	155390	11
F. sambucinum	Fuckel	160850	10
F. semitectum	Berkley & Ravenel	157845b	11

The maximum storage period of the isolates retrieved from Booth's soil collection was 15 years (3 isolates), a further 15 isolates had been stored for 14 years and the remainder had been kept for periods longer than 6 years. All isolates retrieved had retained their morphological characteristics according to Booth (1971). In particular their pigment production and sporulation had remained normal whereas CMI records had shown the loss of these characteristics when isolates had been retrieved from storage in oil.

4.03 A comparison of silica gel storage with oil storage, centrifugal freeze drying and liquid nitrogen storage

Onions (1977) successfully stored 87 (75%) of the isolates of a variety of Ascomycetes, Basidiomycetes, Deuteromycetes and Zygomycetes tested in silica gel (2.08) from 1971 to 1972. Twenty five of the isolates died during storage, the remainder were retrieved and grown on selected media at suitable incubation temperatures (Appendix III). Of the isolates that remained viable some had been preserved by centrifugal freeze drying (2.10), oil (2.05) and liquid nitrogen storage (2.13). These were retrieved, inoculated on to the same media used previously and their growth and sporulation were compared with the recoveries from silica gel (Table, 4.031).

Forty two of the isolates were in better condition after recovery from silica gel storage than when retrieved from oil. Twelve isolates were of similar condition and 8 were in a poorer state. When compared with the same isolates retrieved from centrifugal freeze drying, 12 were in a better condition after silica gel, 35 were in a similar condition and 15 poorer. A

comparison with isolates stored in liquid nitrogen showed that 3 were in better, 14 similar and 9 in poorer condition.

On recovery from centrifugal freeze drying 38 of the isolates were in better condition than when removed from oil, 15 were in similar and 9 in poorer condition. When centrifugal freeze drying recoveries are compared with liquid nitrogen storage 7 were in better, 11 in the same and 8 in a poorer condition. On recovery from liquid nitrogen 14 were in better condition, 10 were similar and 2 were poorer than after recovery from oil storage.

Twenty eight (45%) of the 62 isolates remaining viable in silica gel grew more slowly and produced fewer spores. Seven isolates retrieved from oil were non-viable though 3 of these had survived longer periods than the 10 or 11 years storage of the same isolates in silica gel. Forty four (71%) did not grow at a normal rate or sporulate typically, 1 of which, Penicillium notatum IMI 15378, lost the ability to produce pigment. One of the freeze dried isolates, Aspergillus sejunctus IMI 168779, died in storage, and 24 (40%) had reduced growth and sporulation after retrieval. None of the 26 isolates tested in liquid nitrogen died and 10 (38%) showed reduced growth and sporulation.

Table 4.031 The periods of viability and condition in culture of isolates retrieved from storage by silica gel, oil, freeze drying and liquid nitrogen. Storage periods were 10 or 11 years in silica gel and the maximum storage period in other techniques were 28 years in oil, 15 freeze dried and 12 years in liquid nitrogen

Name	IMI Number	Survival period (years) and condition (see below)			
		SG	Oil	FD	LN
Alternaria alternata	89343	11R	20F	15N	NT
A. brassicae	151659	10F	8F	11A	NT
A. chlamydospora	156426	10R	11R	10R	NT
Ascobolus viridulus	85125	10R	6D	7R	NT
Aspergillus avenaceus	16140	11N	10N	13N	NT
A. candidus	127260	11R	11A	15R	NT
A. giganteus	112341	10N	17N	10N	12N
A. nidulans	134679	11N	22N	15N	13N
A. nidulans var. echinulatus	141181	11N	13F	14N	NT
A. niger	17454	11N	24N	14N	NT
A. sejunctus	168779	10N	10F	9D	NT
Aureobasidium pullulans	45533	11N	19F	15N	NT
Beltraniella humicola	155820	10N	11F	10N	9N
Botryotrichum piluliferum	49832	11N	24F	13R	NT
Byssochlamys fulva	163641	10N	10R	10N	10N
B. nivea	163642	10N	10F	10N	10N
Chaetomium cuniculorum	155487	10F	11F	10R	9F

Growth and sporulation normal, N; Reduced growth and sporulation, R; Further reduced growth and sporulation, F; Abnormal growth and sporulation, A; Vegetative growth only, V; Died in storage (period of viable storage is given), D; Not tested by the technique, NT;

Table 4.031 (continued) The periods of viability and condition in culture of isolates retrieved from storage by silica gel, oil, freeze drying and liquid nitrogen. Storage periods were 10 or 11 years in silica gel and the maximum storage period in other techniques were 28 years in oil, 15 freeze dried and 12 years in liquid nitrogen

Name	IMI Number	Survival period (years) and condition (see below)			
		SG	Oil	FD	LN
Chaetomium elatum	17424a	10R	20A	11N	NT
C. globosum	16203	11R	11A	14N	NT
Colletotrichum dematium	80025	10N	21R	4A	7N
C. dematium f. sp. spinaciae	156656	10F	11N	12A	10N
C. musae	83256	10N	10A	10N	NT
C. typhae	86896b	10N	10R	10A	NT
Coprinus hexagonosporus	161417	10R	10V	10N	10R
C. utrifer	161422	10A	11D	10R	10V
Cryphonectria parasitica	59815	10F	28A	15R	NT
Curvularia lunata var.aeria	96846	10F	13F	11A	11F
Eremascus fertilis	86727	11N	20F	5N	NT
Fusarium solani	68412	11N	25R	15N	NT
Gelasinospora cerealis	76253a	10N	23V	9N	NT
Gliocladium roseum	101020h	10A	19R	12N	NT
Humicola grisea var. thermoidea	126329	10N	15D	15A	13N
Isaria felina	159339	10N	10F	10N	2N
Mucor hiemalis (+)	21216	11N	19F	15N	NT

Growth and sporulation normal, N; Reduced growth and sporulation, R; Further reduced growth and sporulation, F; Abnormal growth and sporulation, A; Vegetative growth only, V; Died in storage (period of viable storage is given), D; Not tested by the technique, NT;

Table 4.031 (continued) The periods of viability and condition in culture of isolates retrieved from storage by silica gel, oil, freeze drying and liquid nitrogen. Storage periods were 10 or 11 years in silica gel and the maximum storage period in other techniques were 28 years in oil, 15 freeze dried and 12 years in liquid nitrogen

Name	IMI Number	Survival period (years) and condition (see below)			
		SG	Oil	FD	LN
Mucor hiemalis (-)	21217	11N	20N	13N	NT
Myceliophthora thermophila	158756	11N	1D	11N	NT
Paecilomyces variotii	108007	10N	18R	12N	NT
Penicillium baarnense	40590	11A	25V	14R	12F
P. brefeldianum	153725	10A	11R	3R	10R
P. chrysogenum	26210	11N	25F	4N	NT
P. claviforme	44744	11N	25V	15N	NT
P. luteum	95152	11R	20R	10R	10F
P. notatum	15378	11N	24R*	6N	11R
P. wortmannii	40047	10R	15V	10R	10N
Pestalozziella parva	124039	10F	10N	10N	10R
Peziza ostracoderma	60977	10R	25D	10N	NT
Phoma herbarum var. medicaginis	156652	10R	10D	5N	9N
P. violacea	49948ii	11N	15R	14N	3N
Piptocephalis virginiana	70910ii	11N	9R	7N	12R
Pycnoporus sanguineus	75002	11N	18F	12N	NT
Rhizopus homothallicus	89714	11N	20N	11N	10N

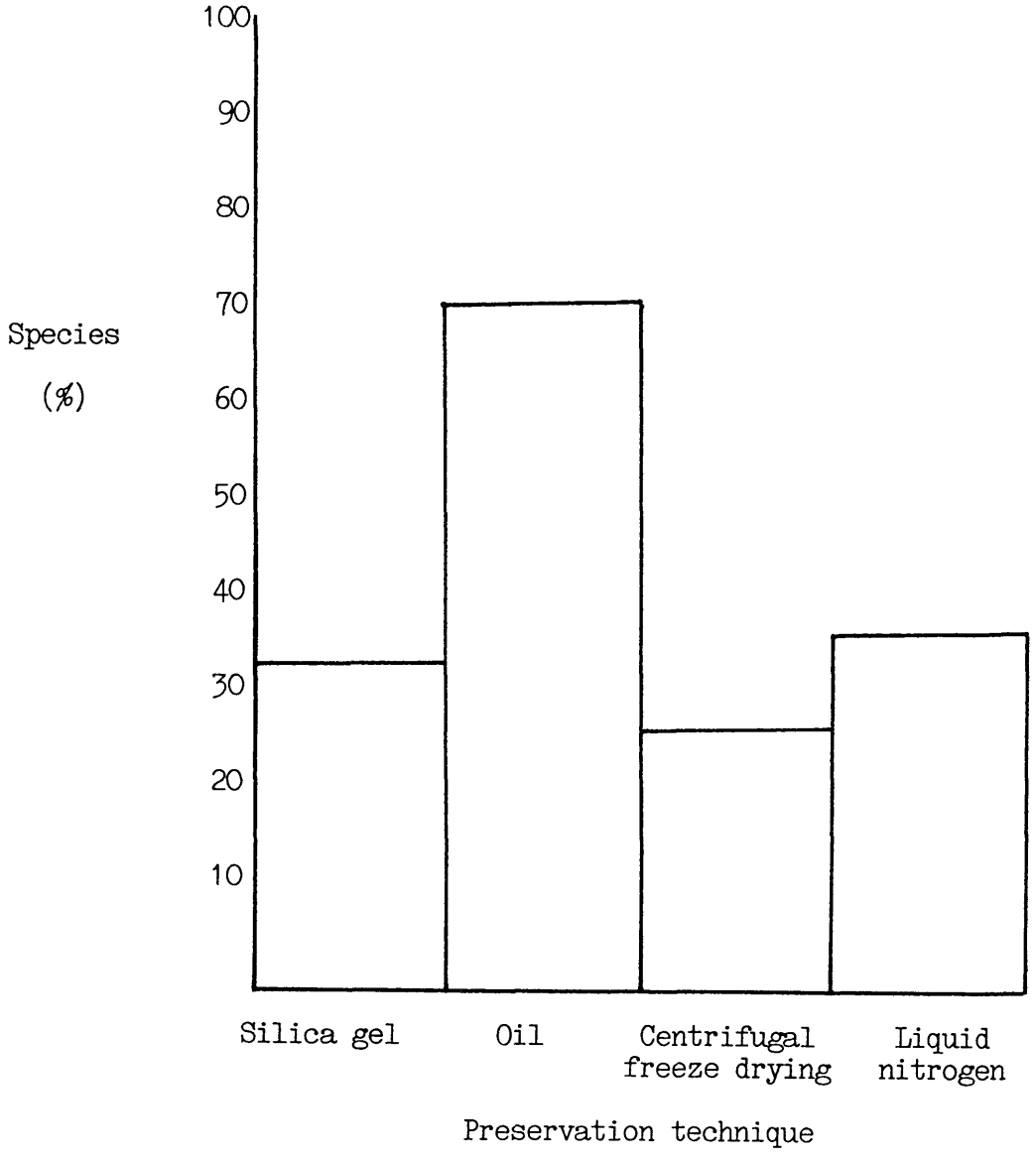
Growth and sporulation normal, N; Reduced growth and sporulation, R; Further reduced growth and sporulation, F; Abnormal growth and sporulation, A; Vegetative growth only, V; Died in storage (period of viable storage is given), D; Not tested by the technique, NT; *, Lost pigmentation.

Table 4.031 (continued) The periods of viability and condition in culture of isolates retrieved from storage by silica gel, oil, freeze drying and liquid nitrogen. Storage periods were 10 or 11 years in silica gel and the maximum storage period in other techniques were 28 years in oil, 15 freeze dried and 12 years in liquid nitrogen

Name	IMI Number	Survival period (years) and condition (see below)			
		SG	Oil	FD	LN
Saccharomyces cerevisiae	140023	11A	13A	13N	NT
Schizophylum commune	89295ii	10F	13A	7A	8N
S. radiatum	90347	11R	20N	7R	NT
Sordaria fimicola (+)	105390	11F	17N	15F	12N
S. fimicola (-)	105391	11N	11N	6N	NT
Sporormiella intermedia	148830	11N	12R	12F	NT
Stachtbotrys atra	82021	11R	22F	13F	NT
Thermoascus crustaceus	158741	11N	1D	11N	NT
Thielavia albomyces	126326	10N	15R	10N	NT
T. terricola	104951	11V	18F	4R	8N
Verticillium dahliae	81822	11F	22R	8R	NT

Growth and sporulation normal, N; Reduced growth and sporulation, R; Further reduced growth and sporulation, F; Abnormal growth and sporulation, A; Vegetative growth only, V; Died in storage (period of viable storage is given), D; Not tested by the technique, NT;

Fig. 4.03a The proportion of species that grew more slowly and had reduced sporulation after storage in silica gel, oil, centrifugal freeze drying and liquid nitrogen



Many of the isolates showed deterioration during storage (Table, 4.031). Oil storage was accompanied by more deterioration than any of the other techniques compared (Fig. 4.03a). The stability of the isolates stored in silica gel is similar to that after preservation by the centrifugal freeze drying and liquid nitrogen storage techniques. Twenty six percent of the isolates freeze dried, 38% in liquid nitrogen and 32% in silica gel showed deterioration in growth and sporulation.

For the preservation of these fungi silica gel storage, centrifugal freeze drying and liquid nitrogen should be used in preference to oil which caused more instability.

Table 4.032 The isolates that failed (-) to survive the initial drying in silica gel and those that died during storage after recovery from storage under oil, after centrifugal freeze drying and in liquid nitrogen. The condition in culture is normal unless qualified (see below). The number of years they survived is given.

Name	IMI number	Period of successful storage (years)	Survival period (years) and condition after storage		
			Oil	FD	LN
Allomyces arbuscula	129543	0	14	-	7
A. arbuscula	152201	5	7D	NT	11
A. javanicus	144364	0	4D	NT	NT
Armillariella mellea	61755	0	27	NT	NT
Arnium arizonense	169785	0	8	8	8
Arthrobotrys oligospora	102121	<3	16	6	NT
Ascochyta fabae	135517	0	7A	12A	NT
Ascotricha lusitanica	147693	0	11	11	11
Aspergillus candidus	130667	<3	14	8	NT
A. citrisporus	25285	4	11A	13	NT
A. ochraceus	16247iii	2	11	11A	NT
A. restrictus	127782	<3	14	14	NT
A. ustus	100391	3	18	8	NT
A. wentii	162039	4	11A	10	NT
Basidiobolus meristosporus	108476	3	12	5	NT
Botryodiplodia theobromae	125847	4	9	8	NT
Calonectria quinquiseptata	136139	<3	11	-	11
Candida lipolytica	93743	0	20	15	NT

Growth and sporulation :- Abnormal, A; Vegetative only, V; Died during storage after given years storage, D; Not tested, NT.

Table 4.032 (continued) The isolates that failed (-) to survive the initial drying in silica gel and those that died during storage after recovery from storage under oil, after centrifugal freeze drying and in liquid nitrogen. The condition in culture is normal unless qualified (see below). The number of years they survived is given.

Name	IMI number	Period of successful storage (years)	Survival period (years) and condition after storage		
			Oil	FD	LN
Ceratocystis ulmi	147188	2	9A	1	NT
C. ulmi	173135	0	9	6	NT
C. ulmi	173136	3	9	9	NT
Cercospora beticola	77043	-	4A	NT	NT
C. sesami	111779	-	12A	12V	12
Chytridium olla	86666	-	7D	NT	-
Cochliobolus sativus	166172	-	9	9	9
C. sativus	166173	-	9	9	9
Coemansia formosensis	170166	-	9	9	9
C. mojavensis	140079	1	NT	NT	NT
C. pectinata	142377	2	10A	9	10
Colletotrichum gossypii	82269	2	5V	12	12
C. tabacum	54048	4	10	NT	NT
C. trichellum	82378	<4	15	10	NT
C. truncatum	86431	1	21	5	NT
Conidiobolus coronatus	68174	2	13	-	NT
C. heterosporus	102043	-	18	NT	NT
C. lobatus	138635	-	12	-	NT

Growth and sporulation :- Abnormal, A; Vegetative only, V; Died during storage after given years storage, D; Not tested, NT.

Table 4.032 (continued) The isolates that failed (-) to survive the initial drying in silica gel and those that died during storage after recovery from storage under oil, after centrifugal freeze drying and in liquid nitrogen. The condition in culture is normal unless qualified (see below). The number of years they survived is given.

Name	IMI number	Period of successful storage (years)	Survival period (years) and condition after storage		
			Oil	FD	LN
Conidiobolus mycophagus	113701	3	6	-	9
Coprinus luteocephalus	161421	-	NT	9	NT
C. semitalis	161423	-	NT	9	NT
Corticium rolfsii	77445a	-	22	5	NT
Cryptospora suffusa	173497	<3	9	9	9
Culicinomyces clavosporus	177011	-	17	5	5
Dictyostelium discoideum	69094ii	-	27	15	11
Exobasidiella culmigerum	136517	-	13	7D	NT
Fusarium culmorum	175485	<7	9	9	NT
F. solan1	76761	1	23D	15	NT
F. solani	172507	-	NT	7	NT
Gaeumannomyces graminis	160145	-	11	2	2
Geosmithia lavendula	40570	1	27	15	NT
Gnomonia fructicola	164147a	-	9	9	NT
Helicodendron tubulosum	92743	<3	12	6	10
Helicosporina veronae	114458	-	16	4	8
Heliscus submersus	82609	<3	16	9	9
Heterocephalum aurantiacum	131684	8	12A	12	14

Growth and sporulation :- Abnormal, A; Vegetative only, V; Died during storage after given years storage, D; Not tested, NT.

Table 4.032 (continued) The isolates that failed (-) to survive the initial drying in silica gel and those that died during storage after recovery from storage under oil, after centrifugal freeze drying and in liquid nitrogen. The condition in culture is normal unless qualified (see below). The number of years they survived is given.

Name	IMI number	Period of successful storage (years)	Survival period (years) and condition after storage		
			Oil	FD	LN
Hypoxylon mediterraneum	75991	<3	22	NT	NT
H. nummularium	146051	0.2	6	-	NT
Leptosphaeria doliolum	199777	<4	6	NT	5
Martensiomyces pterosporus	60573	-	26	2	2
Metarhizium anisopliae	98375	<1	13A	10	NT
Micromonospora vulgaris	126892	5	15A	11	NT
Monacrosporium salinum	109555	-	17	5	5
Mortierella bainieri	167609	-	9	9	NT
Mycosphaerella deightonii	119431	-	7	-	NT
Mycovellosiella ferruginea	124973	-	15	9	NT
Neurospora crassa	19419	5	12	6	NT
N. crassa	68614ii	-	12	12	12
N. crassa	147001	2	5D	NT	NT
Nomuraea atypicola	186963	<1	8	7	NT
Penicillium brevicompactum	17456	<3	25	15	NT
P. canescens	149218	-	11	11	NT
P. coryophilum	101082	2	19	11A	NT
P. corymbiferum	68414	<3	22	12	NT

Growth and sporulation :- Abnormal, A; Vegetative only, V; Died during storage after given years storage, D; Not tested, NT.

Table 4.032 (continued) The isolates that failed (-) to survive the initial drying in silica gel and those that died during storage after recovery from storage under oil, after centrifugal freeze drying and in liquid nitrogen. The condition in culture is normal unless qualified (see below). The number of years they survived is given.

Name	IMI number	Period of successful storage (years)	Survival period (years) and condition after storage		
			Oil	FD	LN
Penicillium cyclopium	19759	<3	19	15	NT
P. digitatum	91956	-	14	14	NT
P. digitatum	92217	<3	12	14	NT
P. expansum	191205	2A	7	7	NT
P. helicum	197479	2A	7	6	NT
P. idahoense	148393	-	12	12	12
P. janthinellum	108033	<4	18	15	NT
P. luteum	112513	-	5D	11	NT
Phoma epicoccina	164070	-	9	9	9
Phycomyces blakesleeanus	118496	5	12A	8	NT
P. blakesleeanus	118497	11	11	12	NT
Phytophthora cactorum	21168	-	32	-	10
Piptocephalis xenophila	156650	4	10A	5A	11
Pleospora infectoria	173200	3	9	9	9
Pyrenopeziza brassicae	204290	-	NT	3	NT
Pyrenophora graminea	129760	<1	15V	10D	NT
Pyronema domesticum	57472	-	27	15	2
Pythium debaryanum	48558	-	28	NT	NT

Growth and sporulation :- Abnormal, A; Vegetative only, V; Died during storage after given years storage, D; Not tested, NT.

Table 4.032 (continued) The isolates that failed (-) to survive the initial drying in silica gel and those that died during storage after recovery from storage under oil, after centrifugal freeze drying and in liquid nitrogen. The condition in culture is normal unless qualified (see below). The number of years they survived is given.

Name	IMI number	Period of successful storage (years)	Survival period (years) and condition after storage		
			Oil	FD	LN
Pythium flevoense	176046	-	8	NT	7
P. middletonii	42098	-	31	NT	NT
Rhizoctonia carotae	162910	-	10	NT	NT
R. solani	20697	1	32A	NT	5
Rhizophidium sphaerotheca	143633	-	12	-	-
Rhizopus rhizopodiformis	158738	-	11	11	NT
Rhodotorula rubra	38784	4	12	5	NT
Ryparobius polysporus	75299	-	13	10	NT
Saprolegnia ferax	146489	-	11	-	3
Sclerotinia sclerotiorum	147201	-	12	NT	12
Serpula lacrimans	152233	-	11	NT	NT
Sclerotium delphinii	159926	-	10	4	9
Sphaerobolus stellatus	155101	-	10	NT	10
Sporobolomyces roseus	43529	-	31	11	NT
Stachybotryna columnare	158980	-	10	NT	NT
Stilbum macrosporum	163252	-	9	9	NT
Streptomyces griseus	50967	-	29	15	NT
S. lisandri	137178	-	12	10	NT

Growth and sporulation :- Abnormal, A; Vegetative only, V; Died during storage after given years storage, D; Not tested, NT.

Table 4.032 (continued) The isolates that failed (-) to survive the initial drying in silica gel and those that died during storage after recovery from storage under oil, after centrifugal freeze drying and in liquid nitrogen. The condition in culture is normal unless qualified (see below). The number of years they survived is given.

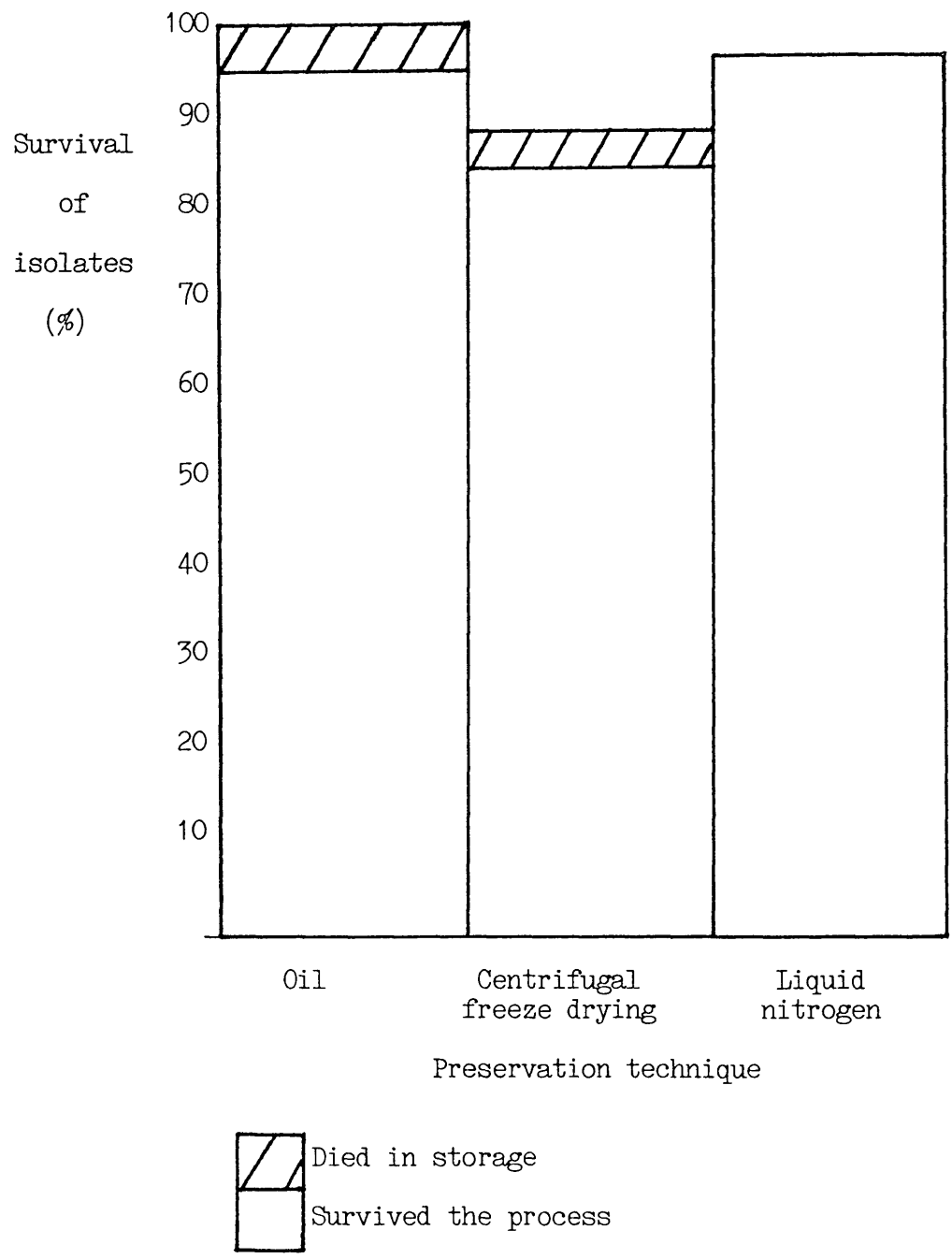
Name	IMI number	Period of successful storage (years)	Survival period (years) and condition after storage		
			Oil	FD	LN
Syzygites megalocarpus	231978	2	NT	2	4
Thielavia fimeti	116692	-	16	10	NT
T. terricola	153731	-	1D	11	11
Trichoderma viride	57421	5	25	4	NT
Trichothecium roseum	129425	-	14	14	NT
Tritirachium roseum	169856	3	10	10	NT
Zalerion maritima	81620	3	14	-	10
Zopfiella leucotricha	153733	-	-	10	10

Growth and sporulation :- Abnormal, A; Vegetative only, V; Died during storage after given years storage, D; Not tested, NT.

Analysis of records at CMI showed that 116 isolates of 426 tested failed or died during storage in silica gel during the storage period of 11 years ending in 1983 (Table, 4.032). Some of the 116 isolates had been previously stored in oil, freeze drying and liquid nitrogen and were retrieved from storage and their condition in culture examined (Table, 4.032). Of the 116 isolates 96 were centrifugally freeze dried of which 85 survived the inital process and the remainder failed to survive. Two of the survivors died during storage. Only 42 of the isolates that failed to survive silica gel storage were stored in liquid nitrogen of these, 40 survived 2 or more years and still remain viable. The two isolates that failed were Chytridium olla and Rhizophydium sphaerotheca.

Most of the 110 isolates stored under mineral oil remained viable for periods exceding 6 years, 76 survived 10 or more years and 20 of these were viable after 20 years without transfer. Seven isolates died during storage in oil, one in the first year. On recovery, 14 isolates sporulated poorly or not at all. In every case, isolates that had survived the initial drying in silica gel but that had died during storage had survived longer periods in oil storage. Of the 85 freeze dried isolates that remained viable only 5 showed any deterioration in growth and sporulation after recovery from storage. None of the forty isolates retrieved from liquid nitrogen showed any form of deterioration. As can be seen many of those that failed the initial drying in silica gel or died in subsequent storage were successfully preserved by other techniques (Fig. 4.03b).

Fig. 4.03b The survival of isolates that failed the initial drying or died during storage in silica gel (Table, 4.032) when stored in oil, by freeze drying and in liquid nitrogen storage



4.04 General conclusions drawn from comparison of these preservation techniques

1. All isolates tested of Fusarium in soil storage were viable and were found to be morphologically typical after storage periods of between 6 and 15 years.

2. Many fungi belonging to the Mastigomycotina, Ascomycotina, Deuteromycotina and Zygomycotina failed to survive silica gel storage. Although silica gel storage retains a level of morphological stability similar to that obtained by freeze drying and liquid nitrogen storage, only the sporulating fungi survived. Of the 116 isolates tested that failed to survive the initial drying or storage in silica gel, many survived in oil, by centrifugal freeze drying and in liquid nitrogen (Fig. 4.03b).

3. Of the organisms that survived oil storage 71% of isolates that also survived silica gel and 14% (14 from 103) of those that had failed in silica gel showed deterioration in their growth and sporulation. Centrifugal freeze drying caused less deterioration of the isolates during storage. Forty percent of the isolates that had also survived in silica gel and 6% (5 from 85) of those that had failed, showed deterioration. Deterioration was shown by 45% of the survivors in silica gel and 38% in liquid nitrogen storage. However, none of those surviving liquid nitrogen storage but failing silica gel deteriorated.

4. The freeze drying and liquid nitrogen storage techniques are more reliable and preserve a wider range of fungi than silica gel storage. The results show that silica gel storage is a suitable technique for preservation of some fungi, viz the sporulating Hyphomycetes, the Coelomycetes and the Ascomycetes (Smith &

Onions 1983b). However, both liquid nitrogen storage and freeze drying allow a wider range of fungi to survive than silica gel storage (Table, 4.032).

CHAPTER 5

INVESTIGATION OF LIQUID NITROGEN STORAGE TECHNIQUE

Contents

5.01 Introduction

As a result of an analysis of the CMI records of the results of liquid nitrogen storage (1.037) it was seen that the Mastigomycotina were the least well preserved. Experiments were therefore designed to determine whether failure in this subdivision was due to damage incurred during preparation for preservation. In particular the effect of various cryoprotectants and cooling rate were investigated.

5.02 Investigation of some techniques that avoid excessive manipulation prior to freezing and storage in liquid nitrogen

Some fungi that failed initial attempts at storage in liquid nitrogen or survived in poor condition were stored using methods to avoid excessive manipulation. The original method involved the preparation of suspensions by adding the cryoprotectant to agar slopes and scraping colonies from the agar surface. Such a technique could cause mechanical damage therefore new procedures were used to reduce this effect (2.13). These procedures involved cutting agar pieces from the edge of a colony without dislodging the fungus, or growing the fungus on small amounts of agar medium in polypropylene screw capped ampoules and adding the cryprotectant when the culture has matured. The surviving propagules were enumerated by transfer to fresh agar medium and counting the typical colonies developed. The results of storage are compared with previous attempts (Table, 5.021).

Table 5.021 Viabilities of some examples of the Mastigomycotina after 2 years storage in liquid nitrogen avoiding excessive manipulation compared with results of previous trials (1.037)

Name	IMI Number	Growth in previous trials	Viability after new treatments			
			Growth	Germination of propagules		
				tested	viable	%
Achlya ambisexualis	143524	-	AN	9	2	22
A. ambisexualis	143525	-	R	12	7	58
A. americana	191108	-	N	10	10	100
A. racemosa	110079	-	N	12	11	92
A. racemosa	137396	F 11/24*	R	9	7	78
Aphanomyces brassicae	188398	-	F	20	9	45
A. euteiches	155842	-	R	11	7	64
Phlyctochytrium arcticum	143636	-	R	12	7	58
Phytophthora botryosa	189728	-	N	11	9	82
P. cactorum	62471	-	R	10	8	80
P. capsici	40502	-	F	13	6	46
P. capsici	130937	-	N	31	29	94
P. cryptogea	21278	-	N	23	20	87
P. heveae	208224	-	R	24	17	71
P. heveae	210111	-	N	29	28	97

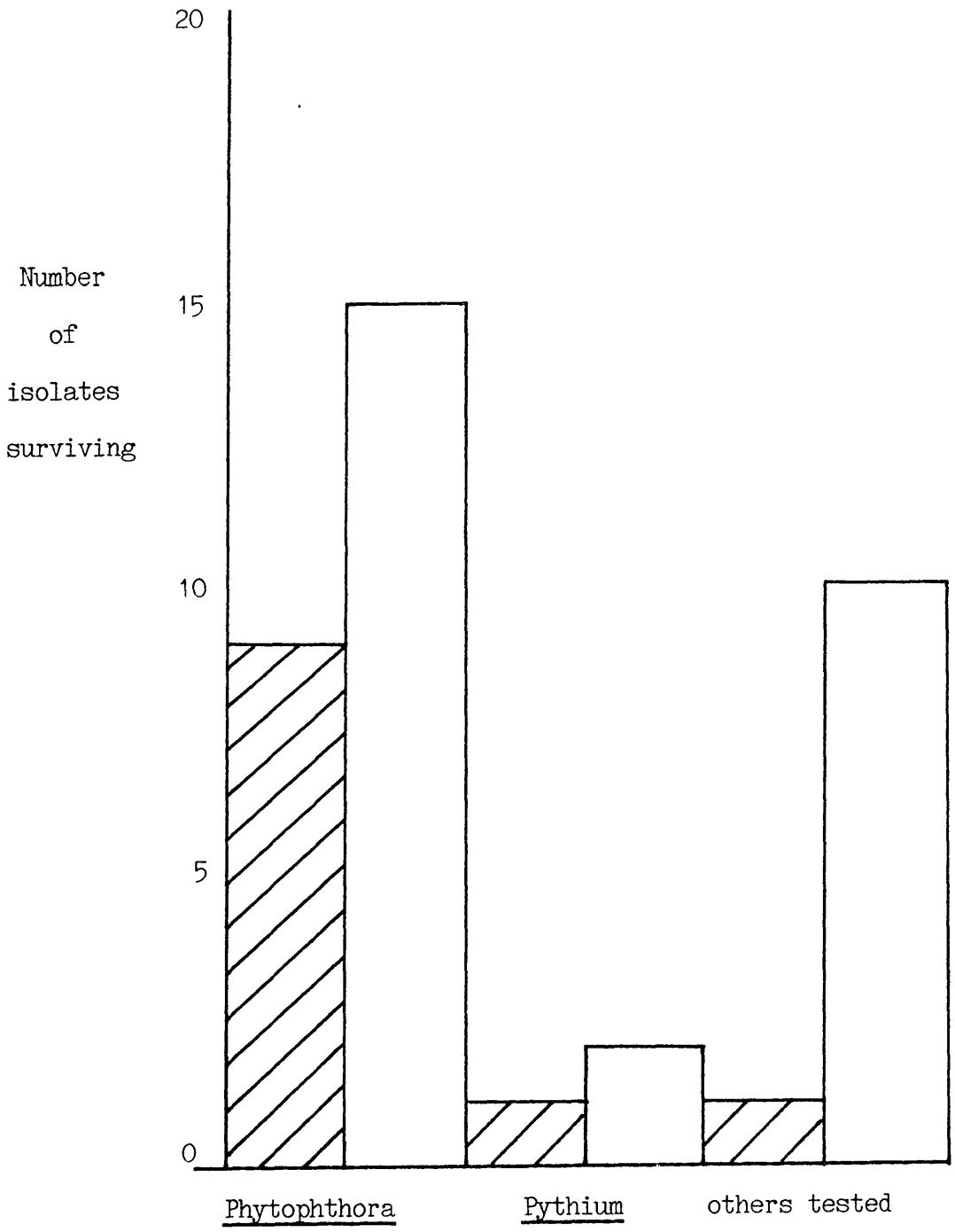
Growth and sporulation normal, N; Reduced growth and sporulation, R; Further reduced growth and sporulation, F; Abnormal growth and sporulation, AN; *The fungus was frozen on onion seed and figures given are number of seeds issuing fresh growth against the number thawed and inoculated; -, failed to survive;

Table 5.021 (continued) Viabilities of some examples of the Mastigomycotina after 2 years storage in liquid nitrogen avoiding excessive manipulation compared with results of previous trials (1.037)

Name	IMI Number	Growth in previous trials	Viability after new treatments			
			Growth	Germination of propagules		
				tested	viable	%
Phytophthora infestans	259084	NT	F(D)	25	18	72
P. nicotianae	207770	NT	R	23	16	70
P. palmivora	189724	R	N	25	25	100
P. palmivora	189727	N	N	22	19	86
P. palmivora	198928	F	N	26	23	88
P. palmivora	202542	F	N	25	23	92
P. palmivora	202544	F	F	22	14	64
P. palmivora	203531	NT	AN	21	5	24
P. palmivora	203532	-	-	23	0	0
P. palmivora	203536	F	F	23	11	48
P. palmivora	203538	F	F	24	13	54
P. palmivora	270386	R	R	25	19	76
Pythium aristosporium	209670	-	-	23	0	0
P. irregulare	203387	-	N	24	24	100
Rhizophydium biporosum	170357	-	N	23	22	96
Saprolegnia parasitica	191668	-	F	24	15	63

Growth and sporulation normal, N; Reduced growth and sporulation, R; Further reduced growth and sporulation, F; Abnormal growth and sporulation, A; Not tested by the technique, NT; Viable after 2 years but were non-viable when tested after further storage, D; -, failed to survive;

Fig. 5.02a A comparison of survival of isolates after 2 years storage preserved by the original and improved liquid nitrogen storage methods (2.13)



Using the technique avoiding excessive manipulation, 17 of the 19 isolates that had failed previous attempts survived freezing and storage in liquid nitrogen (Fig. 5.02a). Four isolates that had survived previous trials gave rise to better growth and sporulation when preserved by the improved technique. A further 3, not previously tested, also survived. Most isolates (83%) showed high recovery rates but in 5 of 29 cases less than half of the propagules tested survived. Both Chytridiomycetes survived the procedure, 96% of the propagules of Rhizophydium biporosum being recovered. Only 2 of the 21 Oomycetes tested, both of which were reprocessed, failed to survive and in each instance, other species of the genera were recovered.

5.03 Investigation of the effect of different cryoprotectants at various concentrations

As previously discussed (1.029) the cryoprotectant used for the preservation of organisms by freezing and storage in liquid nitrogen may be quite critical (Meryman, 1966; Calcott, 1978). Traditionally glycerol has been selected as the cryoprotective medium for the preservation of fungi. This has proved successful but may not be the best available. Many other compounds have been successfully used to protect other organisms during freezing (1.029) but a comparative study of cryoprotectants for the preservation of filamentous fungi has never been undertaken. In this study cryoprotectants and cryoprotectant mixtures were used as suspending media in the preparation for freezing 16 test organisms. To determine if any of the cryoprotectants inhibited growth, four test organisms were incubated in 10% glycerol, 10% PVP, 5, 10 and 15% DMSO, 5 and 10% glucose and the mixtures of

glucose and DMSO and samples transferred at 1, 5 and 24 h intervals, without washing, onto fresh medium to test for viability. The fungi tested were Erynia exitalis IMI 155908, Pythium sylvaticum IMI 248394, P. sylvaticum IMI 248395 and Phytophthora palmivora IMI 202528. All fungi resumed normal growth after all incubation times in each cryoprotectant preparation following 7-14 days on suitable growth media (Appendix III) at 25 °C.

The results of freezing and thawing 16 test fungi in different cryoprotectants or cryoprotectant mixtures are presented (Table, 5.031). The results obtained for each fungus after storage for 4 days and 6 months were similar. All samples of Aspergillus carbonarius and Cunninghamella elegans, and 1 from 6 samples of Wallemia sebi survived without any suspending medium. A. carbonarius survived all treatments whereas C. elegans did not survive when 5% glucose or a mixture of 10% glucose and 15% DMSO were used as cryoprotectants.

Table 5.031 Comparison of cryoprotectants used in the freezing and liquid nitrogen storage of 16 test fungi after 6 months storage. The results are expressed as the number of ampoules giving survival of spores or mycelial propagules.

Name	IMI Number	Cryoprotectants													Overall survival (%)
		0	1	2	3	4	5	6	7	8	9	10	11	12	
Aspergillus carbonarius	186307	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	100
Cercospora xanthosomatis	179717	0/8	3/3	4/4	0/8	0/8	0/8	3/4	4/4	4/4	4/4	4/4	4/4	4/5	50
Corynespora cassiicola	56007	0/6	4/4	0/6	NT	NT	NT	3/4	3/4	4/4	4/4	4/4	NT	0/6	52
Cunninghamella elegans	200332	4/4	4/4	5/5	0/7	3/4	4/5	4/4	4/5	4/5	4/4	4/4	3/4	0/8	68
Diplosclerophoma ceratoniae	180575	0/6	4/5	0/5	NT	0/6	NT	4/5	NT	4/4	NT	NT	NT	NT	39
Emericellopsis terricola	68332	0/6	4/4	3/4	0/5	0/5	5/5	3/5	4/4	4/4	4/4	3/4	5/5	0/5	58
Erynia excitalis	155908	0/8	0/7	NT	0/8	0/8	0/7	0/7	0/6	3/4	3/5	3/4	1/8	0/8	13
Myxotrichum thaxteri	83470	0/7	4/4	0/6	NT	0/6	NT	2/3	NT	4/4	NT	NT	NT	NT	33
Penicilliopsis clavariaeformis	60372	0/5	4/4	5/5	NT	4/6	NT	4/4	4/4	4/4	4/4	5/5	0/5	0/6	65
Phycomyces blakesleeanus	118496	0/8	4/4	6/6	NT	4/4	NT	4/4	NT	4/4	NT	NT	NT	NT	73

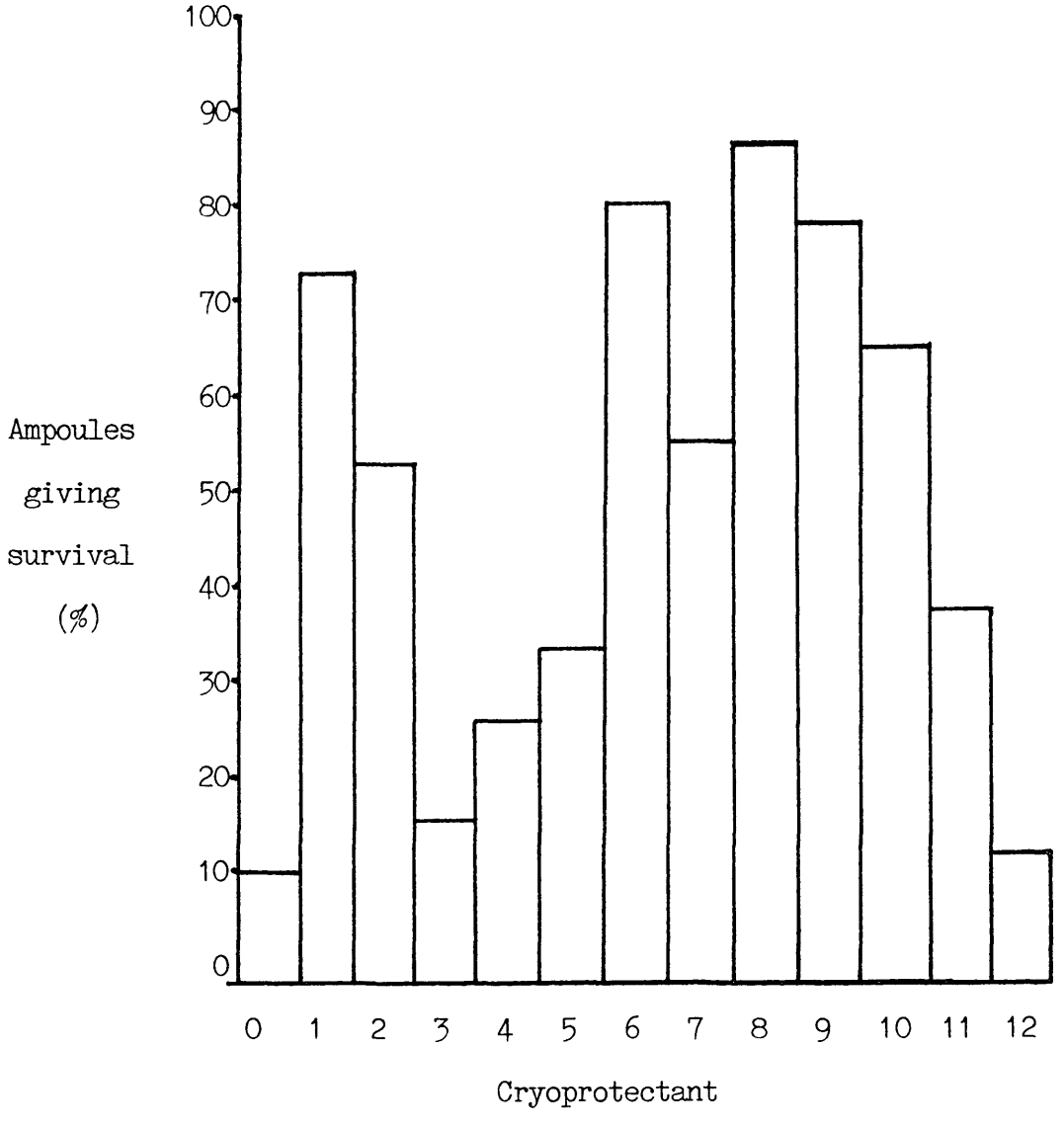
Cryoprotectants
0 - No cryoprotectant; 1 - 10% glycerol; 2 - 10% polyvinyl pyrrolidone 3 - 5% glucose;
4 - 10% glucose; 5 - 5% DMSO; 6 - 10% DMSO, 7 - 5% glucose + 10% DMSO;
8 - 8% glucose + 10% DMSO; 9 - 10% glucose + 10% DMSO; 10 - 10% glucose + 5% DMSO;
11 - 10% glucose + 8% DMSO; 12 - 10% glucose + 15% DMSO; Not tested, N;
The ratios given represent the proportion of ampoules with viable spores or mycelial propagules.

Table 5.031 Comparison of cryoprotectants used in the freezing and liquid nitrogen storage (continued) of 16 test fungi after 6 months storage. The results are expressed as the number of ampoules giving survival of spores or mycelial propagules.

Name	IMI Number	Cryoprotectants													Overall survival (%)
		0	1	2	3	4	5	6	7	8	9	10	11	12	
Phytophthora palmivora	202528	0/5	4/5	0/6	0/6	0/6	0/5	0/6	0/6	4/6	NT	4/5	1/6	0/6	19
Pseudophaeolus baudonii	ODA641	0/5	0/4	NT	NT	0/5	NT	0/4	0/5	3/6	3/6	0/5	0/6	0/5	12
Pythium sylvaticum	248394	0/6	4/4	0/5	0/6	0/6	0/6	0/6	3/4	4/4	4/4	0/6	0/6	0/6	22
P. sylvaticum	248395	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/5	3/6	0/6	0/6	0/6	0/6	4
Setosphaeria prolata	181085	0/8	4/4	4/4	NT	3/3	NT	4/4	NT	4/4	NT	NT	NT	NT	70
Wallemia sebi	86292	1/6	4/4	7/7	4/4	3/3	4/4	4/4	4/4	4/4	4/4	4/4	4/4	0/6	81
Fraction of isolates surviving		13%	81%	50%	22%	40%	44%	69%	67%	100%	91%	75%	45%	17%	
Fraction of ampoules germination		10%	73%	52%	15%	26%	34%	53%	55%	86%	78%	64%	38%	11%	

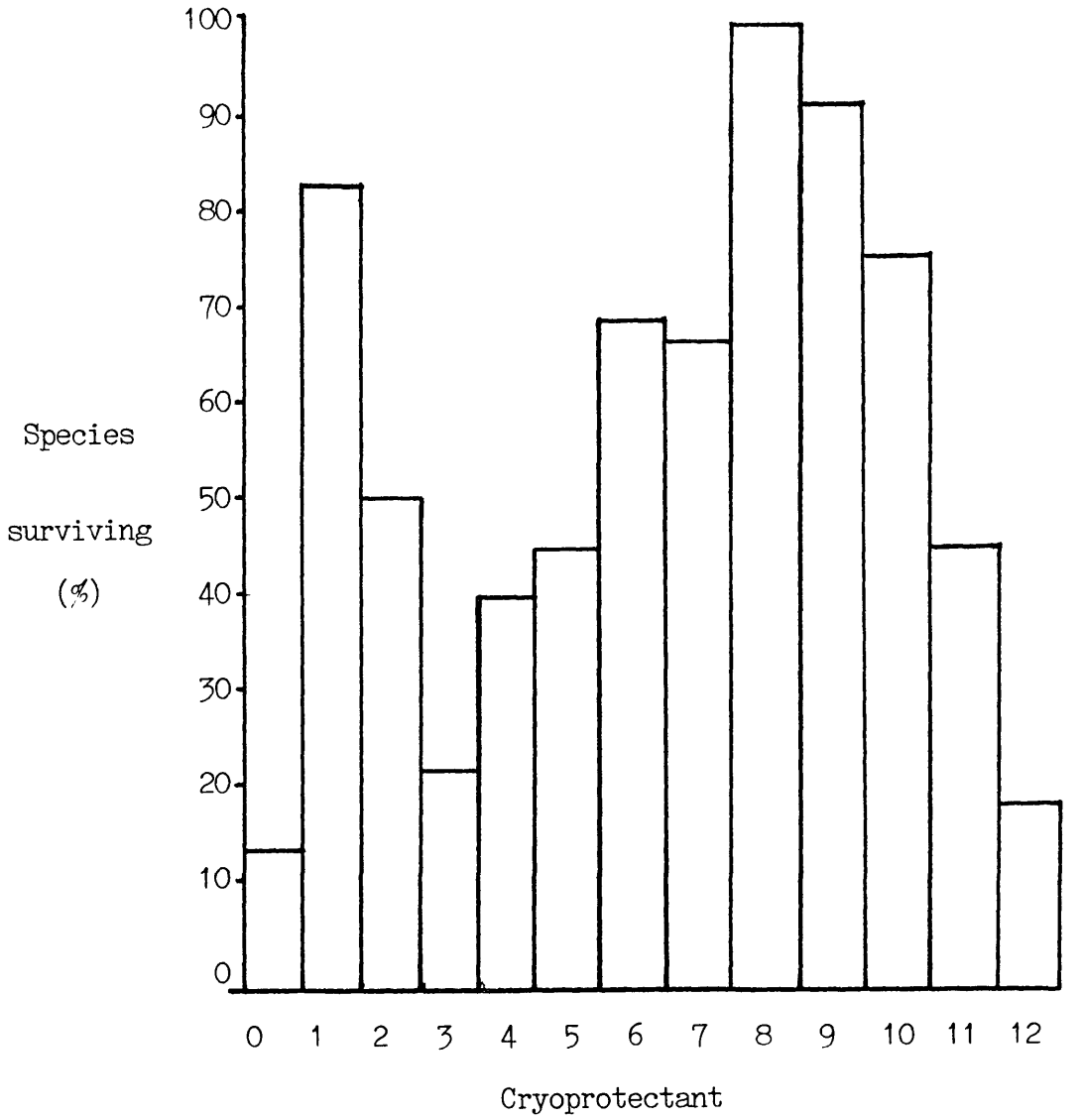
Cryoprotectants
0 - No cryoprotectant; 1 - 10% glycerol; 2 - 10% polyvinyl pyrrolidone 3 - 5% glucose;
4 - 10% glucose; 5 - 5% DMSO; 6 - 10% DMSO, 7 - 5% glucose + 10% DMSO;
8 - 8% glucose + 10% DMSO; 9 - 10% glucose + 10% DMSO; 10 - 10% glucose + 5% DMSO;
11 - 10% glucose + 8% DMSO; 12 - 10% glucose + 15% DMSO; Not tested, N;
The ratios given represent the proportion of ampoules with viable spores or mycelial propagules.

Fig. 5.03a Percentage of ampoules giving survival for each cryoprotectant under test from Tables 5.031 an 5.032



Cryoprotectants
0 - No cryoprotectant, 1 - 10% glycerol, 2 - 10% polyvinyl pyrrolidone
3 - 5% glucose, 4 - 10% glucose, 5 - 5% DMSO, 6 - 10% DMSO
7 - 5% glucose + 10% DMSO, 8 - 8% glucose + 10% DMSO
9 - 10% glucose + 10% DMSO, 10 - 10% glucose + 5% DMSO,
11 - 10% glucose + 8% DMSO, 12 - 10% glucose + 15% DMSO

Fig. 5.03b The percentage of species surviving freezing and storage in the 12 cryoprotectants tested from Tables 5.031 and 5.032



Cryoprotectants
0 - No cryoprotectant, 1 - 10% glycerol, 2 - 10% polyvinyl pyrrolidone
3 - 5% glucose, 4 - 10% glucose, 5 - 5% DMSO, 6 - 10% DMSO
7 - 5% glucose + 10% DMSO, 8 - 8% glucose + 10% DMSO
9 - 10% glucose + 10% DMSO, 10 - 10% glucose + 5% DMSO,
11 - 10% glucose + 8% DMSO, 12 - 10% glucose + 15% DMSO

All the isolates tested survived 6 months storage when frozen and stored in the mixture of 8% glucose and 10% DMSO although only 86% of the ampoules thawed gave rise to normal growth and sporulation of the fungi. Ten of the 11 (91%) isolates frozen in 10% glucose-10% DMSO mixture survived. Both these mixtures were more successful than 10% glycerol which allowed the preservation of 81% of the isolates tested (Fig. 5.03b) but only 73% of the ampoules thawed gave rise to normal growth and sporulation (Fig. 5.03a). The glucose-DMSO mixtures were more successful than DMSO on its own although using the mixture of 10% glucose- 15% DMSO only 17% of the isolates tested survived and only 11% of the ampoules thawed gave rise to normal growth and sporulation.

The overall survival, which was the proportion of ampoules with viable fungi for all cryoprotectants, gave an indication of how necessary the cryoprotectant was to the fungus during freezing. Aspergillus carbonarius was the only fungus to give 100% survival and therefore the choice of cryoprotectant was not an important factor in survival. This was confirmed by its survival without a cryoprotectant. Wallemia sebi and Phycomyces blakesleeanus were the only other isolates that had overall survivals above 70%. The cryoprotectant was critical for survival of Erynia excitialis (13% overall survival), Phytophthora palmivora (19% overall survival), Pseudophaeolus baudonii (12% overall survival), Pythium sylvaticum IMI 248394 (22% overall survival) and IMI 248395 (4% overall survival). The use of 8% glucose/10% DMSO here as a cryoprotectant enabled the preservation of some species of Erynia and Pythium that would

otherwise not have survived liquid nitrogen storage. Tests have shown that the cryoprotectants can be left in contact with the fungi and their viability is unaffected. Four test isolates were able to resume normal growth after 24 h incubation in all cryoprotection preparations of glucose, DMSO and glycerol.

5.04 Cryomicroscopic observations of the effects of cooling rates

Observations were made of hyphae of three test organisms cooled at different rates on the stage of a cryomicroscope (2.092). Penicillium expansum IMI 174158 and Phytophthora nicotianae IMI 158733 were cooled at rates of 1, 5, 10, 15, 20, 50, 70 and 100 $^{o}C\ min^{-1}$ both with and without 10% v/v glycerol. The hyphae of both organisms and the spores of Penicillium expansum had survived trials using the original liquid nitrogen storage technique (2.13). Observations were also made on Phytophthora citrophthora IMI 129906 at cooling rates of 10, 20 and 100 $^{o}C\ min^{-1}$ without and at 100 $^{o}C\ min^{-1}$ with, 10% v/v glycerol. This organism had previously failed to survive the liquid nitrogen storage technique. Hyphae recorded on video tape were examined for granularity of the contents, shrinkage, gas bubbles rupture and movement of contents. The granular appearance of the hyphae is assumed to be caused by the formation of intracellular ice. This phenomenon has been observed with other cell types (Morris & McGrath, 1981). To determine the effect of shrinkage on the viability of Penicillium expansum and Phytophthora nicotianae their hyphae were immersed in 2.75M sodium chloride. This is the concentration the hyphae were exposed to when cooled to -10 ^{o}C in a solution isotonic to the

hyphal contents as water is removed as ice (Morris, 1981)

The viability of the hyphae of P. expansum IMI 174158 and P. nicotianae IMI 158733 was measured at cooling rates similar to those used in the cryomicroscopy (2.094) both with and without glycerol.

Observations on P. expansum showed that cooling rates greater than 50 ^{o}C min^{-1} induced granularity in all hyphae (Fig. 5.04a). The change from the normal opacity of the hyphae to the granular appearence occurred rapidly indicating intracellular ice formation. It occurred within a narrow range of temperatures between -11.5 and -14 ^{o}C without glycerol and between -25 and -30 ^{o}C in its presence.

At slow cooling rates (<15 ^{o}C min^{-1}) extensive shrinkage of the diameter of the hyphae was observed (Plate 1). Shrinkage decreased, until at 100 ^{o}C min^{-1} it was negligible. Intracellular ice formed in hyphae cooled at >15 ^{o}C min^{-1} and increased in incidence with increased cooling rate. Both features were seen occasionally within the same hyphae. With the addition of glycerol (10% v/v) shrinkage was reduced and intracellular ice formation did not occur until rates of 35 ^{o}C min^{-1} and faster were used (Fig. 5.06a; Plate 2). Shrinkage in the length of the hyphae did not occur as the distance between the septa was unchanged during cooling.

During warming at a rate of 5 ^{o}C min^{-1} of hyphae after cooling at rates where intracellular ice formed localised melting occurred (Plate 3). However, warming at faster rates (50 ^{o}C min^{-1}) showed gas bubble formation in the hyphae of P. expansum when the intracellular ice melted (Plate 4). The bubbles disappeared on

further warming to 0 °C. Where gas bubbles formed the septa ruptured in all hyphae. Some of the hyphae cooled without glycerol, that had gas bubbles, burst and released their contents after warming above 0 °C.

The hyphae of P. expansum shrank to 58% of their original diameter when cooled without glycerol at a rate of 5 °C min^{-1} and the degree of shrinkage decreased with faster rates to none at all at a cooling rate of 100 °C min^{-1}, at which rate intracellular ice was observed in all hyphae (Plate 2). The hyphae cooled at 5 °C min^{-1} after pretreatment in glycerol shrank to 91% of the original diameter. The smallest mean diameter of hyphae cooled in glycerol (81%) was observed after cooling at 50 °C min^{-1}. In every case on rewarming the shrunken hyphae regained their normal diameter.

When fungi were placed in 2.75 M sodium chloride they shrank to approximately 60% of their diameter and on re-immersion in CZ broth they regained their original diameter. On subsequent subculture to CZ medium all the colonies resumed normal growth.

The viability of P. expansum decreased from 95% when cooled at 25 °C min^{-1} to 83% at 100 °C min^{-1} in the presence of glycerol and rose again to 95% at 200 °C min^{-1}. When cooled without glycerol a loss in viability from 90% to 63% occurred between rates of 16 and 22 °C min^{-1}.

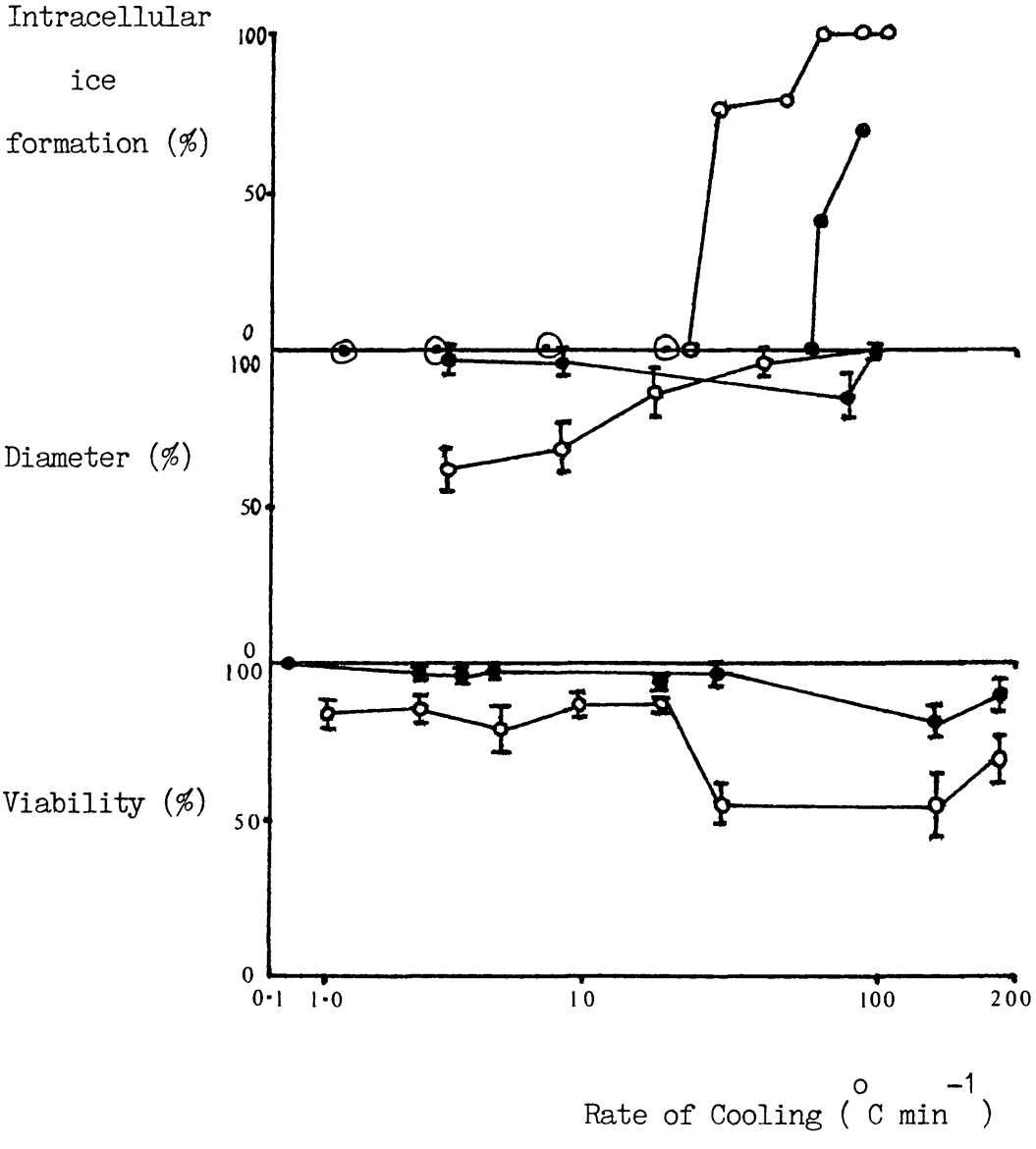
When the hyphae of Phytophthora nicotianae were examined shrinkage was observed at cooling rates of 10 °C min^{-1} to 100 °C min^{-1} (Plate 5). Only 2 hyphae from 57 examined (3.5%) showed the formation of intracellular ice at 70 °C min^{-1} at which rate

viabilities were reduced to approximately 15% (Fig. 5.04b). The hyphae that showed intracellular ice formation did so at approximately $-5^{o}C$ without glycerol and at $-23^{o}C$ with glycerol. The shrinkage increased with increased cooling rate and was reduced by the presence of 10% glycerol (Fig. 5.04b). No hyphae were viable after freezing in the absence of glycerol but with glycerol the viability fell from a high value (75%) after slow cooling to a low value (15%) as the cooling rate increased above $11^{o}C\ min^{-1}$. A limited number of hyphae of P. citrophthora IMI 129906 were observed during cooling. Again shrinkage occurred and only 3 hyphae from the 41 (7%) observed showed intracellular ice at a rate of $100^{o}C\ min^{-1}$. One froze on rewarming, but all did so at similar temperatures between -4 to $-5^{o}C$. The hyphae of both species regained their original diameter on warming.

When the colonies of P. nicotianae were placed in 2.75 M sodium chloride the hyphae shrank to 60% of their original diameter and on re-immersion in onion seed medium they regained their normal size. Only 19% (10/53) of these colonies remained viable.

At a cooling rate of $10^{o}C\ min^{-1}$ the majority of shrinkage had occurred before $-10^{o}C$ was reached both with and without glycerol (Fig. 5.04c). This was also observed at the faster rates of cooling.

Fig. 5.04a Viability, average percentage of original diameter of, and intracellular ice formation in, hyphae of Penicillium expansum IMI 174154 after cooling at different rates to -30 °C both with an without glycerol. Viabilities were counted after further cooling to -196 °C and thawing

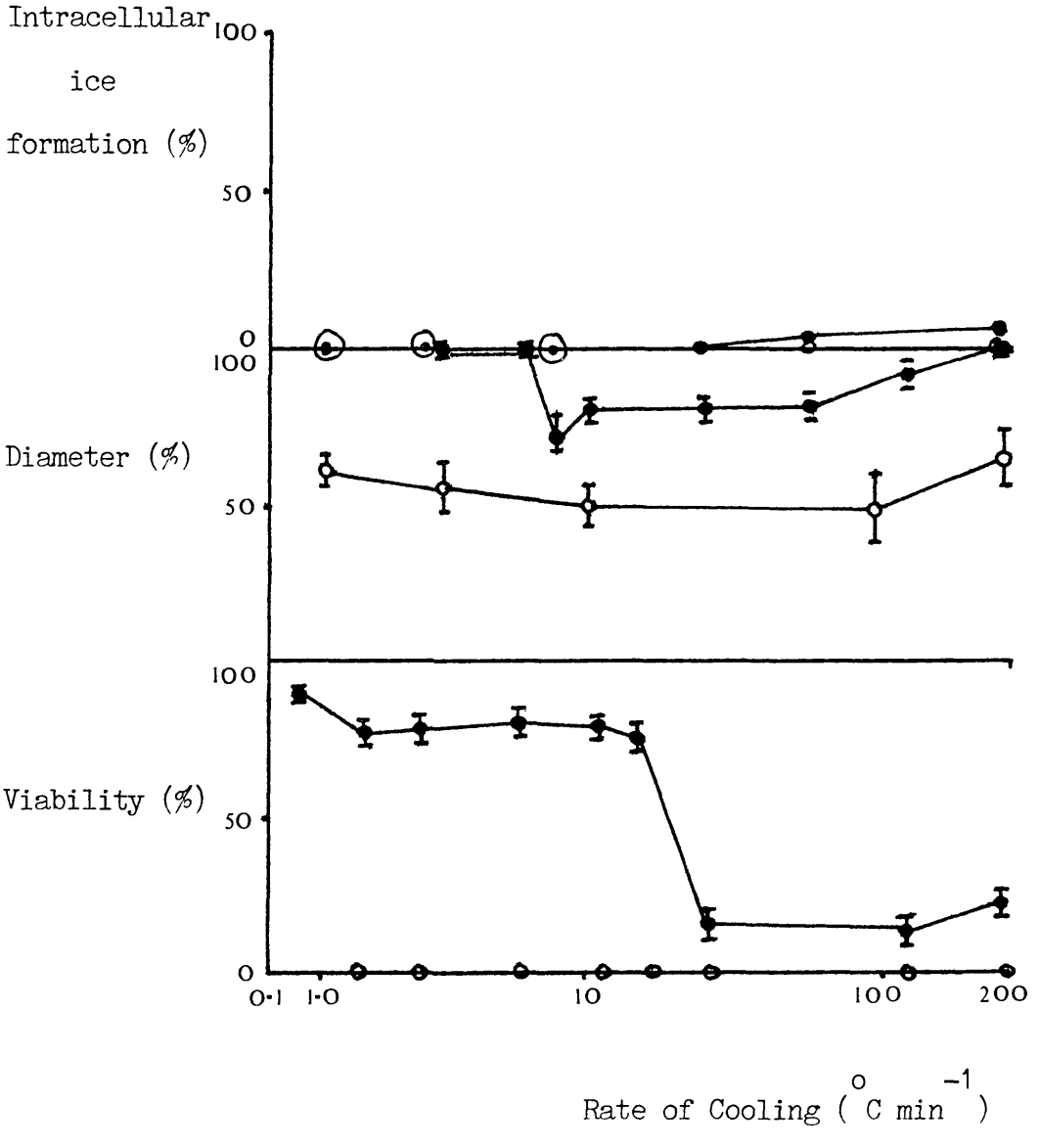


● With 10% (v/v) glycerol

○ Without 10% glycerol

The bars represent 2 standard deviations from the mean

Fig. 5.04b Viability, average percentage of original diameter of, and intracellular ice formation in, hyphae of P. nicotianae IMI 158733 after cooling at different rates to -30 °C both with and without 10% v/v glycerol. Viabilities were counted after further cooling to -196 °C and thawing



● With 10% (v/v) glycerol

o Without 10% glycerol

The bars represent 2 standard deviations from the mean

Fig. 5.04c The percentage of the original diameter of the hyphae of *P. nicotianae* IMI 158733 cooled at 10 °C min^{-1}

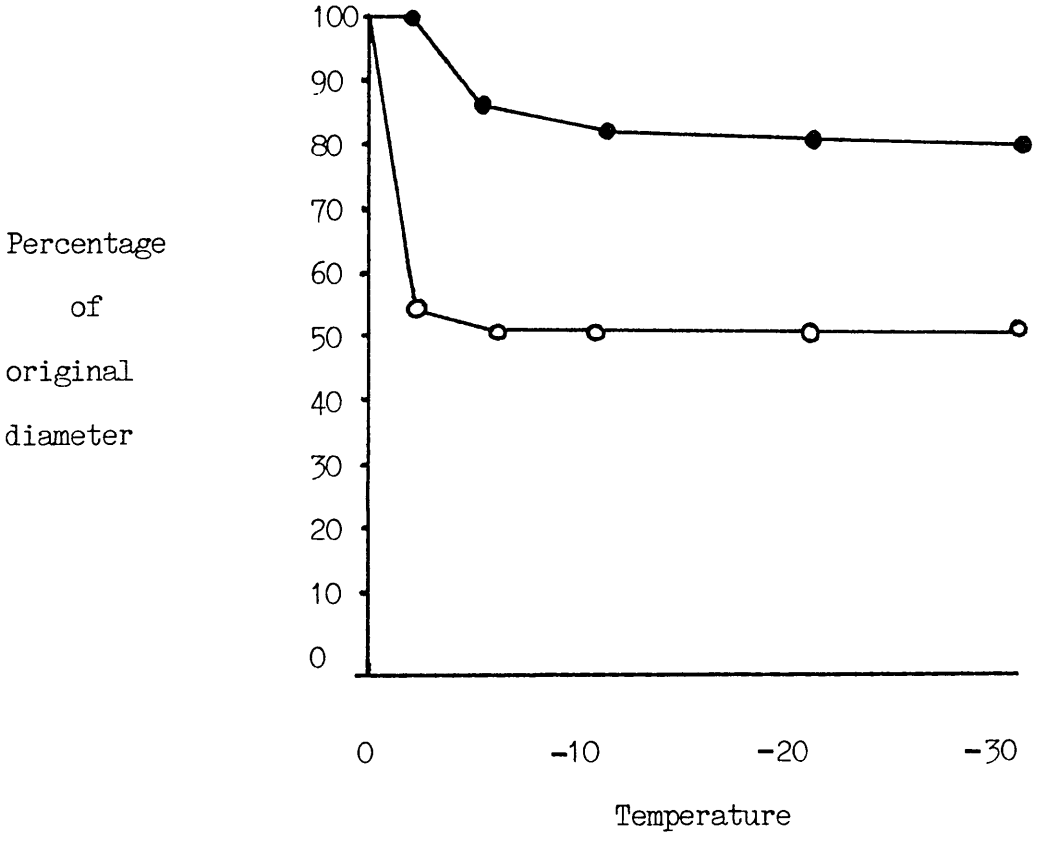


Plate 1 Light microscopy of *P. expansum* during cooling at a rate of 10 °C min^{-1} in Czapek-Dox broth

(a) Unfrozen control.

(b) At -5 °C.

(c) At -10 °C where the hypha diameter has noticeably decreased.

(d) At -15 °C.

(e) At -20 °C.

(f) At -25 °C where the hyphae have shrunk to approximately 60% of their original diameter.

PLATE 1

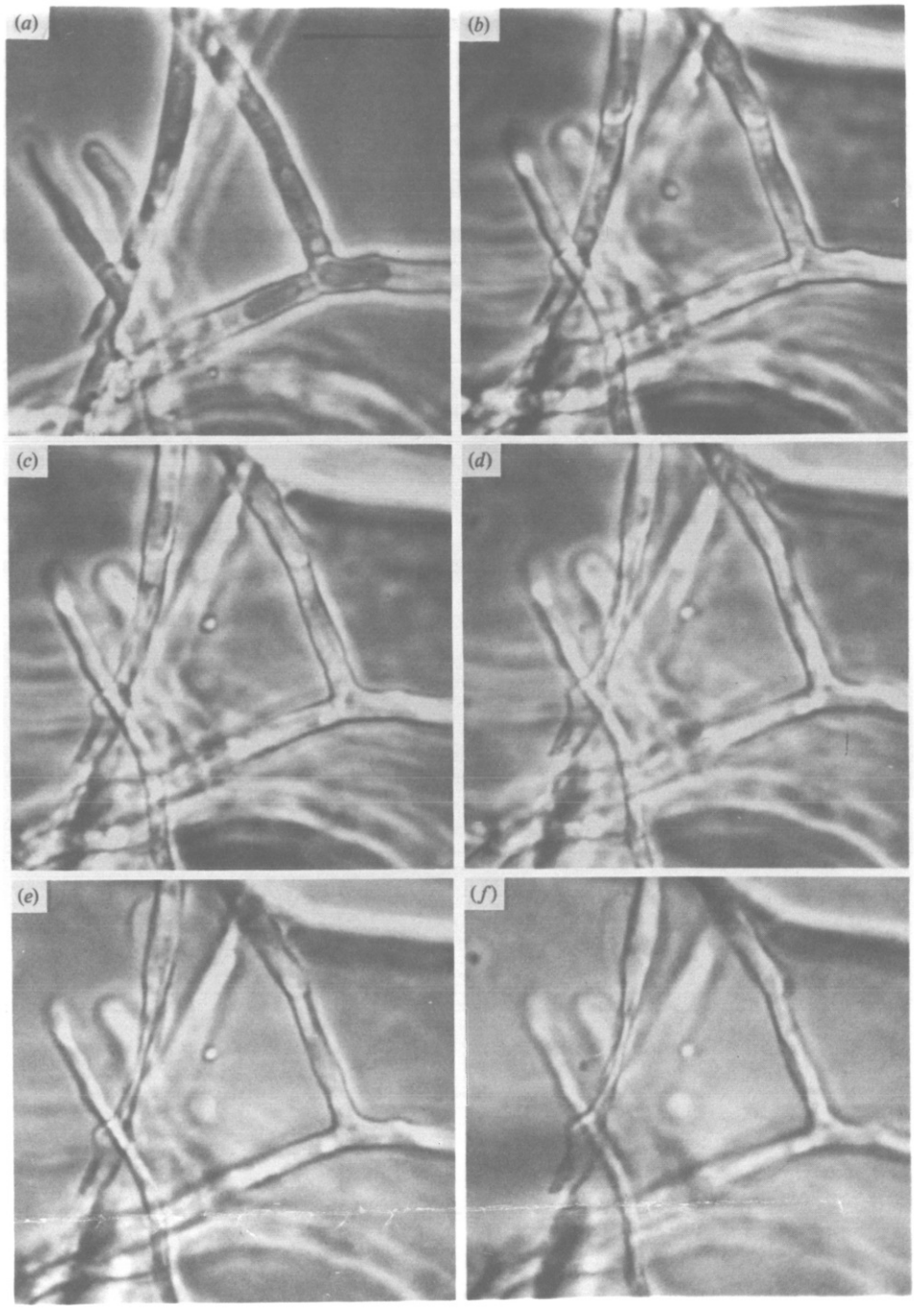


Plate 2 Light microscopy of *P. expansum* during cooling at a rate of 100 °C min^{-1} in Czapek-broth

(a) Unfrozen control.

(b) At -5 °C.

(c) At -10 °C where two hyphae show a granular appearence and have frozen internally.

(d) At -15 °C where all three hyphae have frozen internally.

(e) At -20 °C.

(f) At -25 °C where all hyphae are frozen and little or no shrinkage has occurred.

PLATE 2

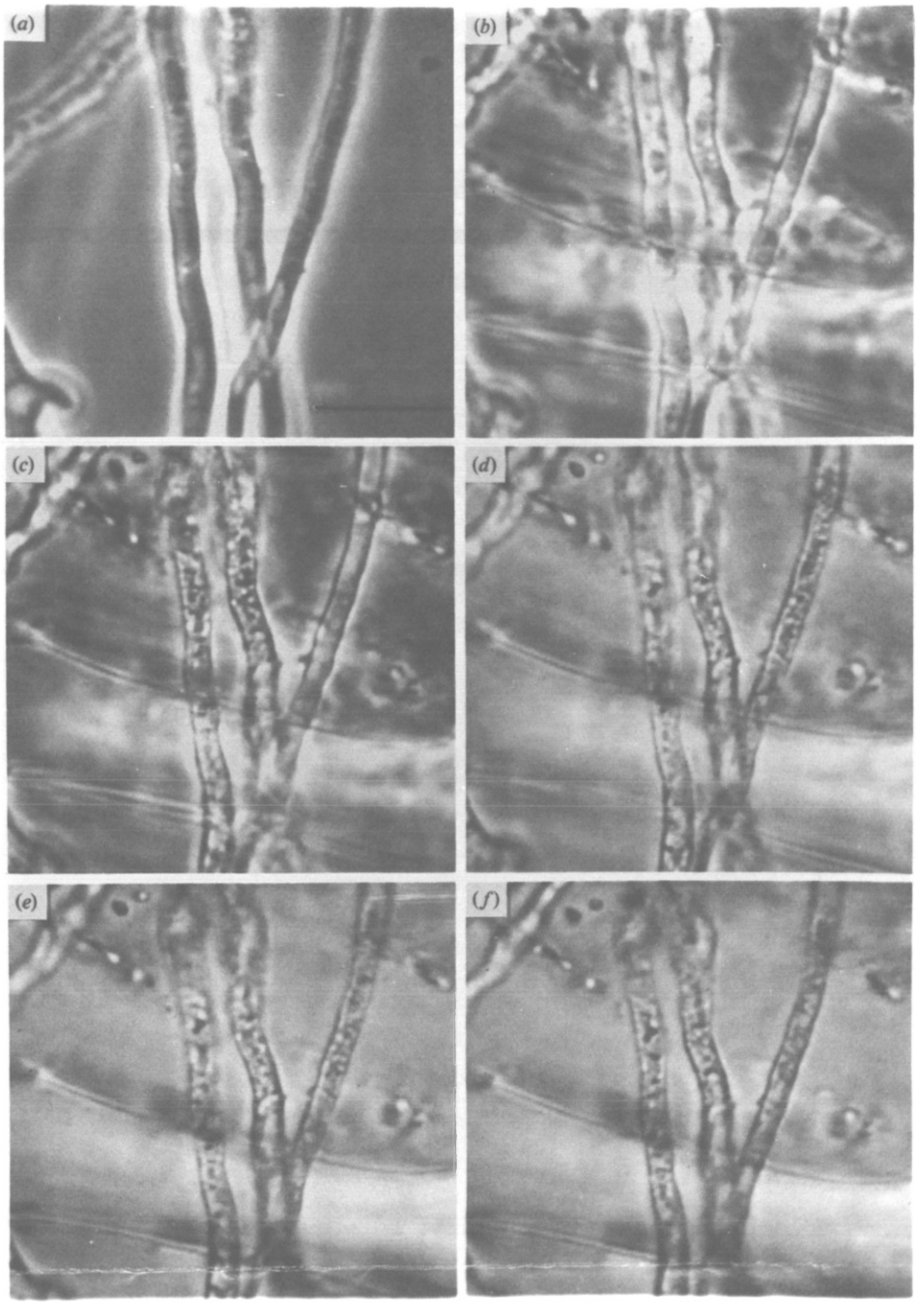


Plate 3 Light microscopy of *P. expansum* during warming at a rate of 5 °C min^{-1} after cooling at 50 °C min^{-1} to -50 °C in Czapek-Dox broth

(a) Unfrozen control.

(b) At -50 °C.

(c) During rewarming from -50 °C at -10 °C where localised melting can be seen.

(d) Thawed.

PLATE 3

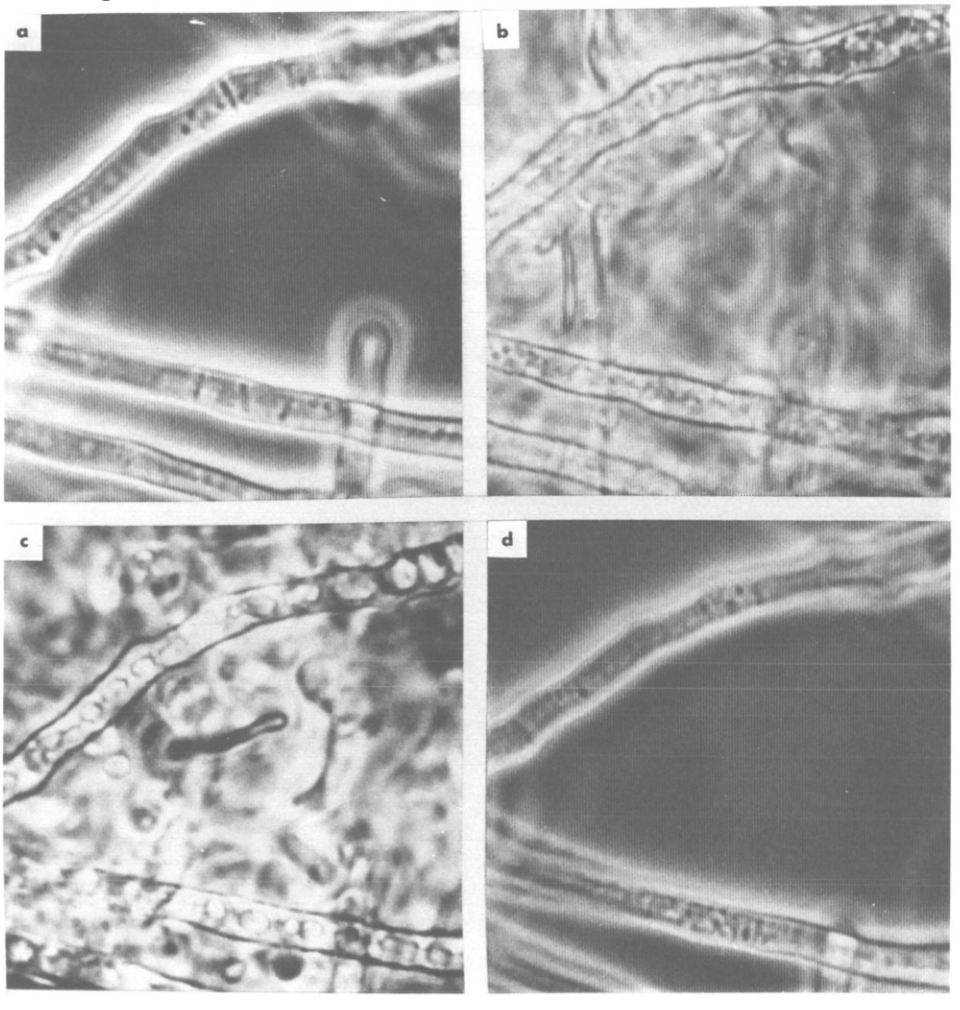


Plate 4 Light microscopy of *P. expansum* during warming at a rate of $50\ ^{o}C\ min^{-1}$ after cooling at $100\ ^{o}C\ min^{-1}$ to $-25\ ^{o}C$ in Czapek-Dox broth

(a) At $-15\ ^{o}C$.

(b) At $-10\ ^{o}C$.

(c) At $-7.5\ ^{o}C$ showing localised melting and intracellular gas bubble formation.

(d) At $-5\ ^{o}C$ showing intracellular melting and enlargement of the gas bubbles.

PLATE 4

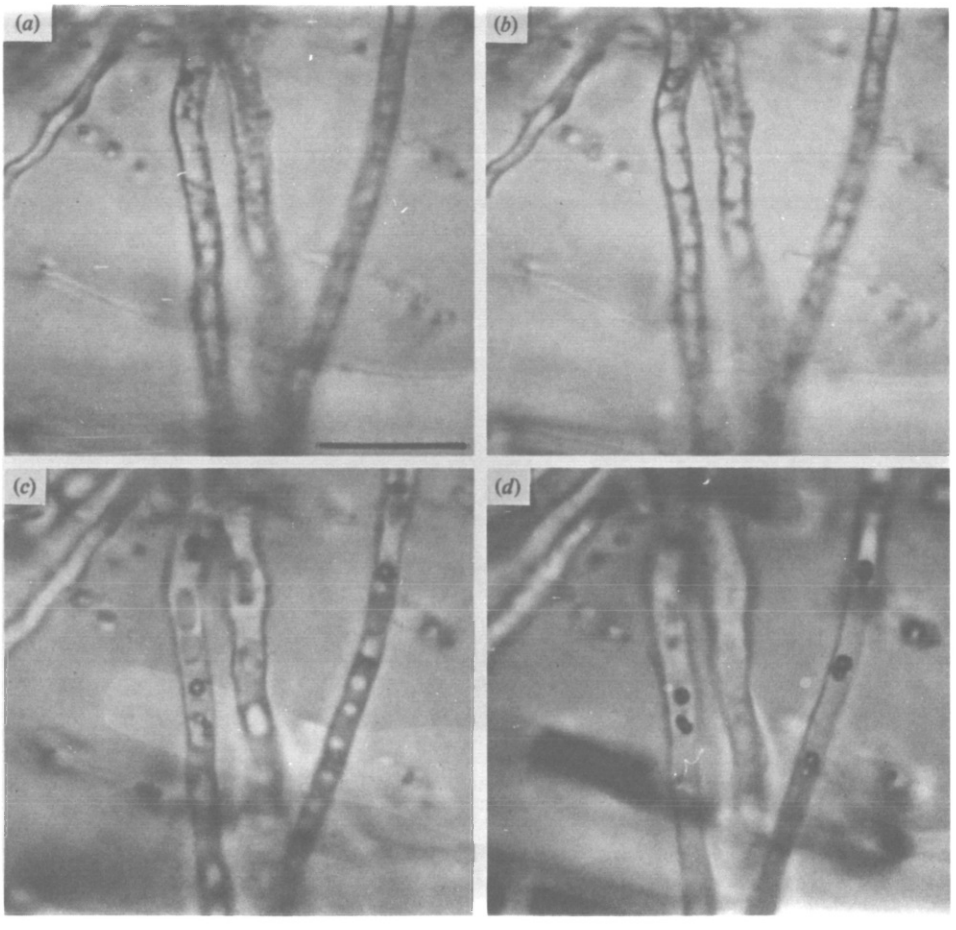


Plate 5 Light microscopy of *P. nicotianae* during cooling at a rate of 100 °C min^{-1} in hemp seed medium

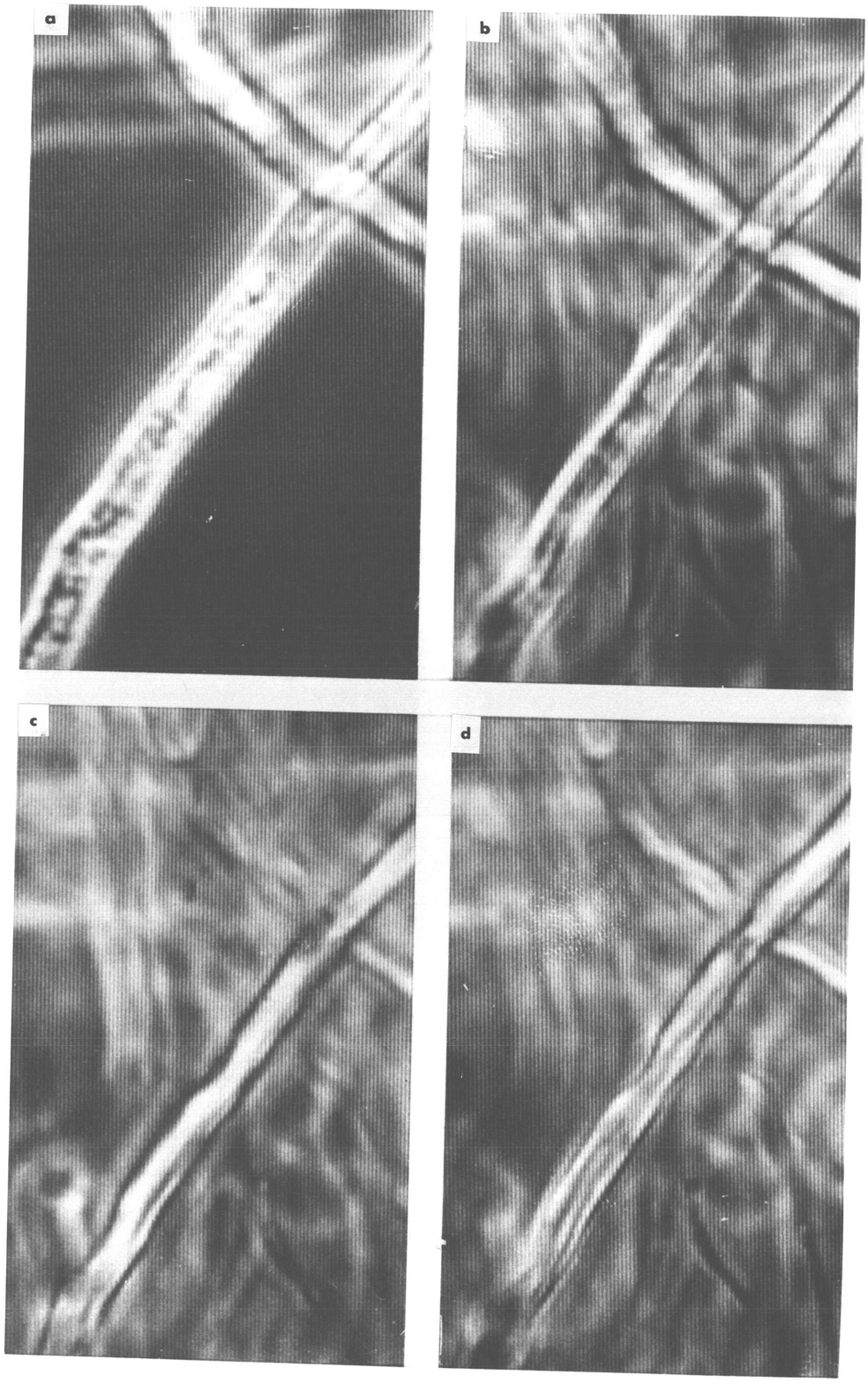
(a) Unfrozen control.

(b) At -10 °C.

(c) At -30 °C where shrinkage and crumpling of the hyphal wall can be seen.

(d) At -50 °C where the plasmamembrane has separated from the hyphal wall.

PLATE 5



5.05 General conclusions drawn from the study of the liquid nitrogen storage technique

1. Some isolates of the Mastigomycotina failed liquid nitrogen storage because they were damaged during preparation for preservation. Seventeen isolates, from 19 that had previously failed, survived when excessive manipulation was avoided.

2. All 16 test fungi survived and 86% of all ampoules opened gave normal growth when an 8% glucose-10% DMSO mixture was used as cryoprotectant. However, 3 isolates failed to survive in 10% glycerol and only 73% (51 from 71) of the ampoules opened gave rise to normal growth and sporulation.

3. Despite improvements made to the liquid nitrogen storage technique some isolates failed to survive. Phytophthora palmivora IMI 203532 and Pythium aristosporum IMI 209670 were non-viable after freezing even when excessive manipulation was avoided (Table, 5.021). A second isolate of P. palmivora IMI 203531 survived but did not grow or sporulate normally after thawing. An isolate of P. infestans died after 2 years storage. The isolates tested had been kept by serial transfer techniques before attempts were made to freeze them. This may account for some of the lack of success and further investigations should employ freshly isolated organisms.

4. Cooling at rates between 1 $^{o}C\ min^{-1}$ and 100 $^{o}C\ min^{-1}$ in the presence of glycerol (10% v/v) had little effect on the viability of Penicillium expansum.

5. Some viability of P. expansum was lost without glycerol.

6. The shrinkage seen in hyphae cooled at slow rates (<10 $^{o}C\ min^{-1}$)

did not occur in the presence of glycerol and at faster rates the shrinkage was reduced; for example without glycerol the hyphae shrank to 58% of their original diameter and in its presence to 87% when cooled at 10 ^{o}C min^{-1}.

7. The viability of hyphae of P. expansum was unaffected by shrinkage at +20 ^{o}C and at slow cooling rates (<10 ^{o}C min^{-1}).

8. The presence of glycerol raised the threshold of the cooling rate at which intracellular ice formation occured from 15 ^{o}C min^{-1} to 35 ^{o}C min^{-1}.

9. Loss in viability of hyphae of P. expansum without glycerol approximately correlated with rates at which intracellular ice formed. However, over 60% of the colonies remained viable at all rates of cooling. Therefore the hyphae of P. expansum survived the formation of intracellular ice.

10. On thawing of intracellular ice gas bubbles were observed in P. expansum after cooling both with and without glycerol at a rate of 100 ^{o}C min^{-1}.

11. The hyphae of Phytophthora nicotianae shrank to 50% and less of their original diameter when cooled without glycerol.

12. The addition of glycerol reduced the shrinkage of hyphae of P. nicotianae.

13. In the absence of glycerol P. nicotianae was killed by cooling at all rates.

14. Intracellular ice formed in less than 3% of the hyphae of the hyphae of P. nicotianae. Therefore it is unlikely that this was responsible for the death of the fungus.

15. The viability of P. nicotianae, when cooled in glycerol (10% v/v), fell at cooling rates faster than 15 ^{o}C min^{-1}. Under these

conditions the original diameter of the hyphae was reduced to below 70% and re-expanded on thawing.

16. There were no differences observed in the behaviour of hyphae of *P. nicotianae*, which remained viable after cooling at rates of between 1 and 11 $^{o}C\ min^{-1}$, and *P.citrophthora* which failed to survive.

CHAPTER 6

FREEZING AND LIQUID NITROGEN STORAGE OF SOME OBLIGATE PLANT PATHOGENS

6.01 Introduction

Previously in this study it has been shown that adjustment to techniques has allowed the preservation of some fungi which were otherwise difficult to preserve. Here the preservation of some examples of obligate plant pathogens Erysiphe pisi, Bremia latucae and Sclerospora graminicola is attempted. It is necessary to maintain races of these pathogens for resistance testing of new varieties of host. They are normally kept on host plants and transferred regularly. A method of preserving these pathogens on their hosts tissue is investigated.

The spores of Erysiphe pisi did not survive direct freezing at 1 ^{o}C min^{-1} when previously tested at CMI (unpublished) and therefore more rapid cooling was investigated.

A technique is also developed for the preservation of the downy mildew fungi Sclerospora graminicola and Bremia lactucae. As discussed above (1.02) it has been shown that when the spores of such fungi have been frozen and stored the results varied widely. The harvesting and preparation of suspensions may cause damage and therefore infected host tissue was frozen to avoid unnecessary manipulations. Such techniques that avoid mechanical damage have already been employed successfully to store some recalcitrant fungi (5.02).

6.02 Freezing and thawing Erysiphe pisi

The spores or infected tissue of E. pisi were frozen in small aluminium foil packets by plunging them into liquid Arcton 22 at or just above its melting point of -150 C (2.14). Eight packets were frozen and immediately thawed in each test and the germination tested by floating the conidia on water. Only low survival (10% of the spores of 1 sample germinated) was observed and all other attempts failed (Table, 6.021).

Table 6.021 Freezing and thawing of conidia or infected leaves of Erysiphe pisi in aluminium foil packets

Cells frozen	Freezing medium	Thawing	Germination
Conidia	Solid/liquid Arcton 22	Metal surface	None
Conidia	Liquid Arcton 22	Metal surface	None (intact conidia)
Infected leaf tissue	Solid/liquid Arcton 22	Metal surface	No intact conidia
Conidia	Solid/liquid	Water immersion	1 conidium but aborted
Conidia	Solid/liquid Arcton 22	Water immersion	4 from 40 (10%) conidia
Conidia in Fluorinert (FC43)	Solid/liquid Arcton 22	Water immersion	None
Conidia in Fluorinert (FC43)	Solid/liquid Arcton 22	Water immersion	None

Control samples gave 88% spore germination

Table 6.022 Freezing *Erisyphe pisi* in aluminium foil packets in solid/liquid isoceon 12 and thawed by immersion in water

Cells frozen	Storage	Germination
Conidia	-	(a) 4/176 (2.3%) (b) 0/557 (c) 0/125 (d) 1/75 (1.3%) (e) 5/291 (1.7%) (f) 0/190 (g) 0/217
Conidia (aluminium foil shinier side in)	-	(a) 0/210 (b) 8/313 (2.6%) (c) 0/93 (d) 0/126
Conidia (double wrapped in aluminium foil)	-	0/73
Infected leaf tissue	-	2/251 (0.8%)
Infected leaf epidermis	-	(a) 0/96 (b) 0/153 (c) 1/67 (d) 3/172 (1.7%) (e) 2/256 (0.8%) (f) 0/325 (g) 0/179 (h) 0/172

Table 6.022 shows the results of freezing Erisyphe pisi in aluminium foil packets in melting Isoceon 12 at or just above its melting point and thawing by immersion in water. Most conidia that failed to germinate were fragmented after freezing Those double wrapped in aluminium foil and which remained intact but did not float when released in water. Only 3 of the tests gave germination in some samples but the percentage survival was extremely low. Conidia frozen in solid/liquid Isoceon 12 germinated at 1.3%, 1.7% and 2.3%; two further attempts gave no germination. In 1 sample from 4 of conidia frozen in aluminium foil with the shinier side innermost in solid/liquid Isoceon 12, 2.6% of the conidia germinated. None of the conidia germinated when frozen double wrapped in aluminium foil and thawed immediately or after 2 h storage. The freezing of infected leaf tissue was unsuccessful as none of the frozen and thawed conidia germinated.

When infected leaf epidermis was frozen only 3 samples of of 8 tested gave germination of conidia, 1.7%, 1.5% and 0.8%. One of the successful trials gave rise to branched mycelium but the remainder of the conidia were fragmented.

6.03 Freezing and storage of Bremia lactucae

Table 6.031 shows the infectivity of Bremia lactucae after freezing and thawing frozen infected host tissue in liquid nitrogen. The proportion of infected cotyledons was recorded after 7 an 14 days incubation. Unfrozen controls gave 100% infection when pieces of infected tissue were immediately inoculated onto cotyledons on moist filter paper and 75% infection after a further 7 days, on the host. Samples frozen in

10% DMSO-8% glucose mixture were not infective after 7 days or subsequent storage. Infectivity of samples thawed immediately after freezing in 8.5% skimmed milk-10% glycerol was reduced to 34%, this was further reduced after 1.5 years storage to 4%. Only 26% of the samples frozen in 10% glycerol remained infective after immediate thawing and although none of the samples tested were able to infect after 7 days and 1 year storage 6% were infective after 1.5 years. Of the samples frozen without a suspending medium, 46% remained infective after immediate thawing and 22% after 1.5 years storage in liquid nitrogen. Fifty four percent of the samples frozen in distilled water were infective after immediate thawing but after 1.5 years storage only 15% were infective.

Table 6.031 Infectivity of Bremia lactucae after storage in liquid nitrogen

Suspending medium	Infection immediately after processing		Infection after storage					
			7 days		1 year		1.5 years	
	No. of infected leaves	%	No. of infected leaves	%	No. of infected leaves	%	No. of infected leaves	%
Unfrozen control	26/26	100	15/20	75	NT		NT	
Frozen samples								
None	13/18	46	9/26	35	6/20	30	8/36	22
10% DMSO + 8% glucose	18/30	60	0/25	0	0/20	0	0/30	0
8.5% skimmed milk + 10% glycerol	10/29	34	6/26	23	5/20	25	2/52	4
10% glycerol	8/31	26	0/20	0	0/25	0	3/50	6
Distilled water	14/16	54	7/19	37	8/20	40	3/20	15

6.04 Freezing and storage of Sclerospora graminicola

A similar technique to that used for the preservation of Bremia lactucae was employed for the preservation of Sclerospora graminicola. The results after immediate thawing are compared with results after several weeks storage (Table, 6.041).

Significant survival occurred with material frozen without any medium and this was increased by including 10% (v/v) glycerol. There is no evidence that viability and infectivity were affected during periods of storage of a few weeks and 1 trial storage for 1 year gave similar results.

Table 6.041 Freezing and storage of Sclerospora graminicola in liquid nitrogen

Suspending medium		Infection after immediate thaw		Storage (weeks)	Infection after storage	
		Ratio	%		Ratio	%
None	(a)	7/20	35	12	7/20	35
	(b)	4/20	20	10	5/20	25
	(c)	9/18	50	6	10/20	50
	(d)	8/20	40	6	8/20	40
10% glycerol	(a)	12/20	60	6	11/18	61
	(b)	13/20	65	10	12/20	60
				52	13/20	65
	(c)	NT		10	11/20	55
	(d)	12/20	60	12	12/20	60
	(e)	12/20	60	6	9/18	50

6.05 General conclusions drawn from the studies of the liquid nitrogen storage of some obligate plant pathogens

1. *Erysiphe pisi* was not preserved well by the methods tested here. Survival was limited to a few percent with both detached conidia and infected leaves. The most successful trial was with conidia wrapped in aluminium foil rapidly cooled in Arcton 22. Storage was for a few minutes only and thawing was effected by water at room temperature.

2. *Bremia lactucae* and *Sclerospora graminicola* infecting host tissues were preserved with the retention of infectivity. Best results, as evidenced by greatest infectivity, were obtained without a suspending medium or in distilled water with *B. lactucae* or by suspension in glycerol with *S. graminicola*.

CHAPTER 7

THE EVALUATION OF THE CENTRIFUGAL FREEZE DRYING TECHNIQUE FOR THE PRESERVATION OF FUNGI

7.01 Introduction

As discussed above (1.027) the centrifugal freeze drying technique has been used for the preservation of sporulating fungi for many years. The analysis of CMI's records (1.038) showed that the fungi which fail to survive the technique are not all taxonomically related. This chapter describes experiments to determine the particular stage in the sequence of centrifugal freeze drying procedure at which viability is lost.

Isolates which had been preserved in the CMI collection by centrifugal freeze drying without a suspending medium prior to the commencement of this study were retrieved from storage to determine if the lack of a suspending medium had affected their viability.

7.02 The determination of the death points of some fungi failing to survive the CMI centrifugal freeze drying method

The 212 isolates that had failed to survive centrifugal freeze drying and 44 of those that had survived initially for short periods but died during storage, were obtained from other sources and reprocessed. The freeze drying process was divided into stages and the viability of the fungi was tested after each stage of the sequence. The stages were cooling prior to freezing (precooling), freezing, the first 3 h of the drying on the primary freeze drier (EF6 machine) and the subsequent 17 h drying (secondary drying) with the secondary drier (30S2). The results (Table 7.02) show the stage after which viable fungus could not be recovered. Some fungi surviving processing but died during storage, in these cases their survival times are given in months. Some isolates survived the whole process but either had

deteriorated, giving abnormal growth and sporulation (AN) or grew vegetatively (V).

Fifteen aliquots were processed for each fungus and at least two ampoules were grown to test viability.

Some of the isolates tested had been stored previously in the CMI liquid nitrogen storage collection. These were recovered and their results recorded.

Table 7.021 Stages in the centrifugal freeze drying procedure after which viable fungi were not recovered. The number of other species in the genus that have survived and the result after recovery from liquid nitrogen storage is given for some fungi

Name	IMI Number	Number attempts	Death point	Number of species in genus survived	Survival in liquid nitrogen
Achaetomium globosum	82626	1	D1	4	NT
A. globosum	82626ii	2	S(V)D2	4	N15
Achlya ambisexualis	93805	1	F2	0	NT
A. ambisexualis	93806	2	F2	0	NT
Allomyces arbuscula	129543	1	D1	0	N9
A. cystogenus	93807	1	F2	0	NT
A. javanicus	86906	1	D1	0	NT
Anthostomella spartii	185019	1	F2	1	-
Arachnomyces nitidus	147447	1	D1	0	N14
Areolospora bosensis	184595	2	D2	0	N6
Arthrocladium caudatum	201693	3	D1	0	NT
Ascocalvatia alveolata	151071	2	D1	0	N11
Ascochyta fimbriata	87300	1	D2	9	NT
Balansia sclerotica	138634	2	F2	0	-
Basidiobolus haptosporus	108126	3	F2	2	N13
B. haptosporus	113159	1	F2	2	NT
B. microsporus	93345	8	S(2)D2	2	N11
Battarraea phalloides	151693	1	F1	0	-

Growth and sporulation, normal, N; normal but reduced, R; Abnormal, AN; Vegetative, V; -, failed to survive.
Stages of centrifugal freeze drying when fungus failed to recover F1, after prefreezing; F2, after freezing; D1, after first 3hrs drying on primary drier; D2, after completion of secondary drying; S, after the number of months storage or reason given in brackets;

Table 7.021 (continued) Stages in the centrifugal freeze drying procedure after which viable fungi were not recovered. The number of other species in the genus that have survived and the result after recovery from liquid nitrogen storage is given for some fungi

Name	IMI Number	Number tries	Death point	Number of species in genus survived	Survival in liquid nitrogen
Beltrania africana	149586	2	D1	2	N5
Biscogniauxia simplicior	245191	1	F2	0	R2
Blastocladiella emersonii	136816	1	D1	0	NT
Calonectria diploa	156281	2	S(1)AN	40	NT
C. ilicicola	237462	2	D1	0	NT
C. ilicicola	237463	2	F2	0	-
C. rigidiuscula	77037a	1	D1	0	NT
C. rigidiuscula	84749	1	D1	0	N11
Calospora arausiaca	80737c	2	S(AN)D2	0	NT
Camarops lutea	146521	1	F2	0	NT
Ceratocystis fimbriata	80787	1	D1	22	NT
C. fimbriata	80795	2	D2	22	NT
C. fimbriata	123637	1	D1	22	NT
C. fimbriata	123638	2	D1	22	R4
C. fimbriata	123677	1	F2	22	NT
Cercospora dulcamarae	161112	1	F2	25	-
C. fusimaculans	166241	2	S(2)D2	25	N12
C. plumbaginea	161116	1	F2	25	N12

Growth and sporulation, normal, N; normal but reduced, R; Abnormal, AN; Vegetative, V; -, failed to survive.
Stages of centrifugal freeze drying when fungus failed to recover F1, after prefreezing; F2, after freezing; D1, after first 3hrs drying on primary drier; D2, after completion of secondary drying; S, after the number of months storage or reason given in brackets;

Table 7.021 (continued) Stages in the centrifugal freeze drying procedure after which viable fungi were not recovered. The number of other species in the genus that have survived and the result after recovery from liquid nitrogen storage is given for some fungi

Name	IMI Number	Number tries	Death point	Number of species in genus survived	Survival in liquid nitrogen
Cercospora rautensis	161117	1	D1	25	N12
Cercosporidium bolleana	161111	1	F2	25	N6
Chaetomium amberpetense	144976	4	S(1)D2	88	NT
C. spinigerum	73515	2	D2	88	NT
Chytridium olla	86666	2	F2	0	-
Cladobotryum chlamydosporum	98099	1	F2	2	NT
Conidiobolus bangalorensis	118284	4	F2	4	NT
C. couchii	128727	2	D1	4	N14
C. heterosporus	102043	2	F2	4	N6
C. humicola	92300	2	F2	4	N11
C. khandalensis	102045	2	F2	4	N1
C. lichenicola	113700	9	F2	4	R2
C. lobatus	138635	2	F2	4	NT
C. mycophagus	113701	2	F2	4	N11
C. mycophilus	113698	3	S(1)F2	4	NT
C. nodosus	118285	1	F2	4	NT
Coprinus alkalinus	133856	3	S(1)D2	6	N14
C. amphibius	133855	1	D2	6	R5

Growth and sporulation, normal, N; normal but reduced, R; Abnormal, AN; Vegetative, V; -, failed to survive.
Stages of centrifugal freeze drying when fungus failed to recover F1, after prefreezing; F2, after freezing; D1, after first 3hrs drying on primary drier; D2, after completion of secondary drying; S, after the number of months storage or reason given in brackets;

Table 7.021 (continued) Stages in the centrifugal freeze drying procedure after which viable fungi were not recovered. The number of other species in the genus that have survived and the result after recovery from liquid nitrogen storage is given for some fungi

Name	IMI Number	Number tries	Death point	Number of species in genus survived	Survival in liquid nitrogen
Coprinus atramentarius	132648	1	F2	6	-
C. macrocephalus	182008	5	F2	6	-
Coriolus versicolor	79126	2	S(2)	0	N14
Cunninghamella vesiculosa	93346	2	F2	10	-
C. vesiculosa	93346ii	2	F2	10	NT
C. vesiculosa	130775	1	D1	10	NT
Cylindrocarpon pseudocandidum	171060	2	F2	17	NT
Cylindrocladium oumaiensis	167983	7	S(1)F2	5	NT
C. quinqueseptatum	78332	7	F2	5	NT
C. quinqueseptatum	136139	4	D1	5	NT
Cytospora eriobotryae	136523	2	S(1)F2	3	N14
Dactuliophora tarrii	102794	1	D1	0	N14
Dactylaria junci	131540	2	S(C)D1	2	NT
Didymella exitialis	183209	2	S(36)F2	5	-
Didymosphaeria donacina	187053	5	S(V)D2	1	NT
Dimargaris bacillospora	130774	1	D1	1	NT
D. xerosporica	113067	1	F2	1	NT
Echinopodospora jamaicensis	135507	4	S(2)D2	1	NT

Growth and sporulation, normal, N; normal but reduced, R; Abnormal, AN; Vegetative, V; -, failed to survive.
Stages of centrifugal freeze drying when fungus failed to recover F1, after prefreezing; F2, after freezing; D1, after first 3hrs drying on primary drier; D2, after completion of secondary drying; S, after the number of months storage or reason given in brackets;

Table 7.021 (continued) Stages in the centrifugal freeze drying procedure after which viable fungi were not recovered. The number of other species in the genus that have survived and the result after recovery from liquid nitrogen storage is given for some fungi

Name	IMI Number	Number tries	Death point	Number of species in genus survived	Survival in liquid nitrogen
Echinopodospora sacchari	141542	2	S(1)D1	1	N11
Eleutherascus tuberculatus	45134ii	2	D1	2	NT
Eremomyces bilateralis	151076	1	D1	0	N13
Erynia excitiales	155908	1	F2	0	N12
Flammulina velutipes	144603	1	F2	0	NT
Fomes fraxineus	81803	1	F2	0	NT
F. ulmarius	82772	2	D1	0	N7
F. ulmarius	82773	1	F2	0	NT
F. ulmarius	86444	1	F2	0	NT
Gabarnaudia betae	72913	3	D2	0	NT
Ganoderma applanatum	157818	4	S(1)F2	0	R5
G. philippii	108700	1	F2	0	NT
Gelasinospora longispora	130041	1	S(1)	0	N14
Genicularia bogoriensis	109554	1	S(V)	0	NT
Gloeosporium kaki	86556	3	D1	2	NT
Gnomonia leptostyla	77378	3	D2	4	NT
Hypoxylon argillaceum	146527	1	D1	8	N7
H. cohaerans	146054	1	D1	8	N7

Growth and sporulation, normal, N; normal but reduced, R; Abnormal, AN; Vegetative, V; -, failed to survive.
Stages of centrifugal freeze drying when fungus failed to recover F1, after prefreezing; F2, after freezing; D1, after first 3hrs drying on primary drier; D2, after completion of secondary drying; S, after the number of months storage or reason given in brackets;

Table 7.021 (continued) Stages in the centrifugal freeze drying procedure after which viable fungi were not recovered. The number of other species in the genus that have survived and the result after recovery from liquid nitrogen storage is given for some fungi

Name	IMI Number	Number tries	Death point	Number of species in genus survived	Survival in liquid nitrogen
Hypoxylon cohaerans	198632	2	D1	8	N6
H. confluens	146053	1	F2	8	-
H. fuscum	146525	1	D2	8	N7
H. multiforme	146526	1	F2	8	N7
H. multiforme	198631	5	D1	8	NT
H. nummularium	146051	3	F2	8	NT
H. nummularium	198629	7	D1	8	N7
H. nummularium	198630	5	F2(D1)	8	N7
H. udum	198633	3	S(V)D1	8	NT
Ingoldiella fibulata	177453	4	F2	1	NT
Iodophanus carneus	70912ii	3	F2	0	NT
Kretzschmaria clavus	245190	2	D1	0	N4
Lacellinopsis sacchari	143987	2	F2	0	AN7
Lasiobolidium spirale	151083	4	S(V)	0	V5
Lentinus degener	110525	1	F2	0	N7
Lenzites betulina	144608	1	F2	0	NT
Leptoporus albellus	144609	1	F2	0	V7
Leptosphaeria nodorum	86734	1	S(V)	8	NT

Growth and sporulation, normal, N; normal but reduced, R; Abnormal, AN; Vegetative, V; -, failed to survive.
Stages of centrifugal freeze drying when fungus failed to recover F1, after prefreezing; F2, after freezing; D1, after first 3hrs drying on primary drier; D2, after completion of secondary drying; S, after the number of months storage or reason given in brackets;

Table 7.021 (continued) Stages in the centrifugal freeze drying procedure after which viable fungi were not recovered. The number of other species in the genus that have survived and the result after recovery from liquid nitrogen storage is given for some fungi

Name	IMI Number	Number tries	Death point	Number of species in genus survived	Survival in liquid nitrogen
Leptosphaeria nodorum	190918a	1	D2	8	NT
Lophiostoma angustilabrum	143287	1	D2	2	NT
Marasmius palmivorus	123937	1	F2	0	V7
Melanconis modonia	80245	2	S(V)D2	0	NT
M. modonia	80246	1	D2	0	NT
Monacrosporium oxysporum	78728	3	S(12)D2	6	NT
M. rutgeriense	129960	3	D2	6	AN7
Monilinia laxa	133266	3	D1	6	NT
Monosporascus eutypoides	226000	1	D2	1	NT
Monotosporella setosa	139138	7	F2(D1)	0	N12
Mortierella ambigua	149024	2	D1	34	N4(V1)
M. minutissima	146672	1	D1	34	NT
M. vesiculosa	140982	1	D2	34	NT
M. vesiculosa	140983	1	D1	34	NT
Mucor azygospora	101213	7	D1	31	N8
Mycoleptodiscus sphaericus	159038	2	S(V)D2	1	N12
Mycosphaerella concentrica	119431	2	D2	25	NT
Mycovellosiella vaglnae	107865	1	D2	3	NT

Growth and sporulation, normal, N; normal but reduced, R; Abnormal, AN; Vegetative, V; -, failed to survive.
Stages of centrifugal freeze drying when fungus failed to recover F1, after prefreezing; F2, after freezing; D1, after first 3hrs drying on primary drier; D2, after completion of secondary drying; S, after the number of months storage or reason given in brackets;

Table 7.021 (continued) Stages in the centrifugal freeze drying procedure after which viable fungi were not recovered. The number of other species in the genus that have survived and the result after recovery from liquid nitrogen storage is given for some fungi

Name	IMI Number	Number tries	Death point	Number of species in genus survived	Survival in liquid nitrogen
Myrothecium leucotrichum	152595	2	D2	11	NT
Nectria dealbata	53165	1	S(AN)	37	NT
N. freycinetiae	208153	2	S(AN)	37	AN7
N. peristomialis	208155	2	S(AN)	37	R7
N. pertusa	208156	1	D2	37	NT
Nodulisporium argillaceum	107226	3	D2	7	NT
Nummularia dennisii	245192	1	D1	0	R4
N. discreta	233095	2	F2	0	N5
Oidiodendron chlamydosporicum	131498	3	S(C)	12	NT
Olpitrichum tenellum	89327	2	D1	1	N12
Ophiobolus oryzinus	88663	1	S(AN)	1	NT
Ophionectria trichospora	166077	3	S(C)D2	0	N11
Otthia lisae	122089	1	D2	0	NT
Panus stypticus	144612	1	F2	0	NT
Penicillifer pulcher	134023	1	F2	0	NT
P. pulcher	162653	7	D1(F2)	0	N12
Pezicula alba	68994	2	S(V)	2	AN2
Peziza brunneoatra	137181	1	D2	2	NT

Growth and sporulation, normal, N; normal but reduced, R; Abnormal, AN; Vegetative, V; -, failed to survive.
Stages of centrifugal freeze drying when fungus failed to recover F1, after prefreezing; F2, after freezing; D1, after first 3hrs drying on primary drier; D2, after completion of secondary drying; S, after the number of months storage or reason given in brackets;

Table 7.021 (continued) Stages in the centrifugal freeze drying procedure after which viable fungi were not recovered. The number of other species in the genus that have survived and the result after recovery from liquid nitrogen storage is given for some fungi

Name	IMI Number	Number tries	Death point	Number of species in genus survived	Survival in liquid nitrogen
Peziza ostracoderma	61802	1	D2	2	NT
Phacidiopycnis tuberivora	79162	1	D1	24	NT
Phaeoisariopsis griseola	137804	1	D1	0	NT
P. griseola	144489	2	D2	0	NT
Phomopsis sclerotioides	151823	1	D1	24	-
Phytophthora arecae	62656	1	F2	0	NT
P. boehmeriae	32199	2	F2	0	NT
P. cactorum	21168	1	F2	0	N14
P. cactorum	129909	1	F2	0	V14
P. cambivora	40505	2	F2	0	NT
P. cinnamomi	40506	2	F2	0	NT
P. citricola	45571	2	F2	0	NT
P. cryptogea	45168	2	F2	0	V14
P. drechsleri	40500	2	F2	0	NT
P. erythroseptica	34684	1	F2	0	N14
P. meadii	36529	2	F2	0	NT
P. megasperma	56348	1	F2	0	N11
P. nicotianae	21279	2	F2	0	N8

Growth and sporulation, normal, N; normal but reduced, R; Abnormal, AN; Vegetative, V; -, failed to survive.
Stages of centrifugal freeze drying when fungus failed to recover F1, after prefreezing; F2, after freezing; D1, after first 3hrs drying on primary drier; D2, after completion of secondary drying; S, after the number of months storage or reason given in brackets;

Table 7.021 (continued) Stages in the centrifugal freeze drying procedure after which viable fungi were not recovered. The number of other species in the genus that have survived and the result after recovery from liquid nitrogen storage is given for some fungi

Name	IMI Number	Number tries	Death point	Number of species in genus survived	Survival in liquid nitrogen
Phytophthora nicotianae	35087	3	F2	0	N5
P. nicotianae	77972	3	F2	0	N7
P. palmivora	63555	2	F2	0	NT
Piedraia quintanilhae	101644	1	F2	0	NT
Platystoma compressum	143286	3	D1	0	NT
Pleospora scirpicola	117653	2	D1	8	NT
Polyporus betulina	144618	1	F2	2	N14
Polyschema terricola	114592	12	S(AN)	2	NT
Poria vaillantii	146444	1	D1	1	-
Pseudocercospora karaka	166178	1	F2	25	N11
Puccinia graminis	174499	1	F2	0	-
P. graminis	174509	1	F2	0	-
P. paupercula	121021	1	F2	0	-
Pyrenochaeta lycopersici	136604	2	D1	8	NT
Pyrenophora dictyoides	135811	1	D1	7	V14
Pythium debaryanum	48558	2	F2	0	NT
P. graminicola	34768	1	F2	0	-
P. helicoides	61433	2	F2	0	NT

Growth and sporulation, normal, N; normal but reduced, R; Abnormal, AN; Vegetative, V; -, failed to survive.
Stages of centrifugal freeze drying when fungus failed to recover F1, after prefreezing; F2, after freezing; D1, after first 3hrs drying on primary drier; D2, after completion of secondary drying; S, after the number of months storage or reason given in brackets;

Table 7.021 (continued) Stages in the centrifugal freeze drying procedure after which viable fungi were not recovered. The number of other species in the genus that have survived and the result after recovery from liquid nitrogen storage is given for some fungi

Name	IMI Number	Number tries	Death point	Number of species in genus survived	Survival in liquid nitrogen
Pythium middletonii	42098	3	F2	0	V14
P. polymorphon	48559	2	F2	0	N12
P. splendens	61523	2	F2	0	NT
P. ultimum	82514	2	F2	0	NT
P. undulatum	35558	2	F2	0	N14
Quaternaria dissepta	146056	2	D1	0	NT
Ramularia deusta	101370	2	D1	3	NT
Ramulispora sorghi	81785	1	D1	4	NT
R. zonata	129672	1	F2	4	NT
Rhizoctonia lamellifera	83473	1	D1	1	NT
R. solani	20697	2	F2	1	N4
Rhizomucor tauricus	137380	3	F2	1	N14
Rigidoporus lignosus	83027	2	D1	0	NT
Rosellinia aquila	107929	1	F2	1	N14
R. aquila	146522	1	D1	1	NT
R. arcuata	107930	2	D1	1	NT
R. bunodes	107931	1	F2	1	NT
R. buxi	198634	4	F2	1	-

Growth and sporulation, normal, N; normal but reduced, R; Abnormal, AN; Vegetative, V; -, failed to survive.
Stages of centrifugal freeze drying when fungus failed to recover F1, after prefreezing; F2, after freezing; D1, after first 3hrs drying on primary drier; D2, after completion of secondary drying; S, after the number of months storage or reason given in brackets;

Table 7.021 (continued) Stages in the centrifugal freeze drying procedure after which viable fungi were not recovered. The number of other species in the genus that have survived and the result after recovery from liquid nitrogen storage is given for some fungi

Name	IMI Number	Number tries	Death point	Number of species in genus survived	Survival in liquid nitrogen
Rosellinia mammiformis	146524	1	D1	1	NT
R. necatrix	107934	1	F2	1	NT
R. necatrix	108006	1	D1	1	NT
R. necatrix	108336	1	D1	1	NT
R. pepo	107935	1	F2	1	V5
Saprolegnia parasitica	140977	1	F2	0	NT
S. parasitica	169621	1	F2	0	NT
Sclerotium wakkeri	103548	1	F2	2	V14
Scopulariopsis canadensis	86938	1	D1	19	NT
Searchomyces caprophiloides	91832	1	D1	0	NT
S. caprophiloides	138638	1	D1	0	NT
Selinia pulchra	67947	1	D1	0	NT
Septofusidium elegantulum	147205	1	D2	2	NT
Septoria menthae	100278	1	S(V)	11	NT
Setosphaeria turcica	77392	2	S(1)	0	-
S. turcica	113848	2	D1	0	NT
Sigmoidea marina	239282	2	S(AN)	0	NT
Sordaria bosensis	90324	2	D1	8	NT

Growth and sporulation, normal, N; normal but reduced, R; Abnormal, AN; Vegetative, V; -, failed to survive.
Stages of centrifugal freeze drying when fungus failed to recover F1, after prefreezing; F2, after freezing; D1, after first 3hrs drying on primary drier; D2, after completion of secondary drying; S, after the number of months storage or reason given in brackets;

Table 7.021 (continued) Stages in the centrifugal freeze drying procedure after which viable fungi were not recovered. The number of other species in the genus that have survived and the result after recovery from liquid nitrogen storage is given for some fungi

Name	IMI Number	Number tries	Death point	Number of species in genus survived	Survival in liquid nitrogen
Sordaria bosensis	110455	1	D1	8	NT
Sphaceloma embeliae	92304	1	F2	6	NT
S. ixorae	92303	1	F2	6	V12
Sphaerobolus stellatus	155101	1	D1	0	N14
S. stellatus	155102	1	D1	0	N14
Sphaerostilbe repens	84360	1	D1	0	NT
S. repens	135503	4	D1	0	V9
Stemphylium triglochinicola	135460	1	S(1)	13	NT
Stereum purpureum	62469	1	F2	0	NT
S. purpureum	145560	1	F2	0	NT
Sympodiella multiseptata	158984	4	S(2)	0	N14
Syzigites megolocarpus	122577	2	F2	0	NT
Tetranacrium gramineum	83001	1	D1	0	NT
Thanatephorus solani	44226	1	D1	4	NT
T. solani	70816	1	F2	4	NT
T. solani	70827	1	F2	4	NT
T. solani	78354	2	F2	4	NT
T. solani	78358	1	F2	4	NT

Growth and sporulation, normal, N; normal but reduced, R; Abnormal, AN; Vegetative, V; -, failed to survive.
Stages of centrifugal freeze drying when fungus failed to recover F1, after prefreezing; F2, after freezing; D1, after first 3hrs drying on primary drier; D2, after completion of secondary drying; S, after the number of months storage or reason given in brackets;

Table 7.021 (continued) Stages in the centrifugal freeze drying procedure after which viable fungi were not recovered. The number of other species in the genus that have survived and the result after recovery from liquid nitrogen storage is given for some fungi

Name	IMI Number	Number tries	Death point	Number of species in genus survived	Survival in liquid nitrogen
Thanatephorus solani	82071	1	F2	4	NT
T. solani	172732	1	D1	4	N8
Thielavia setosa	21599	2	S(12)	23	NT
T. trichorobusta	130230	2	S(C)	23	NT
Torula elaeodes	157787	5	S(V)	2	NT
Tubakia japonica	157600	2	S(V)	3	NT
T. subglobosa	157596	3	S(12)	3	N14
Ustilaginoidea virens	165999	1	F2	0	V12
Ustilago scitaminea	35616	2	S(6)	3	NT
Ustulina deusta	108703	1	D1	0	NT
U. deusta	121510	4	D1	0	V4
U. deusta	146057	2	F2	0	NT
U. deusta	193239	1	F2	0	NT
Volvariella esculenta	63833ii	1	F2	0	NT
V. esculenta	70680	4	F2	0	NT
Xylaria carpophila	146428	1	D1	1	V13
X. longipes	146055	1	D1	1	V7
X. polymorpha	146529	3	D1	1	-

Growth and sporulation, normal, N; normal but reduced, R; Abnormal, AN; Vegetative, V; -, failed to survive.
Stages of centrifugal freeze drying when fungus failed to recover F1, after prefreezing; F2, after freezing; D1, after first 3hrs drying on primary drier; D2, after completion of secondary drying; S, after the number of months storage or reason given in brackets;

Table 7.021 (continued) Stages in the centrifugal freeze drying procedure after which viable fungi were not recovered. The number of other species in the genus that have survived and the result after recovery from liquid nitrogen storage is given for some fungi

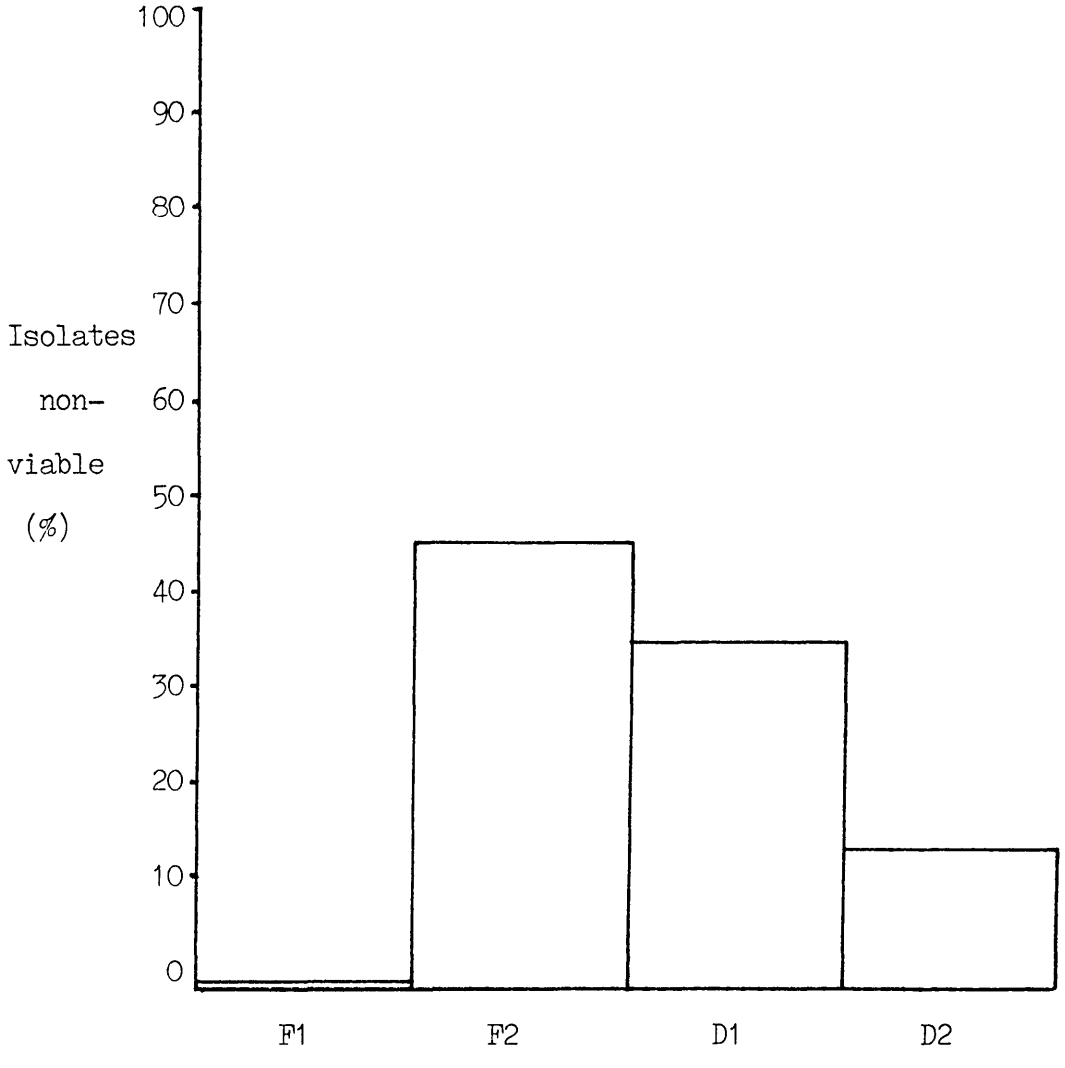
Name	IMI Number	Number tries	Death point	Number of species in genus survived	Survival in liquid nitrogen
Xylaria polymorpha	193238	3	D1	1	NT
X. sicula	73034	2	D1	1	NT
Zopfiella vehmii	185021	1	F2	0	NT

Growth and sporulation, normal, N; normal but reduced, R; Abnormal, AN; Vegetative, V; -, failed to survive.
Stages of centrifugal freeze drying when fungus failed to recover F1, after prefreezing; F2, after freezing; D1, after first 3hrs drying on primary drier; D2, after completion of secondary drying; S, after the number of months storage or reason given in brackets;

Table 7.022 The isolates that failed to recover after centrifugal freeze drying (data from Table 7.021) and the number of species in the same genera of the failures. Arranged taxonomically after Hawksworth, Sutton & Ainsworth, 1983

	No. of genera failed	No. of isolates failed	Number of species		
			failed	viable in genus	%
MASTIGOMYCOTINA					
Chytridiomycetes	3	5	5	0	0
Oomycetes	4	29	24	0	0
ZYGOMYCOTINA					
Zygomycetes	9	28	22	71	76
ASCOMYCOTINA					
Clavicipitales	2	6	5	1	17
Diaporthales	3	4	3	4	57
Diatrypales	1	1	1	0	0
Dothideales	11	13	11	31	74
Gymnoascales	2	2	2	0	0
Helotiales	2	2	2	8	80
Hypocreales	5	12	9	37	80
Ophiostomatales	1	5	1	22	96
Pezizales	4	5	5	4	44
Sordariales	7	12	10	125	93
Sphaeriales	8	32	22	10	31
BASIDIOMYCOTINA					
Hymenomycetes	15	31	21	13	38
Gasteromycetes	2	3	2	0	0
Urediniomycetes	1	3	2	0	0
Ustilaginomycetes	1	1	1	3	81
DEUTEROMYCOTINA					
Coelomycetes	8	12	12	66	85
Hyphomycetes	36	50	45	187	81

Fig. 7.02a Loss of viability in 255 isolates tested after 4 stages of the centrifugal freeze drying procedure.



F1, after precooling; F2, after freezing; D1, after primary drying D2, after secondary drying;

One hundred and forty seven of the isolates which lost viability have other representatives of their genus that have survived the whole centrifugal drying procedure. One hundred and fifteen isolates (45%) died during the freezing stage (F2) of the method. One further isolate, Battarraea phalloides IMI 151693 died during the initial cooling prior to the freezing (F1). Eight isolates survived the technique in poor condition, 5 lost the ability to sporulate and 3 grew and sporulated abnormally.

One hundred and nine of the isolates were frozen and stored in liquid nitrogen of which 88 survived. Of these, 41 isolates had died during the freezing stage (F2) of centrifugal freeze drying.

7.03 Determination of the viabilities of some fungi freeze dried without suspending medium

The isolates freeze dried without suspending media were inoculated onto selected agar media (Table, 7.031). Only one isolate, Aspergillus amstellodami, died during storage. Otherwise 50% was the lowest recorded survival of the freeze dried agar blocks of Sporidesmium flexum; over 60% of all other freeze dried isolates survived.

Table 7.031 Survival periods of isolates freeze dried by the centrifugal method without suspending medium

Name	IMI Number	Survival period (years)	Growth	Viable blocks	
				ratio	%
Ascocoryne sarcoides	68130	14	F	9/10	90
Aspergillus amstelodami*	71295	8	-	0/10	0
A. candidus	73074	14	N	10/10	100
A. carneus	73777	14	N	10/10	100
A. nidulans	61454ii	14	R	10/10	100
A. niger	75353ii	14	N	9/9	100
A. ostianus	93445	14	R	9/10	90
A. quadrilineatus	72733	14	N	10/10	100
Chaetomium abuense	114513	14	N	10/10	100
Curvularia trifolii	75377	13	N	8/10	80
Cylindrocarpon congoense	69504	14	N	10/10	100
Embellisia chlamydospora	67737	14	N	7/10	70
Fusarium graminearum	69695	14	F	7/10	70
Nectria gliocladioides	71095	14	N	10/10	100
Paecilomyces dactylethromorphus	65752	14	N	10/10	100
Penicillium cyclopium	68236	14	R	10/10	100
P. nigricans	96660	14	R	9/9	100
P. paraherquei	68220	14	F	10/10	100
P. raperi	71625	13	N	10/10	100
P. roquefortii	129207	14	R	10/10	100

Growth and sporulation, normal, N; Reduced growth and sporulation but typical morphology R; Further reduced growth and sporulation but typical morphology, F; Failed to survive, -; * Died after 8 years successful storage;

Table 7.031 Survival periods of isolates freeze dried by the (continued) centrifugal method without suspending medium

Name	IMI Number	Survival period (years)	Growth	Viable blocks	
				ratio	%
Penicillium spinuloramigenum	68617	14	N	10/10	100
P. stecki	72029	14	R	10/10	100
Pestalotiopsis gracilis	69749	14	N	7/10	70
Phaeotrichoconis crotalariae	69755	14	N	8/10	80
Phialomyces macrosporus	110130	14	R	8/10	80
Phomopsis oncostoma	68344	14	R	9/10	90
Pycnoporus sanguineus	75002	9	N	6/10	60
Sagenomella griseoviridis	113160	13	N	8/10	80
Scopulariopsis carbonaria	86941	14	N	9/10	90
Sporidesmium flexum	246524	1	F	5/10	50

Growth and sporulation, normal, N; Reduced growth and sporulation but typical morphology R; Further reduced growth and sporulation but typical morphology, F; Failed to survive, -;

7.04 General conclusions drawn from the studies of the centrifugal freeze drying technique

1. Fungi failed to survive the freezing stage of the centrifugal freeze drying technique.

2. Fungi which survived the initial precooling and freezing stages were more likely to die during the primary drying than during the secondary drying period.

Many sporulating and non-sporulating fungi do not survive the centrifugal freeze drying technique. It has been shown that 45% had died during the freezing stage of the process, 34% during the primary drying and 12% during the secondary drying. Therefore improvements enabling more fungi to survive the freezing stage may lead to more fungi surviving the technique. The liquid nitrogen storage technique allows many fungi to survive. The initial freezing rate could be employed in the freeze drying technique and give the required survival.

Isolates of the same species respond differently to the centrifugal freeze drying method. Many isolates will survive without a cryoprotectant whereas others did not. Results of preservation by this particular method of freeze drying can be unpredictable.

CHAPTER 8

THE DEVELOPMENT OF A FREEZE DRYING TECHNIQUE FOR THE PRESERVATION OF FUNGI

8.01 Introduction

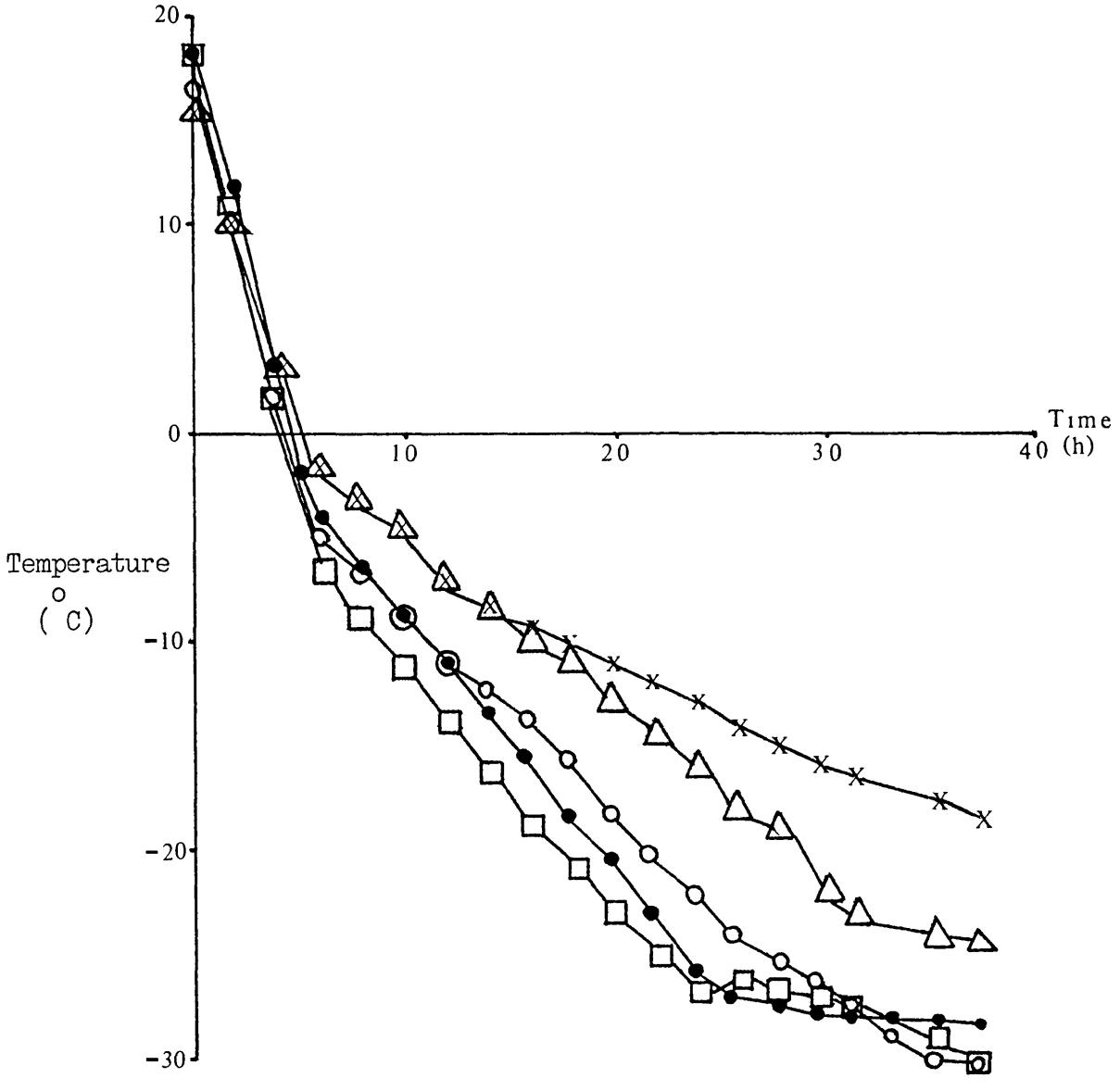
The results of investigating the stage of the centrifugal freeze drying procedure at which death occurred showed that 1 isolate (<0.4%) had died during cooling prior to freezing, 45% during freezing, 34% during the first 3 h of drying and 12% during the second drying stage. Therefore the effects of freezing and drying procedures on the residual water content and viability of fungi were investigated with the objective of developing a method of freeze drying to preserve organisms that are not preserved by the present technique.

8.02 The determination of the cooling rates in liquid nitrogen and in the shelf freeze drier

The cooling rate achieved by gaseous phase cooling in the LR 320 liquid nitrogen refrigerator had proved successful in the liquid nitrogen storage. It was therefore considered to be suitable to employ this cooling in the initial stages of freeze drying. The rate was measured and compared with cooling rates achieved on the shelf freeze drier.

The cooling rates achieved above 0 °C in the shelf freeze drier set at -35 and -45 °C and evacuating the chamber at -20 °C were similar to those achieved in the gaseous phase above liquid nitrogen (Fig. 8.02a). Below -4 °C the curves differed slightly but the cooling rates were similar. Therefore each of the settings, -35 and -45 °C, was equally suitable for investigation of the initial stages of the freeze drying technique.

Fig.8.02a The cooling curves in the gaseous phase of liquid nitrogen and on the shelf freeze drier



x Minifast -20
○ Minifast -35
□ Minifast -45
△ Two step (held at -20 for 10 min then cooled to -35)
● Liquid nitrogen gaseous phase

8.03 The effect of the freezing procedures during freeze drying on the viability of selected fungi

To investigate the effect of the cooling obtained in the gaseous phase of a liquid nitrogen refrigerator on the viability of fungi 13 isolates representing the Mastigomycotina, Zygomycotina, Ascomycotina and Deuteromycotina were selected and prefrozen. Fifty ampoules containing 0.5 ml of suspension in skimmed milk-inositol medium were prepared for each isolate and placed in the gaseous phase at -35^{o}C. After 1 h they were transferred to liquid nitrogen at -196^{o}C for 5 min when cooling was complete. At least 2 ampoules of each frozen isolate were thawed at approximately 190^{o}C min^{-1} in a water bath at 37^{o}C, and opened to test viability (2.16). The remainder were transferred to the chamber of the centrifugal freeze drier which was subsequently evacuated and suspensions freeze dried (2.102). On completion of the freeze drying process the ampoules were stored at 15^{o}C. The freeze drying procedure was repeated on at least 2 occasions for each isolate. After 1 week, 3 months and 6 months storage a further 2 or 3 ampoules were opened to test viability.

All the isolates tested were viable after the initial freezing (thawed from -196^{o}C) stage. The results in table 8.031 show that all remaining ampoules of Pythium sylvaticum, IMI 248394 and IMI 248395, and Saprolegnia parasitica IMI 169621 gave no viable growth after 1 week of storage following drying under vacuum. Phytophthora palmivora IMI 202528 survived 3 months storage after freeze drying but was not viable after 6 months storage. All ampoules tested of the remaining 9 isolates were viable after storage.

Table 8.031 Viability of fungi in skimmed milk inositol-medium following two stage cooling in the gaseous phase at -35°C for 1 h and liquid nitrogen (-196°C) for 5 min and vacuum drying in the centrifugal freeze drier (2.102)

Name	IMI Number	Propagules tested	No. of occasions tested	No. of ampoules giving normal growth after storage against the number tested		
				1 week	3mths	6mths
Basidiobolus ranarum	89715	Conidia	4	12/12	12/12	12/12
Ceratocystis paradoxa	77668	Conidia & Ascospores	2	4/4	4/4	4/4
Cercospora xanthosomatis	179717	Conidia	3	9/9	9/9	9/9
Chaetomium globosum	16203	Ascospores	3	9/9	9/9	9/9
Coniophora puteana	79127	Mycelium	3	9/9	9/9	9/9
Coprinus hexagonosporus	161417	Basidia	3	9/9	9/9	9/9
Cunninghamella elegans	200332	Conidia	3	9/9	9/9	9/9
Hypoxylon fragiforme	192581	Conidia	2	6/6	6/6	6/6
Macrophomina phaseolina	179649	Conidia	3	9/9	9/9	9/9
Phytophthora palmivora	202528	Sporangia & Mycelium	3	6/9	6/9	0/132
Pythium sylvaticum	248394	Sporangia & Mycelium	3	0/144	NT	NT
Pythium sylvaticum	248395	Sporangia & Mycelium	3	0/144	NT	NT
Saprolegnia parasitica	169621	Sporangia & Mycelium	3	0/144	NT	NT

NT, not tested.

8.04 The determination and effect of residual water content after different drying periods on the viability of selected fungi

It was established above that the cooling rate during freezing prior to vacuum drying influences survival of some fungi. As the residual water content after freeze drying is critical for the retention of viability during storage and 1 to 2% is considered to be best (Fry, 1954; Fry and Greaves, 1951) drying procedures were investigated in order to achieve this.

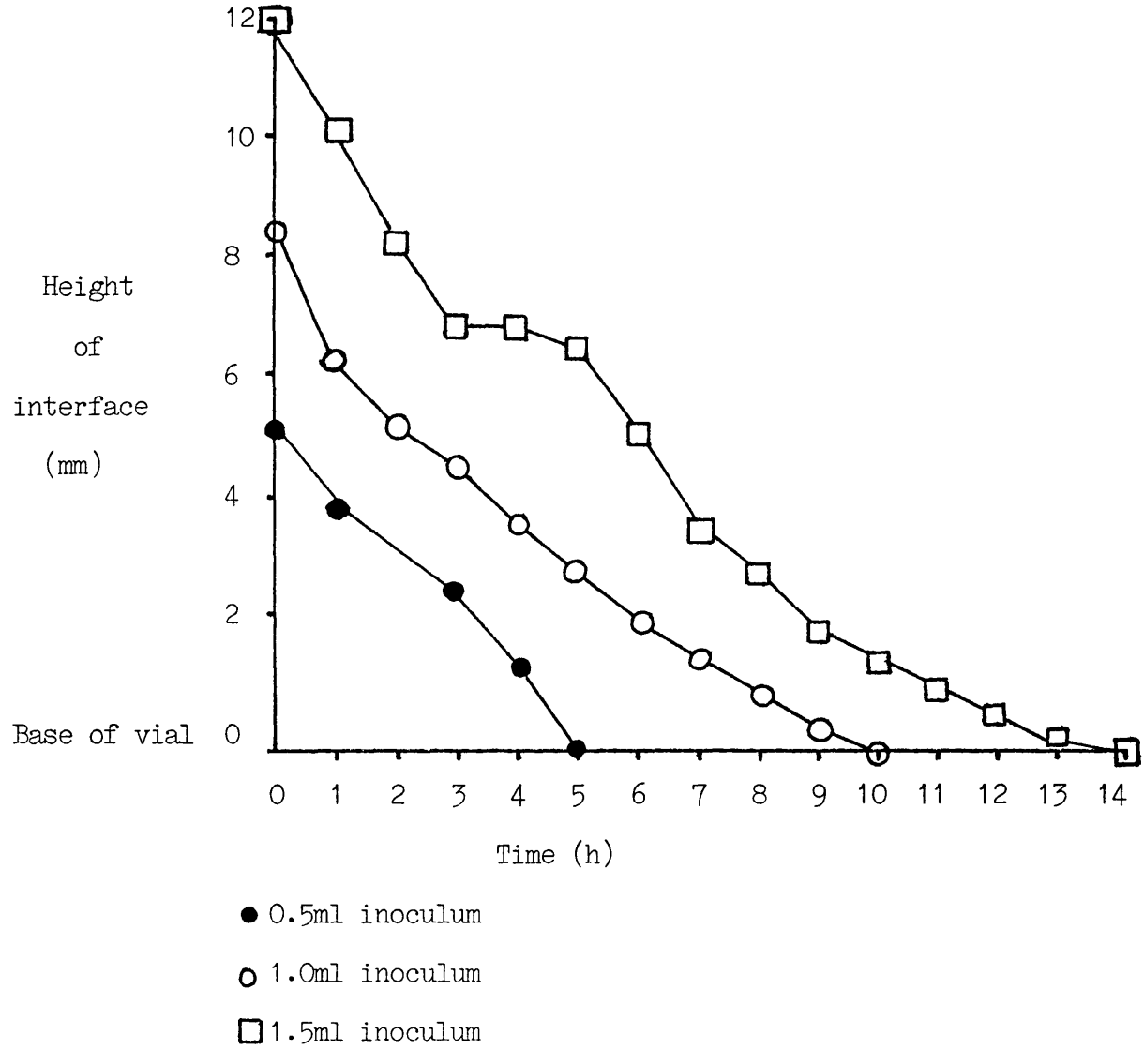
(i) The effect of volume of spore suspension on the recession of the ice interface and residual water content during freeze drying

In each test at least 10 vials were freeze dried for residual water content determinations. Each vial contained initially 0.5, 1.0 or 1.5 ml of suspension of Aspergillus niger conidia in skimmed milk-inositol medium which was freeze dried for selected periods of time. When ice forms it excludes all other material from its structure and as it sublimed under reduced pressure it left a porous structure with a powdery appearance. The ice/dried product interface was the junction of the glassy appearance of the ice with powdery appearance of the freeze dried product. During the initial period of drying the progress of the ice/dried product interface was observed at 1h intervals and the height read from the scale marked on the vials (2.11ii).

When 0.5 ml of suspension is dried the interface reaches the base of the vial at 5 h, with 1ml of suspension it reaches the base at 10 h and with 1.5 ml at 13 h. A lag is seen in the recession of the interface through the frozen material in the 1.5 ml volume between 3 and 5 h and the rate of recession is

slower than the initial rate from 5 to 13 h. This is less obvious with the 1.0 ml volume, although the recession is faster over the first hour, and lacking in the 0.5 ml volume (Fig.8.04a). This is caused by the impedance of the passage of water vapour through the dried material.

Fig 8.04a Recession of the ice/dried product interface with different volumes of suspension of Aspergillus niger IMI 91855ii in skimmed milk-inositol medium in vials freeze dried in the shelf freeze drier

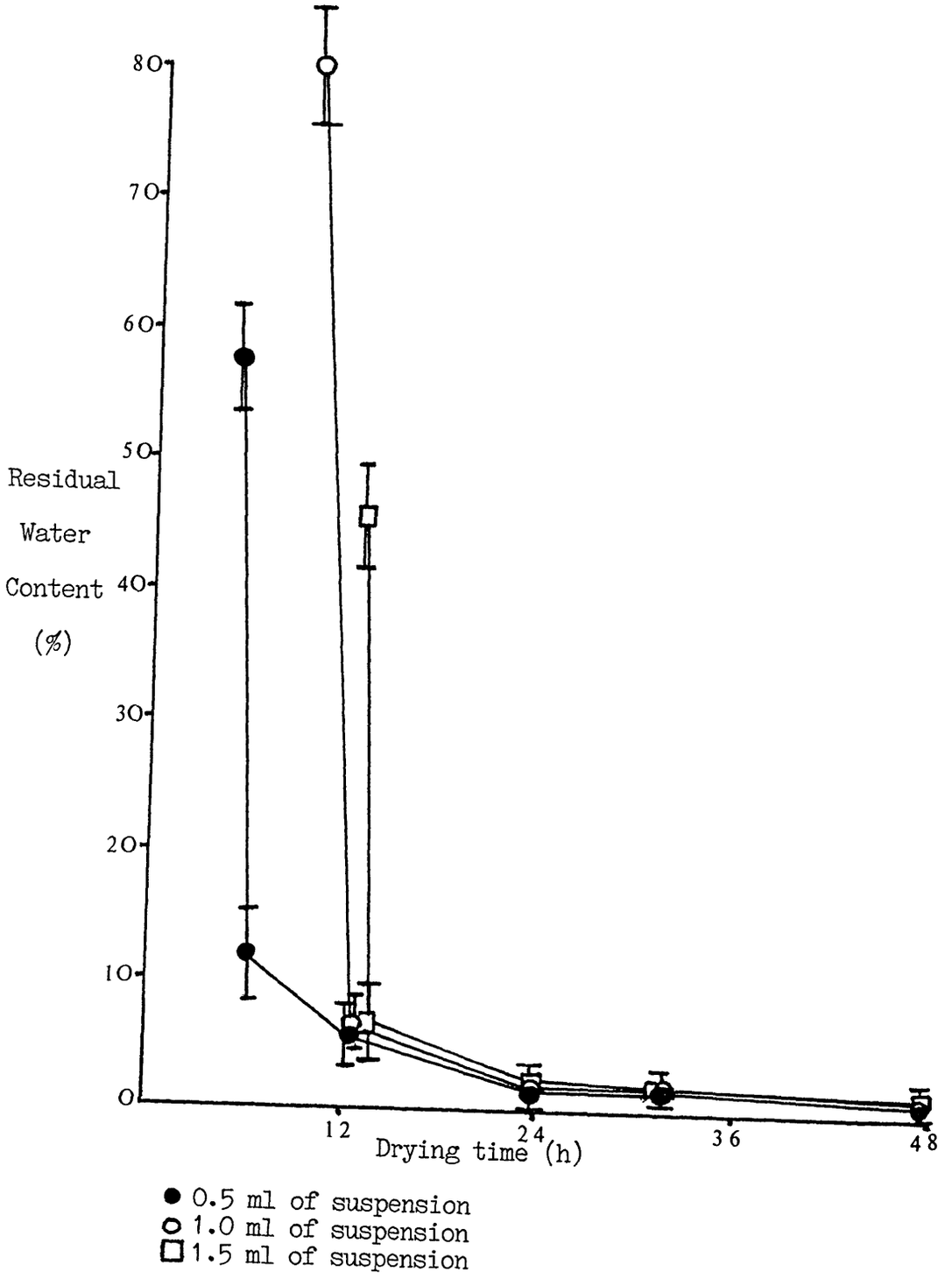


Ten vials of each set containing 0.5, 1.0 and 1.5 ml were opened after 4, 5, 12, 13, 14, 24, 32 and 48 h periods of freeze drying and the residual water content measured by weight (2.12). The residual water content of the three volumes differed during the first 12 hours drying but at 24 h all were between 1 and 2%. Thus the different volumes of suspension, between 0.5 and 1.5 ml, did not influence the final residual water content when drying for a period of 24 h.

(ii) The effect of different final residual water contents on the viability of selected fungi.

Fifty vials containing approximately 1 ml of suspension were prepared for each of 4 test organisms and were cooled in the shelf freeze drier with the shelf temperature set at $-45^{o}C$. The chamber was evacuated when the suspension temperature reached $-20^{o}C$ and the cooling continued to $-45^{o}C$. The vials were warmed, while still under vacuum, at $0.2^{o}C\ min^{-1}$ for selected periods of time. Ten vials of each organism were opened and the water content of the freeze dried product determined by dry weight (2.12). A further 3 vials were opened for each viability test of each fungus. The rehydrated suspension was streaked onto suitable agar media and incubated (see 2.16). Viabilities were estimated immediately after freeze drying and again after 1 year storage. The proportion of the propagules germinating was plotted against residual moisture content for each test organism, viz. Armillariella mellea IMI 158162 (Fig. 8.04c), Aspergillus niger IMI 91855ii (Fig. 8.04d), Chaetomium sp. IMI 260186 (Fig. 8.04e) and Penicillium ochrochloron IMI 61271 (Fig. 8.04f).

Fig. 8.04b Residual water content (% of weight of freeze dried product) of various volumes of suspensions of conidia of Aspergillus niger suspended in skimmed milk-inositol medium and dried in the shelf freeze drier



The bars through each point represent 2 standard deviations from the mean

Viability of isolates of fungi after freeze drying 1 ml aliquots of suspension in the shelf freeze drier to various residual water contents (% of weight of the freeze dried product)

Fig. 8.04c Armillariella mellea

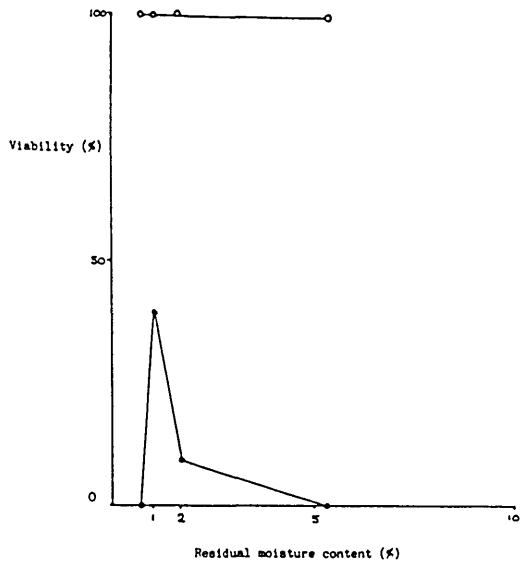


Fig. 8.04d Aspergillus niger

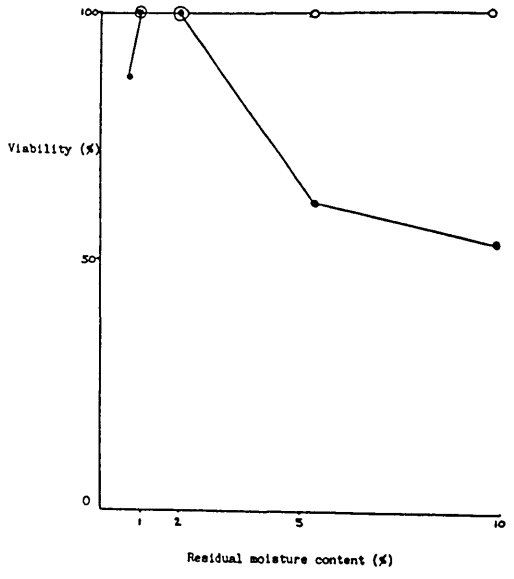


Fig. 8.04e Chaetomium sp.

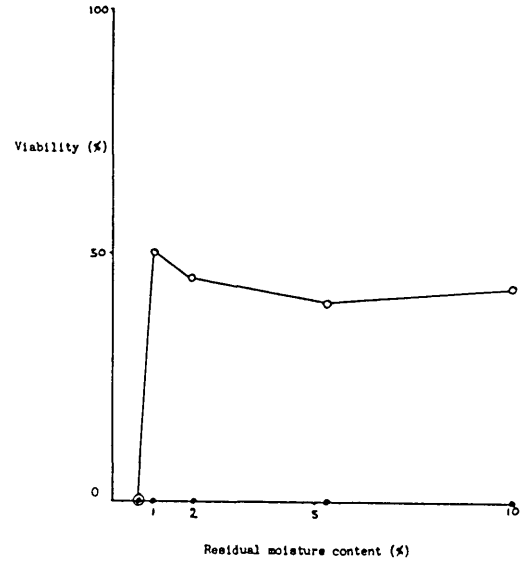
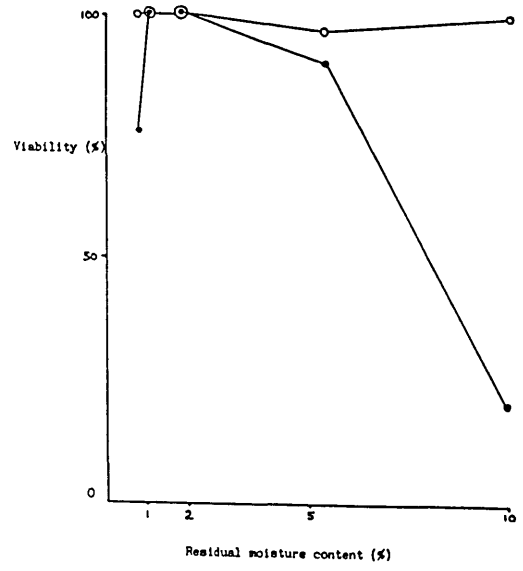


Fig. 8.04f Penicillium ochrochloron



○ Immediate viability

● Viability following 1 year of storage

Initially Armillariella mellea retained 100% viability when dried to residual water contents between 0.8 and 10% (Fig. 8.04c). However, only samples with water contents of between 1 and 2% survived storage for 1 year and even then the maximum survival was only 40% at 1% water content.

At residual water contents of between 1 and 2% all samples before and after storage of Aspergillus niger were 100% viable. When samples were dried to less than 1% they remained 100% viable but viability was reduced to 87% after 1 year storage. At higher water contents the viabilities were reduced but even after 1 year samples with 10% water content were 55% viable. The shapes of the viability plots of Penicillium ochrochloron closely resembled those of Aspergillus niger except that at 10% water content the viability was initially higher (100%) but lower (<20%) after 1 year storage.

The samples of Chaetomium all died during 1 year storage. Immediate tests of the freeze dried material showed that drying to 0.8% water content killed the organism whereas drying to levels between 1 and 10% retained up to 50% viability.

8.05 The effect of different drying procedures on the viability of selected fungi

It was established above that cooling in the shelf freeze drier set at $-45\ ^{o}C$ and drying to between 1 and 2% residual water content enables some fungi to retain their viability. Therefore it was investigated whether different drying procedures resulting in the same water contents would influence viability. The cooling rate of $1.2\ ^{o}C\ min^{-1}$ was employed to cool the fungal suspensions to $-20\ ^{o}C$ when the chamber of the shelf freeze drier

was evacuated and the cooling continued to -45 °C. The drying procedures from -45 °C to to +10 °C were as follows:-

1. Immediate warming at 0.3 °C min^{-1}.
2. Immediate warming at 0.2 °C min^{-1}.
3. Immediate warming 0.13 °C min^{-1}.
4. Held for 1 h at -45 and warming at 0.13 °C min^{-1}.
5. Held for 2 h at -45 and warming at 0.13 °C min^{-1}.
6 Held for 3 h at -45 and warming 0.13 °C min^{-1}.
7. Held for 3 h at -45 and warmed 0.08 °C min^{-1}.

Thirty two isolates with either known death points or had survived when processed by centrifugal freeze drying were dried by these procedures and the results are presented in table 8.051. Each drying procedure was repeated 4 times and the vials sealed at the completion of each stage. Stage 1, was after 25 min, when the suspension had cooled to -10 °C; stage 2 was after the application of vacuum when suspension temperature was -45 °C; stage 3 was after 3 h drying under vacuum and stage 4 was at the end of the process. Eight vials of each fungus were processed on each occasion. An additional 2 vials of each organism were included on the last run and were opened for residual water content determinations to ensure that the residual water content at the end of freeze drying was between 1 and 2%. In each case when the suspension was at temperatures below 0 °C the sealed vials were rapidly warmed in a water bath at 37 °C until the last ice crystals had melted. The 8 vials of each organism were opened to test viability at the end of each stage. The fungus was recorded as viable if normal growth was resumed. During

determination of death points the recovery of samples from the frozen state involved thawing which could obviously influence viabilities. This stage could not be avoided and its effect alone could not be assessed.

Table 8.051 Stages in centrifugal and shelf freeze drying causing death of fungi. In shelf freeze drying the ampoules were heated at various rates from -45 to +10 C either immediately or after a delay

Name	IMI Number	Death points: Centrifugal freeze drying	Shelf freeze drying: Heating rate ($^{o}C\ min^{-1}$) 0.3	0.2	0.13				0.08
			Delay (h) before heat applied: 0	0	0	1	2	3	3
Achlya ambisexualis	93805	F2	2	2	2	2	2	2	2
Arachnomyces nitidus	147447	D1	3	3	NT	NT	NT	3	3
Armillariella mellea	158162	F2	3	3	3	3	4	4	S
Aspergillus niger	91855ii	N	N	N	N	N	N	N	N
Basidiobolus haptosporus	108126	F2	2	NT	NT	NT	NT	NT	2
Biscogniauxia simplicior	245191	F2	3	3	3	NT	3	NT	3
Calonectria rigidiuscula	84749	D1	3	3	3	3	NT	NT	3
Ceratocystis fimbriata	80795	D2	3	3	NT	NT	NT	4	4
Cercosporidium bolleana	161111	F2	3	3	3	4	4	4	V
Cladosporium cladosporoides	45534	N	4	4	4	S	S	N	N

Stages of centrifugal freeze drying when the organism failed
F2, after freezing; D1, after first 3h drying; D2, after completion of secondary drying

Minifast stages after which the organism could not be recovered
1, After 25 min, suspension cooled to -10; 2, After application of vacuum when suspension temperature -45; 3, After 3 h drying under vacuum; 4, End of run.

Results of shelf freeze drying
S, Survived but died during first 6 months storage; N, gave normal growth after 6 months storage; V, vegetative growth after 6 months storage; NT, Not tested.

Table 8.051 (continued) Stages in centrifugal and shelf freeze drying causing death of fungi. In shelf freeze drying the ampoules were heated at various rates from -45 to +10 either immediately or after a delay

Name	IMI Number	Death points						
		Centrifugal freeze drying	Shelf freeze drying					
			Heating rate (^{o}C min^{-1})					
			0.3	0.2	0.13			0.08
			Delay (h) before heat applied					
			0	0	0	1	2	3	3
Conidiobolus couchii	128727	D1	3	3	NT	NT	3	4	4
Coniophora puteana	79127	F2	4	3	NT	NT	NT	4	V
Coprinus amphibius	133855	D2	3	3	NT	NT	NT	3	3
Erynia excitiales	155908	F2	3	3	NT	NT	3	3	3
Fusarium avenaceum	272884	N	4	4	NT	NT	S	N	N
Hypoxylon confluens	146053	F2	2	2	NT	NT	2	2	2
Kretzschmaria clavus	245190	D1	3	3	NT	NT	NT	3	3
Lentinus degener	110525	F2	3	3	NT	NT	NT	NT	3
Nummularia discreta	233095	D1	4	4	NT	NT	4	4	4
Penicillium ochrochloron	61271	N	N	N	N	N	N	N	N
Phytophthora cactorum	21168	F2	2	2	NT	NT	NT	NT	2

Stages of centrifugal freeze drying when the organism failed
F2, after freezing; D1, after first 3h drying; D2, after completion of secondary drying

Minifast stages after which the organism could not be recovered
1, After 25 min, suspension cooled to -10; 2, After application of vacuum when suspension temperature -45; 3, After 3 h drying under vacuum; 4, End of run.

Results of shelf freeze drying
S, Survived but died during first 6 months storage; N, gave normal growth after 6 months storage; V, vegetative growth after 6 months storage; NT, Not tested.

Table 8.051 (continued) Stages in centrifugal and shelf freeze drying causing death of fungi. In shelf freeze drying the ampoules were heated at various rates from -45 to +10 either immediately or after a delay

Name	IMI Number	Death points: Centrifugal freeze drying	Shelf freeze drying: Heating rate (°C min^{-1}) 0.3	0.2	0.13				0.08
			Delay (h) before heat applied 0	0	0	1	2	3	3
Phytophthora nicotianae	77972	F2	2	2	NT	NT	NT	2	2
P. palmivora	202528	F2	4	4	4	NT	NT	4	S
Podospora curvispora	175246	N	4	4	NT	NT	S	N	N
Pythium middletonii	42098	F2	3	3	NT	NT	NT	3	3
Saprolegnia parasitica	169621	F2	2	2	2	NT	2	2	2
Sclerotium wakkeri	103548	F2	4	4	NT	NT	NT	4	V
Thanatephorus solani	172732	D1	2	2	NT	NT	2	2	2
Trichoderma viride	110138	N	S	S	S	NT	NT	N	N
Ustilina deusta	121510	D1	3	3	NT	NT	NT	3	3
Wallemia sebi	156385a	N	4	4	4	NT	NT	4	N
Xylaria carpophila	146428	D1	4	4	NT	NT	4	4	V

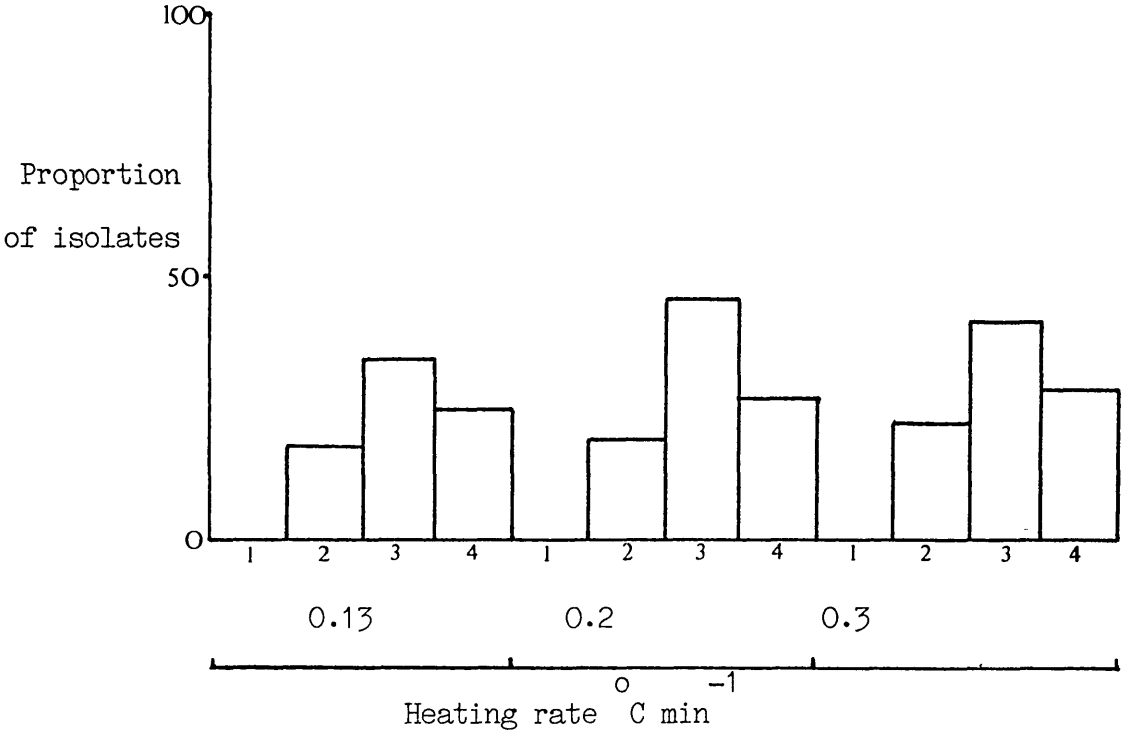
Stages of centrifugal freeze drying when the organism failed
F2, after freezing; D1, after first 3h drying; D2, after completion of secondary drying

Minifast stages after which the organism could not be recovered
1, After 25 min, suspension cooled to -10; 2, After application of vacuum when suspension temperature -45; 3, After 3 h drying under vacuum; 4, End of run.

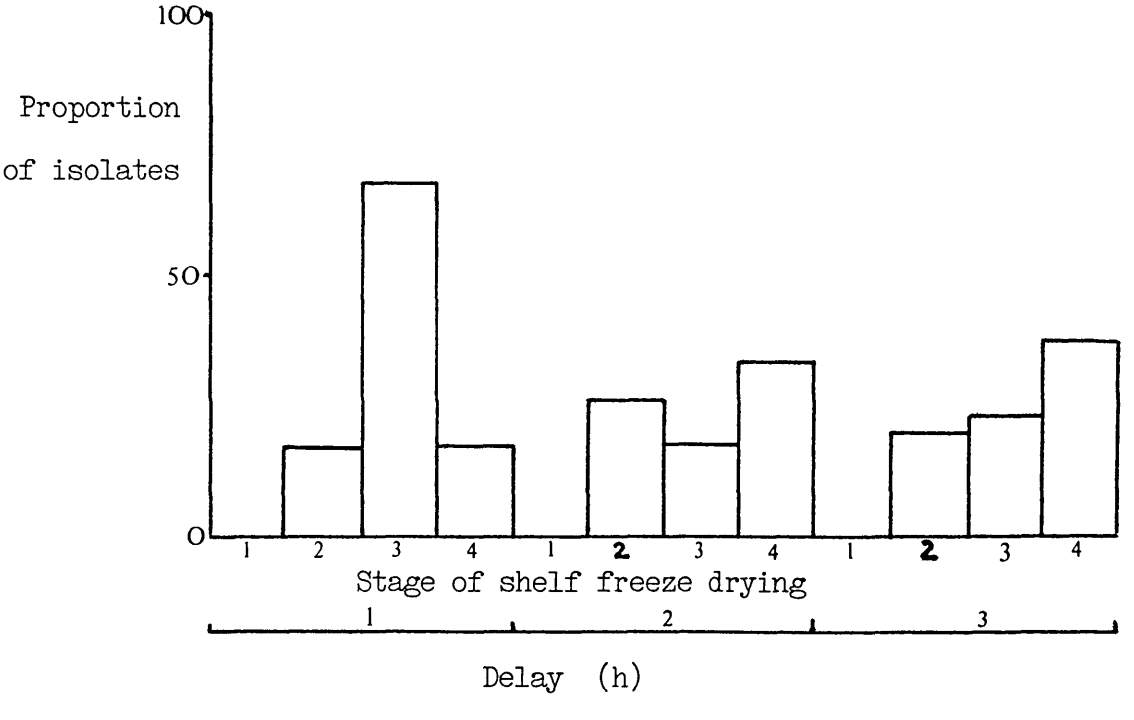
Results of shelf freeze drying
S, Survived but died during first 6 months storage; N, gave normal growth after 6 months storage; V, vegetative growth after 6 months storage; NT, Not tested.

Fig 8.05a Stage of shelf freeze drying process where death occurs

(i) Immediate heating



(ii) Heating after a delay



Isolates that failed to survive the cooling stage of the shelf freeze drying were not tested by all subsequent procedures. As the initial results were similar with warming procedures 1-3 and the procedures 4-7 these were not employed with all the isolates.

Seven of the isolates tested had survived the centrifugal freeze drying method and the remaining 25 isolates had failed, 15 after the freezing stage (F2) and 10 during the drying stages. When the shelf freeze drying technique was applied none of the isolates died in the initial cooling to $-10\ ^{o}C$ (1) although after the application of the vacuum and further cooling to $-45\ ^{o}C$ 7 isolates failed to survive. When these isolates were cooled to $-45\ ^{o}C$ at $1.2\ ^{o}C\ min^{-1}$ and thawed at approximately $190\ ^{o}C\ min^{-1}$ without evacuating the chamber of the shelf freeze drier these isolates remained viable. However, the growth rate and sporulation of these isolates were reduced but the morphology was typical. Normal growth rate and sporulation were recovered after the isolates were transferred to fresh agar media.

Only 3 isolates survived treatments 1 (32 tested), 2 (31 tested), 3 (12 tested) and 4 (7 tested); 5 isolates survived treatments 5 (14 tested) and 6 (27 tested) and 13 survived treatment 7 (32 tested).

Only 6 of the 25 isolates that had failed centrifugal freeze drying were viable at the end of the shelf freeze drying process involving warming after a 3 h holding time at $-45\ ^{o}C$ and warming at $0.08\ ^{o}C\ min^{-1}$ to $10\ ^{o}C$. However, 2 of these, Armillariella mellea IMI 158162 and Phytophthora palmivora IMI 202528 died during the first 6 months storage. Cercosporidium bolleana IMI

161111, Sclerotium wakkeri IMI 103548 and Xylaria carpophila IMI 146428 grew vegetatively after resusitation. Coniophora puteana IMI 79127 grew vegetatively both before and after preservation.

Of the 7 isolates that had survived the centrifugal freeze drying method only Aspergillus niger IMI 91855ii and Penicillium ochrochloron IMI 61271 survived all treatments in the shelf freeze drier. Trichoderma viride IMI 110138 survived warming without a holding period at -45 °C (treatments 1, 2 and 3) but died during the first 6 months of storage. This isolate survived the warming procedure which involved holding at -45 °C for 2 and 3 h before heating and remained viable after 1 year storage. The remaining isolates all failed the treatments which did not include a holding period at -45 °C before heating. Cladosporium cladosporioides IMI 45534, Fusarium avenaceum IMI 272884 and Podospora curvispora IMI 175246 survived warming procedures involving a 2 and 3 h holding period (treatments 6 and 7). Wallemia sebi IMI 156385a only survived after a holding period of 3 h at -45 °C before drying (treatment 7).

8.06 The effect of different freeze drying procedures on water contents

The investigation of selected warming protocol showed that holding the temperature at -45 °C for 3 h and warming at 0.08 °C min^{-1} (treatment 7) gave the optimal results for the fungi under test. The hyphal contents of Penicillium expansum IMI 174158 thaw at -15 °C (5.05). Therefore, in order to establish if loss in viability could be attributed to thawing at high water

contents and further drying of the melted suspension, the residual water contents were measured at 4 points in each drying cycle examined earlier (8.05) viz. after 3, 5, 9, and 24 h. The mean residual water content was calculated, being the mean of the residual water contents of 10 vials (determined by dry weight) which were freeze dried in batches of 5 on 2 separate occasions. The standard deviation was calculated for each set of results. The temperature of the suspension was also recorded. In some cases the suspension melted and the residual water content was then not measured.

At temperatures above -15^{o} C the hyphal contents of Penicillium expansum melt if the ice has not been removed by sublimation (5.04). If the water has been removed in this way the cell contents cannot be rehydrated when the melting point is reached. The water content of a spore suspension of a test fungus, Aspergillus niger was therefore plotted to determine the water content at -15^{o} C (Fig. 8.06a). Low water contents achieved below -15^{o} C correlated with survival (8.05).

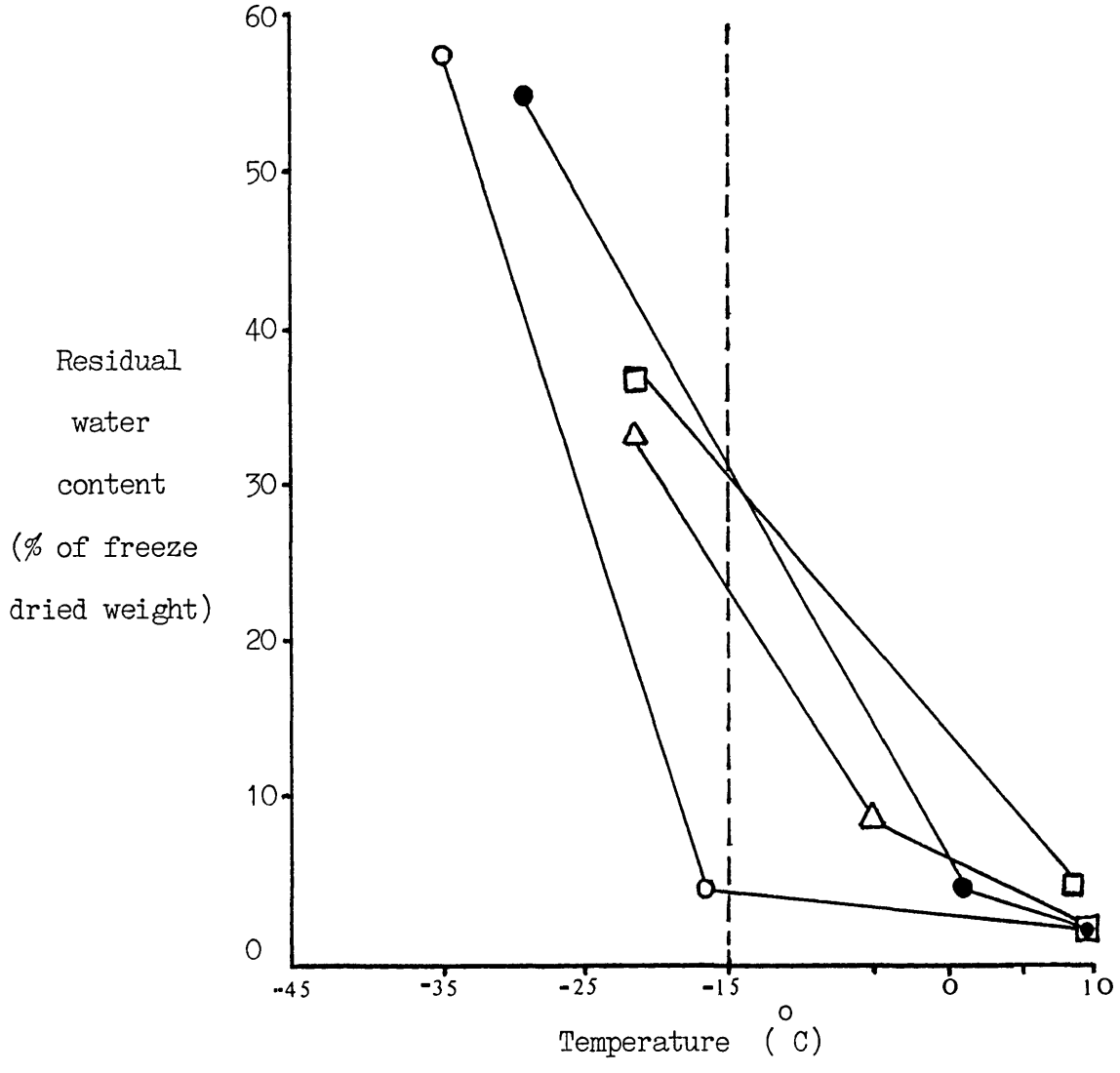
The warming procedure of treatment 7 reduced the residual moisture content at -15^{o} C to 4.8% (Fig. 8.06a). By all other treatments the water contents were not reduced below 24% until the temperature rose above -15^{o} C. More fungi survived the warming protocol of treatment 7 than by any other procedure (8.05).

Table 8.061 Residual water contents (RW, % w/w) and temperatures (°C) measured during shelf freeze drying of suspensions of Aspergillus niger in skimmed milk-inositol medium using different warming procedures

	Warming procedures	Time (h) in freeze drying when RW was determined												Time(h) below −15 °C	RW*
		3			5			9							
		RW	(SD)	Temp.	RW	(SD)	Temp.	RW	(SD)	Temp.	RW	(SD)	Temp.		
1	Immediate warming at 0.3 °C	26.3	(4.62)	+9	5.2	(1.25)	+10	4.9	(0.32)	+10	1.9	(0.41)	+10	1.6	M
2	Immediate warming at 0.2 °C	31.9	(5.82)	−9	5.3	(1.31)	+10	4.9	(0.29)	+10	1.7	(0.71)	+10	2.75	M
3	Immediate warming at 0.13 °C	36.3	(6.73)	−21	10.2	(0.85)	−6	5.1	(0.64)	+10	1.7	(0.08)	+10	3.8	24.8
4	Held for 1 hour at −45 and warmed at 0.13 C	M		−29	22.9	(0.37)	−6	5.7	(0.53)	+10	2.0	(0.41)	+10	4.8	M
5	Held for 2 h at −45 and warmed at 0.13 C	M		−37	38.8	(0.97)	−14	5.9	(0.29)	+9	2.2	(0.07)	+10	5.8	31.2
6	Held for 3 h at −45 and warmed at 0.13	M		−45	57.5	(0.93)	−29	6.2	(1.29)	+2	1.8	(0.76)	+10	6.8	32.8
7	Held for 3 h at −45 and warmed at 0.08 C	M		−45	59.8	(2.01)	−35	5.8	(0.43)	−16	1.9	(0.75)	+10	9.0	4.8

Warming rates are °C min^{-1}; SD - Standard deviation; M - Melted. *Data from fig. 8.06a

Fig. 8.06a The residual water contents at different temperatures of suspensions of conidia of Aspergillus niger IMI 91855ii after shelf freeze drying with different warming procedures



Warming rate from −45 to +10 °C after freezing during drying

△ Immediate warming at 0.13 °C min^{-1} (treatment 3 in table 8.061)

□ Held for 2 h at −45 and warmed 0.13 °C min^{-1} (treatment 5 in table 8.061)

● Held for 3 h at −45 and warmed 0.13 °C min^{-1} (treatment 6 in table 8.061)

○ Held for 3 h at −45 and warmed 0.08 °C min^{-1} (treatment 7 in table 8.061)

8.07 The effect of controlled freeze drying on the viability of selected fungi

It has been shown above that a water content of below 5% when the temperature reaches -15 °C, the melting point of the hyphal contents of Penicillium expansum, can be correlated with survival of fungi. Suspensions of fungal spores or mycelium were freeze dried in the shelf freeze drier by initial cooling to -45 °C, evacuating the chamber and holding the temperature for 3 h followed by warming at a rate of 0.08 °C min^{-1} to 10 °C and holding until the 24 h period was completed (the improved shelf freeze drying technique). Sample vials were tested immediately they were prepared and fungi that survived were retested after 2 years storage at + 15 °C. Spore germination was assessed by both a microscope slide technique (2.16_{I}) and directly on agar media (2.16_{II}). All counts were carried out at x400 magnification on the stage of a light microscope. The spore germination results obtained by the microscope slide technique did not correlate with the subsequent growth test. Some slides showed no germination of spores though colonies were formed on agar. Therefore the results presented in table 8.071 are those obtained by the agar media technique.

Fourteen of the 19 isolates survived the improved shelf freeze drying technique (Table, 8.071), though only 11 survived 2 years storage at 15 °C (Table, 8.072). The isolates, Fusarium culmorum, F. tricinctum and Gaeumannomyces graminis were viable immediately after freeze drying but died during storage. The majority of those that survived showed a decrease in viability immediately after processing of between 2 and 26% (Fig. 8.07a).

A further decrease in viability of between 1 and 11% was observed in most samples over the 2 year storage period (Fig. 8.07a). The majority of the fungi freeze dried by the improved shelf technique had higher viabilities than the samples freeze dried centrifugally (Fig, 8.07b). However, the isolates of F. tricinctum, G. graminis and Infundibura adhaerens survived 2 years storage when centrifugally freeze dried whereas they failed to survive the improved shelf freeze drying technique. The isolate of Petriellidium fusoideum survived 2 years when freeze dried by the shelf technique but failed to survive the same period after centrifugal freeze drying. The hyphae of Cryphonectria euginae and Nigrospora sphaerica failed to survive both freeze drying techniques. The chlamydospores of Fusarium poae survived both techniques although only 4% of the microconidia survived centrifugal freeze drying and none survived the shelf technique.

Table 8.071 Viabilities of fungi immediately after freeze drying by the improved shelf technique

Name	IMI Number	Germination and growth					
		Before freeze drying			After freeze drying		
Arthrocristula hyphenata	272976	5/94	5%	AN	0/74	0%	-
Aspergillus candidus	127259	42/100	42%	R	51/127	40%	R
A. terreus	44243ii	32/34	94%	N	146/158	92%	N
Cladosporium herbarum	49627	57/103	55%	R	9/31	29%	N
Cryphonectria eugeniae	56425a*	11/53	21%	AN	0/75	0%	-
Fusarium avenaceum	272884	94/96	98%	N	80/83	96%	N
F. avenaceum	272887	77/78	99%	N	63/65	97%	N
F. culmorum	272853	80/81	99%	N	67/69	97%	N
F. poae (microconidia)	272890	4/1220	0.33%	N	0/354	0%	-
(chlamydospores)	272890	83/83	100%	N	89/89	100%	N
F. tricinctum	273620	10/34	29%	R	12/48	25%	AN
Gaeumannomyces graminis	272213	39/84	46%	N	23/97	24%	R
Infundibura adhaerens	274154	27/107	25%	R	0/100	0%	-
Myrothecium lachastrae	273160	51/87	59%	R	114/187	61%	N
Nigrospora sphaerica	46685*	15/94	16%	F	0/70	0%	-
Penicillium coryophilum	273248	123/124	99%	R	251/256	98%	N
Petriellidium fusoideum	271728	15/103	15%	N	3/61	5%	N
Polyscytalum pustulans	197208	134/220	61%	R	111/217	51%	N
Ramichloridium subulatum	273184	29/105	28%	R	112/397	28%	R
Trichoderma harzianum	274332	50/52	96%	N	63/69	91%	N

Growth and sporulation, N, grew and sporulated normally; R, reduced growth and sporulation but typical; AN, abnormal growth; -, failed to survive; *, No spores, propagules were fragmented hyphae;

Table 8.072 Viabilities after two years storage of fungi which survived (Table, 8.071) freeze drying by the improved shelf technique

Name	IMI Number	Germination and growth					
		Immediate			After 2 years storage		
Aspergillus candidus	127259	51/127	40%	R	91/249	37%	R
A. terreus	44243ii	146/158	92%	N	119/131	91%	N
Cladosporium herbarum	49627	9/31	29%	N	13/52	25%	AN
Fusarium avenaceum	272884	80/83	96%	N	52/54	95%	N
F. avenaceum	272887	63/65	97%	N	55/56	98%	N
F. culmorum	272853	67/69	97%	N	0/49	0%	-
F. poae (chlamydospores)	272890	89/89	100%	N	67/67	100%	N
F. trincinctum	273620	12/48	25%	AN	0/67	0%	-
Gaeumannomyces graminis	72213	23/97	24%	R	0/86	0%	-
Myrothecium lachastrae	273160	114/187	61%	N	81/109	74%	N
Penicillium coryophilum	273248	251/256	98%	N	319/319	100%	N
Petriellidium fusoideum	271728	3/61	5%	N	5/106	5%	N
Polyscytalum pustulans	197208	111/217	51%	N	217/549	40%	N
Ramichloridium subulatum	273184	112/397	28%	R	182/435	42%	R
Trichoderma harzianum	274332	63/69	91%	N	76/89	85%	R

All counts were carried out at x400 magnification on agar plates; Growth and sporulation, N, grew and sporulated normally; R, reduced growth and sporulation but typical; F, further reduced but normal; AN, abnormal; -, failed to survive;

Fig. 8.07a The viabilities of fungi before and after freeze drying by the improved shelf technique (ex Tables, 8.071 and 8.072)

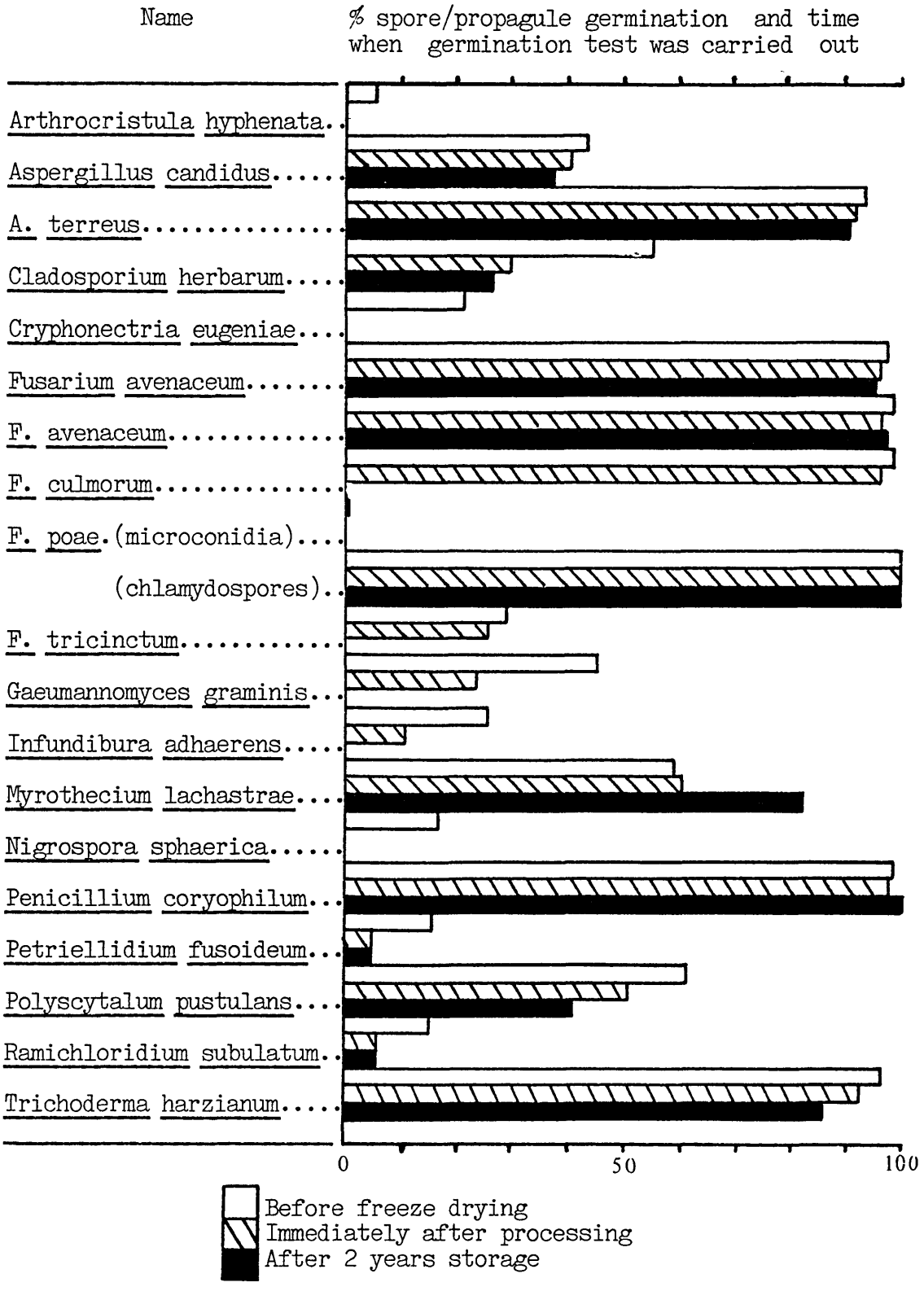
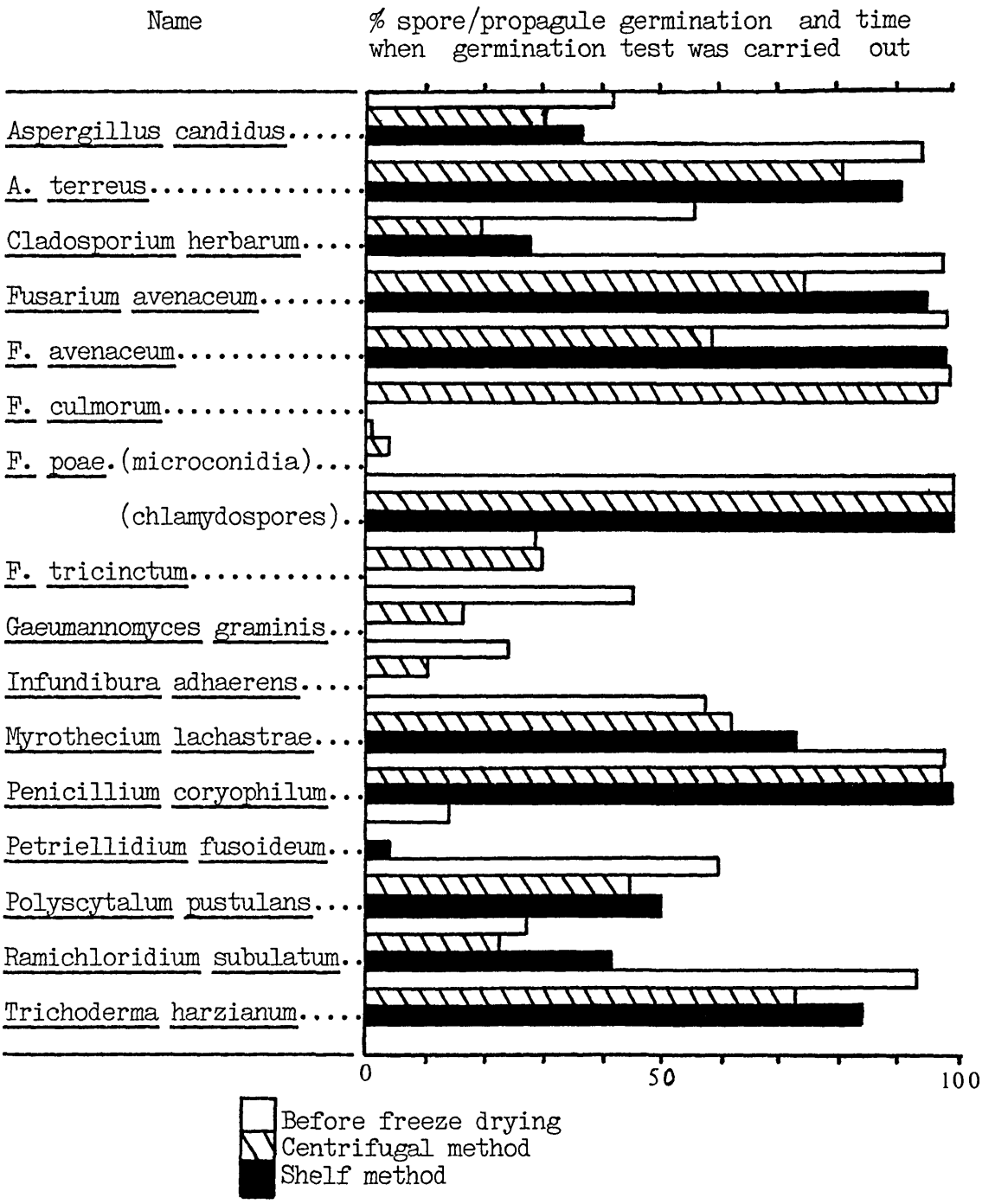


Table 8.073 Viabilities of samples of the fungi that were freeze dried by the improved shelf technique after simultaneously freeze drying by the centrifugal technique and 2 years storage

Name	IMI Number	Germination and growth					
		Before			After		
Aspergillus candidus	127259	42/100	42%	F	25/83	30%	F
A. terreus	44243ii	32/34	94%	N	73/91	80%	N
Cladosporium herbarum	49627	57/103	55%	R	31/159	19%	AN
Fusarium avenaceum	272884	94/96	98%	N	73/98	74%	N
F. avenaceum	272887	77/78	99%	N	57/99	58%	N
F. culmorum	272853	80/81	99%	N	107/112	96%	N
F. poae (microconidia)	272890	4/1220	0.33%	N	5/129	4%	F
(chlamydospores)	272890	83/83	100%	N	77/77	100%	N
F. tricinctum	273620	10/34	29%	R	34/112	30%	N
Gaeumannomyces graminis	272213	39/84	46%	N	15/90	17%	R
Infundibura adhaerens	274154	27/107	25%	R	8/79	10%	R
Myrothecium lachastrae	273160	51/87	59%	R	155/252	62%	R
Penicillium coryophilum	273248	23/124	99%	R	102/104	98%	N
Petriellidium fusoideum	271728	15/103	15%	N	0/97	0%	-
Polyscytalum pustulans	197208	34/220	61%	R	45/110	45%	N
Ramichloridium subulatum	273184	29/105	28%	F	19/81	23%	F
Trichoderma harzianum	274332	50/52	96%	N	66/70	94%	N

All counts were carried out at x400 magnification; N, grew and sporulated normally; R, reduced growth and sporulation but typical; F, Further reduced growth and sporulation; AN, abnormal growth and sporulation;

Fig 8.07b The viability of some fungi after storage of samples freeze dried by the centrifugal and improved shelf techniques (ex Tables, 8.071, 8.072 and 8.073)



To establish if the improved shelf freeze drying technique (2.11) could be used to preserve fungi that had either survived or failed centrifugal freeze drying (2.10) a further 105 isolates were processed that had previously been freeze dried by the centrifugal freeze drying technique (Appendix V). Four of the isolates had failed the centrifugal method and these also failed the shelf method. A further 7 isolates failed which had previously survived the centrifugal method. Three of these isolates Fusarium avenaceum IMI 272884, F. culmorum IMI 272853 and F. poae IMI 272890 survived later attempts. The remaining 4 isolates, Acremonium alternatum IMI 166204, Fusarium culmorum IMI 14764, Martensiomyces pterosporus IMI 60573 and Stilbella buquetii IMI 268568 failed 4 attempts to reprocess. The isolates F. culmorum and M. pterosporus were in a poor condition and failed when reprocessed by the centrifugal method.

Table 8.074 The viability and condition of isolates of fungi after centrifugal and shelf freeze drying. A taxonomic summary of Appendix V according to Hawksworth, Sutton & Ainsworth, 1983.

	Method	Viability		Condition (giving number and proportion of isolates							
		Ratio	%	N	%	R	%	F	%	AN	%
ZYGOMYCOTINA											
Zygomycetes	CFD	19/19	100	16	84	2	11	0	0	1	5
	SFD	18/19	95	15	79	3	16	0	0	0	0
ASCOMYCOTINA											
Diaporthales	CFD	1/1	100	1	100	0	0	0	0	0	0
	SFD	1/1	100	1	100	0	0	0	0	0	0
Dothideales	CFD	2/2	100	1	50	1	50	0	0	0	0
	SFD	2/2	100	2	100	0	0	0	0	0	0
Gymnoascales	CFD	2/2	100	1	50	1	50	0	0	0	0
	SFD	2/2	100	1	50	1	50	0	0	0	0
Hypocreales	CFD	1/1	100	0	0	0	0	1	100	0	0
	SFD	1/1	100	1	100	0	0	0	0	0	0
Sordariales	CFD	4/4	100	2	50	1	25	1	25	0	0
	SFD	4/4	100	3	75	1	25	0	0	0	0
BASIDIOMYCOTINA											
Hymenomycetes	CFD	0/2	0	0	0	0	0	0	0	0	0
	SFD	0/2	0	0	0	0	0	0	0	0	0
DEUTEROMYCOTINA											
Coelomycetes	CFD	5/5	100	4	80	1	20	0	0	0	0
	SFD	5/5	100	2	40	0	0	3	60	0	0
Hyphomycetes	CFD	66/68	97	52	76	10	15	3	4	1	2
	SFD	60/68	88	52	76	7	10	1	2	0	0
Agonomycetes	CFD	1/1	100	1	100	0	0	0	0	0	0
	SFD	1/1	100	1	100	0	0	0	0	0	0
Totals	CFD	101/105	96%	78	77%	16	16%	5	5%	2	2%
	SFD	94/105	90%	78	83%	12	12%	4	4%	0	0%

CFD, centrifugal freeze drying
SFD, improved shelf freeze drying
Growth and sporulation, Normal, N; Reduced but normal, R; Further reduced but normal, F; Abnormal, AN.

Although more fungi survived the centrifugal technique (96%) than the improved shelf technique (90%) more isolates grew more profusely and sporulated better when retrieved from the shelf technique (Table, 8.074). As some isolates that were in a poor condition failed a further 174 isolates were processed by the improved shelf technique. These isolates were those freshly isolated and deposited in the CMI culture collection or those grown in good condition from the collection prior to the trial. Table 8.075 summarizes Appendix V which lists the fungi that have survived the improved shelf freeze drying technique. Of the isolates preserved 97% grew typically although in 18% of samples a slight reduction in growth and sporulation occurred and a further 3% showed a further reduction in these characteristics. From the 319 isolates processed in all only 39 (12%) failed and a further 3 died during the first six months of storage (Table, 8.076).

Table 8.075 Fungi surviving the improved shelf freeze drying technique. A taxonomic summary of Appendix VI according to Hawksworth, Sutton & Ainsworth, 1983)

	Viability		Condition (giving number and proportion of isolates)							
	Number	%	N	%	R	%	F	%	AN	%
ZYGOMYCOTINA										
Zygomycetes	15	100	15	100	0	0	0	0	0	0
ASCOMYCOTINA										
Ascosphaerales	1	100	1	100	0	0	0	0	0	0
Clavicipitales	1	100	0	0	0	0	1	100	0	0
Diaporthales	5	100	3	60	2	40	0	0	0	0
Dothideales	10	100	9	90	0	0	1	10	0	0
Gymnoascales	1	100	1	100	0	0	0	0	0	0
Helotiales	1	100	1	100	0	0	0	0	0	0
Hypocreales	2	100	1	50	1	50	0	0	0	0
Ophiostomatales	3	100	3	100	0	0	0	0	0	0
Sordariales	15	100	14	93	1	7	0	0	0	0
BASIDIOMYCOTINA										
Hymenomycetes	1	100	0	0	0	0	0	0	1	100
DEUTEROMYCOTINA										
Coelomycetes	23	100	15	65	7	30	0	0	1	5
Hyphomycetes	93	100	68	73	20	22	4	4	1	1
Agonomycetes	2	100	0	0	0	0	0	0	2	100

Totals

Genera	Species	Isolates	N	%	R	%	F	%	AN	%
80	148	174	132	76	31	18	6	3	5	3

Table 8.076 Isolates of fungi that failed to survive freeze drying by the improved shelf technique

Name	IMI Number	Maintenance prior to freeze drying Method	Age (yrs)	Condition	Result of CFD	Number and previous storage of other isolates surviving
Achlya ambisexualis	93805	Oil	22	N	-	NT
Acremonium alternatum	166204	Oil	12	F	N	1Sub
A. strictum	276794**	Sub	0.5	F	N	1Sub
Arachnomyces nitidus	147447	Oil	9	F	NT	NT
Armillariella mellea	158162*	Oil	13	F	-	NT
Arthrocristula hyphenata	272976	Sub	0.25	AN	-	NT
Aspergillus ochraceus	225094	Oil	5	V	NT	1Sub
Basidiobolus haptosporus	108126	Oil	12	N	-	NT
Biscogniauxia simplicior	245191	Oil	4	V	NT	NT
Calonectria diploa	278994	LN	1	N	NT	NT
C. rigidiuscula	84749	Oil	15	N(An)	-	NT
Ceratocystis fimbriata	80795	Oil	24	AN	NT	1Sub
Cladosporium allii-cepae	275849	Sub	0.5	F	NT	1Sub
Colletotrichum sublineolum	275718	Sub	0.5	AN	-	4Sub
Conidiobolus couchii	128727	Oil	4	F	-	NT
Coprinus amphibius	133855	Oil	10	V	-	NT
Cryphonectria eugeniae	56425a	Oil	10	V	AN	3Sub
Erynia excitiales	155908	LN	4	N	-	NT

Growth and sporulation, Normal, N; Further reduced growth rate and sporulation but typical, F; Abnormal, AN; Vegetative growth only, V; Failed to grow, -; Anomorph only, An;
Preservation technique, liquid nitrogen, LN; Centrifugal freeze drying, CFD; A fresh subculture, 1 transfer from original material Sub; Not tested, NT;
*, Died during the first 6 months of storage; **, Survived reprocessing;

Table 8.076 The isolates of fungi that failed to survive freeze (continued) drying by the developed shelf technique

Name	IMI Number	Maintenance prior to freeze drying Method	Age (yrs)	Condition	Result of CFD	Number and previous storage of other isolates surviving
Fusarium avenaceum	272884**	Sub	0.5	AN	NT	6Sub
F. culmorum	14764	CFD	5	F	N	1Sub
F. culmorum	272853**	Sub	1	F	NT	1Sub
F. poae	272890**	Sub	0.5	N	NT	2Sub
Gaeumannomyces graminis	272213*	Oil	0.5	AN	NT	NT
Ganoderma applanatum	157816	Oil	11	N	-	NT
Hypoxylon confluens	146053	Oil	14	V	-	NT
Infundibura adhaerens	274154	Sub	0.2	F	N	NT
Kretzschmaria clavus	245190	Sub	1	V	-	NT
Lentinus degener	110525	Oil	20	V	NT	NT
Martensiomyces pterosporus	60573	Oil	29	AN	AN	NT
Nigrospora sphaerica	46685	Oil	26	V	-	NT
Nummularia discreta	233095	Oil	1	AN	-	NT
Phytophthora cactorum	21168	Oil	14	N	NT	NT
P. nicotianae	77972	Oil	10	N	-	NT
P. palmivora	182592	Oil	6	F	NT	1Oil
P. palmivora	202528*	Oil	6	F	-	1Failed
Pythium middletonii	42098	Oil	3	N	NT	NT

Growth and sporulation, Normal, N; Further reduced growth and sporulation but typical, F; Abnormal, AN; Vegetative growth only, V; Failed to grow, -.
Preservation technique, liquid nitrogen, LN; Centrifugal freeze drying, CFD; A fresh subculture 1 transfer from the original material, Sub; Not tested, NT;
*, Died during the first 6 months of storage; **, Survived reprocessing;

Table 8.076 The isolates of fungi that failed to survive freeze (continued) drying by the developed shelf technique

Name	IMI Number	Maintenance prior to freeze drying Method	Age (yrs)	Condition	Result of CFD	Number and previous storage of other isolates surviving
Saprolegnia parasitica	121510	Oil	12	F	NT	NT
Sporodesmium tropicale	275328	Sub	0.5	F	-	NT
Stilbella buquetii	268568	CFD	1	N	N	NT
Thanatephorus solani	172732	LN	7	Scl	N	NT
T. cucumeris	230993	Sub	1	F	-	NT
Ustulina deusta	121510	LN	3	F	-	NT

Growth and sporulation, Normal, N; Further reduced grwth rate but typical F; Abnormal, AN; Vegetative growth only, V; Failed to grow, -.
Sclerotia mycelium only, Scl;
Preservation technique, liquid nitrogen, LN; Centrifugal freeze drying, CFD; A fresh subculture 1 transfer from the original material, Sub; Not tested, NT;

Of the 42 isolates that failed to survive the improved shelf technique or died during subsequent storage (Table, 8.076) 12 were represented by other isolates of their species that survived the process and 11 of these were freshly isolated. Three isolates, Armillariella mellea IMI 158162, Gaeumannomyces graminis IMI 272273 and Phytophthora palmivora IMI 202528, survived the processing but died during the first 6 months of storage. Initially 4 isolates, Acremonium strictum IMI 276794, Fusarium avenaceum IMI 272884, F. culmorum IMI 272853 and the chlamydospores of F. poae IMI 272890 were not viable after freeze drying but the fungi survived after further attempts with the same method. These cultures sporulated more profusely for the second attempt. Twenty four of the isolates were freeze dried after storage in oil for periods of 1 to 29 years of these 4 species were represented by fresh isolates which survived. Of the 42 failures, 26 had been centrifugally freeze dried also; 18 of these had failed, 2 had survived in a poor condition and 6 had survived successfully. Of the 8 isolates that had failed the improved technique but survived the centrifugal method 5 had been processed before they showed deterioration. Eight of the isolates that failed did not sporulate in culture.

Erynia exicitiales IMI 155908 only survived the freezing stage of the liquid nitrogen storage technique when a mixture of DMSO and glucose was used as the cryoprotectant (Tables, 5.051 and 5.052). This organism did not survive the freezing stage of the improved technique in skimmed-milk medium.

8.08 General conclusions drawn from the investigation of the effects of the freeze drying techniques on the viability of selected fungi

1. The cooling rate in the gaseous phase of liquid nitrogen was found to be 1.2 $^{o}C\ min^{-1}$. A similar cooling rate was achieved in the shelf freeze drier with a shelf temperature setting of $-45\ ^{o}C$. This cooling rate had proved successful in the liquid nitrogen storage of fungi and was chosen as the cooling rate for the improved freeze drying technique.

2. Using the cooling rate of 1.2 $^{o}C\ min^{-1}$ fungi survived freeze drying which had previously died during evaporative cooling of centrifugal freeze drying.

3. Although there was a significant difference in the time taken for visible ice to sublime from 0.5, 1.0 and 1.5 ml volumes of of suspensions of conidia in skimmed milk-inositol medium (5-6 h, 10 h and 14 h respectively) the residual water contents were similar after a 24 h drying period. Therefore it is not necessary to measure volumes of suspension accurately between 0.5 and 1.5 ml when a 24 h drying period is used to achieve a residual water content between 1 and 2%.

4. The water content of the freeze dried suspension did not decrease between 24 and 32 h but a drying period of 48 h reduces it to below 1%.

5. Viability of some fungi was lost after a 1 year storage period when the water contents were below 1 or above 2%.

6. More isolates survived warming procedures that involved holding the temperature below $-15\ ^{o}C$ until a residual water content below 5% was reached.

7. All test fungi failing to survive the selected warming procedures died during drying.

8. The viability of the fungi decreased immediately after freeze drying by the improved shelf technique.

9. More isolates survived centrifugal freeze drying than the improved shelf technique.

10. The majority of isolates that survived the improved shelf technique had higher viabilities after 2 years storage than the same samples freeze dried by the centrifugal technique.

11. Most of the isolates that failed the improved freeze drying technique grew slowly and gave sparse colonies with low sporulation.

12. When fresh isolates representing the species that had failed were freeze dried many survived.

13. Some vegetative isolates did not survive the improved shelf freeze drying technique.

14. Isolates of fungi which sporulated poorly seldom survived but when sporulation was improved they can be preserved.

15. Some survivors of the improved shelf technique were only viable for periods of less than 6 months.

CHAPTER 9

DISCUSSION

9.01 Introduction

Several techniques have been evaluated in this study to determine their effectiveness and reliability for the preservation of filamentous fungi. There are 64200 accepted species of fungi (Hawksworth, Sutton & Ainsworth, 1983) and the estimated number may be in excess of 250000 (Hawksworth, 1985). It is an impossible task for any one piece of work to cover representatives of all these species but an attempt has been made to test a wide range of examples (Appendix III). The preservation methods explored were in some cases simple and inexpensive, for example water, soil, oil and silica gel storage and others more sophisticated, viz. liquid nitrogen storage and freeze drying.

9.02 Water storage

As discussed above (1.033) water storage techinques have been used for many years for the preservation of fungi with varying degrees of success. This method has been shown here to retain viability of most isolates of Phytophthora and all isolates of Pythium tested for 3 years but after this storage period a rapid loss in viability was observed and many of those that survived showed deterioration in growth and sporulation. For this reason it is recommended only for short term storage.

The CMI records of reports made on isolates from storage in water showed that the virulence of pathogenic organisms decreased despite the retention of viability.

A point that must also be borne in mind is that 10 isolates (18%) of Phytophthora and 4 isolates (33%) of Pythium were contaminated on retrieval from the first sample bottle. The

reserve storage proved to be contaminant free. Contamination is probably a result of transferring large inocula and it is therefore advisable to have a reserve set of stored cultures to be opened only when reprocessing is necessary therefore reducing the chance of contamination.

A comparison of the same isolates stored in liquid nitrogen showed that the latter storage technique was more suitable for long term preservation and stability. However, 4 isolates of the genus Phytophthora and one of Pythium stored in water could not be kept by freezing methods and in such cases water storage can be used as a last resort. It is recommended that cultures stored in water must be subcultured every 2 years and replaced in storage to ensure retention of viability. Therefore this technique would be useful for laboratories preserving isolates for teaching purposes. However, it should not be used for preservation of plant pathogens that are used in circumstances where they are required to have constant infectivity levels with susceptible and resistant varieties of plants. Furthermore, it is not applicable to culture collections which require techniques that retain viability and other properties for many years.

9.03 Soil storage

Another simple technique examined in this study was storage in soil. All isolates of Fusarium retrieved from Booth's collection were found to be morphologically typical when compared with their original descriptions. Despite this successful storage Booth (1971) found that the initial growth period may in some cases allow selective growth of abnormal

strains presumably due to the segregation of a heterokaryon. This method cannot be recommended therefore as the sole method of preservation for this genus. Booth stated that, in some cases, the considerable time lag before the onset of dormancy as the culture dried may be sufficient for variant vegetative strains to overgrow the wild type or for saprophytic strains to overgrow a pathogenic strain. His results showed that 50% of the isolates of Fusarium acuminatum that survived storage in soil had been replaced by variant strains and 67% of the isolates of F. semitectum had been replaced.

As growth is limited to the initial stages of the technique it should be possible to detect any change early in the storage period of the isolates and, if necessary, attempt the storage again. Although this technique can be used to retain viability during long term storage, techniques that retain stability such as freeze drying are recommended for this genus (Hesseltine, Bradle & Benjamin, 1960; also see 9.08 below).

As discussed earlier (section 1.024) soil storage can be used successfully to retain viability of other fungi such as Septoria, Pseudocercosporella and Alternaria which are not so commonly heterokaryotic. Therefore although the technique may not be desirable for the storage of isolates of Fusarium it could be useful for storage of other fungi.

9.04 Comparison of oil storage with silica gel, liquid nitrogen and centrifugal freeze drying techniques

Preservation of fungi representing the Mastigomycotina, Zygomycotina, Basidiomycotina and Deuteromycotina by oil storage has been compared with their storage in silica gel, liquid

nitrogen storage and by centrifugal freeze drying methods. The samples stored under oil showed the greatest degree of change. Over 70% of the isolates showed deterioration in growth and sporulation after storage whereas with the other techniques less than 40% of the isolates deteriorated. Fungi kept under a layer of mineral oil grow continuously and do so in conditions that are artificial and in most cases adverse. The mineral oil prevents dehydration of the culture during growth and also reduces the transmission of oxygen and therefore reduces the growth rate (Fennell, 1960). At CMI some fungi were found to be adversly affected by the conditions under oil and to remain viable regular transfer at intervals of 2 years is required (Smith & Onions, 1983a). It has now been shown that some isolates must be transferred more often (see 1.03 above). Many changes in morphology, seen as an increase in sectoring, and growth rate were observed when isolates were recovered from the CMI oil collection for this study. Many isolates lost the ability to sporulate during the first year of storage. However, other fungi have remained morphologically stable for periods of up to 32 years (Smith & Onions, 1983b). In addition many isolates have survived preservation by this method while failing to survive centrifugal freeze drying or silica gel storage, though some degree of deterioration was usually observed. Oil storage is recommended as a short term storage technique for use in large culture collections while preservation by other techniques is being carried out. This technique is labour intensive because the sensitive cultures require regular transfer. As the fungi

are continuously growing there is a possibility that they may penetrate the surface of the oil and evaporation of moisture will eventually result in dehydration and death. The collection must therefore be monitored for such occurrences. Lastly, as the bottle caps must remain loose during storage to allow difusion of gases there is the possibility of contamination. The use of cotton or foam plugs may reduce this risk during storage. However, on transfer the culture may become contaminated. The constant supervision by personnel with knowledge of the organisms is required to ensure the culture grown for reimmersion is typical. In addition the collection should be regularly checked for cultures showing signs of dehydration. Retrieval may be slow as the organism may not grow well on the first transfer and further culturing may be necessary. Therefore the possibility of culture dehydration and labour intensiveness make this technique unsuitable and not recommended for long term preservation in large culture collections.

The silica gel storage technique has been shown here to preserve many fungi that sporulate when grown on artificial media. However, some sporulating fungi belonging to the Mastigomycotina and Zygomycotina and all that grew vegetatively in culture failed to survive the initial dehydration or died during storage. Many isolates survived liquid nitrogen storage that had failed dehydration in silica gel and the majority remained unchanged and their potential shelf life is thought to be exceptionally long. The majority of the failures (89%) could be preserved by the centrifugal freeze drying technique although 4% of these died in storage. The majority of isolates that

survive dehydration in silica gel remained stable and unchanged in storage. This technique may be of use for the preservation of fungi that sporulate in culture, particularly those belonging to the Deuteromycotina and Ascomycotina, for collections without freeze drying equipment, however, it is not as successful. Large collections are advised to use freeze drying. The fungi stored in silica gel are susceptible to possible contamination. It is advisable for collections using this method and oil storage to keep stock cultures in reserve for use only when the working collection becomes contaminated or when the isolate needs to be represerved. This is also necessary for the techniques of water and soil storage.

9.05 Liquid nitrogen storage

The evidence presented in this study, and by other workers (see section 1.028 iii), has shown that liquid nitrogen storage is a superior technique to any other that is available at present for the preservation of fungi. The advantages of this technique are that cultures of Phytophthora and Pythium are kept more stable with regard to virulence than by water storage. The majority of both sporulating and non-sporulating fungi in culture survive and isolates are completely sealed free of contamination. This study has shown that isolates of Achlya, Aphanomyces, Phlyctochytrium, Phytophthora, Pythium, Rhyzophydium and Saprolegnia that had originally failed could be preserved when minor changes in technique were made. For example techniques that avoid excessive manipulation allow the survival of many recalcitrant fungi. The lack of septa in the vegetative stages of

the fungi belonging to the Mastigomycotina make members of this subdivision more susceptible to mechanical damage and the fact that some isolates are difficult to grow in culture, forming colonies that tend to be weak and non-sporulating may also have contributed to their failure. Eight isolates of Phytophthora species that failed original attempts of storage in liquid nitrogen were successfully preserved by avoiding excessive manipulation. The genus Pythium was a little more difficult to preserve in liquid nitrogen and the failure rate was over 50%. Survival periods at ultra low temperatures range from between 2 and 13 years. Longest survival periods were noted for the sporulating Eumycota including the Zygomycotina, Basidiomycotina and Deuteromycotina. Non-sporulating cultures producing sclerotia or sterile ascocarps survived for similar periods. Shorter successful storage periods were encountered with some of the Acrasiomycetes and some Mastigomycotina.

The maximum cooling rate obtained by plunging 1 ml plastic ampoules containing 0.5 ml of suspension, into liquid nitrogen at -196^{o} C is approximately 200^{o} C min^{-1}. If the temperature of the coolant is closer to its freezing point then there will be more heat needed to vaporize the coolant and conductivity will be improved from the solid through the liquid rather than through the vapour. As solid/liquid nitrogen was not readily available the refrigerant gases freon and isoceon were used in the solid/liquid (melting) phases (2.10). The use of aluminium foil as a supporting medium allowed rapid conductivity of heat and a method was developed to attempt the preservation of Erysiphe pisi but without a great deal of success. Some spores were able to

germinate after freezing at the rapid rates whereas previous attempts of cooling examples of the genus at 1 ^{o}C min^{-1} had killed all spores.

Erysiphe pisi failed to survive when it was frozen as infected leaf epidermis. However, a similar technique using infected host tissue worked well with the preservation of the downy mildews Bremia lactucae and Sclerospora graminicola. The reason the latter succeeded may have been due to the degree of invasion of host tissue. The downy mildews tend to invade the host tissue via stomata, the hyphae grow intracellularly and haustoria penetrate the cells. Invariably the infection of a powdery mildew is on the surface and haustoria penetrate down through the epidermal cell wall. In the case of the downy mildew the host cell tissue may provide some cryoprotection whereas infections on the surface of plant material can be easily damaged or the fungus dislodged. Alternatively it is the sporangium that survives which would not be present among harvested conidia.

These pathogens are normally kept on their host and transferred to fresh plants as the host deteriorates or dies. This is rather time consuming and requires considerable space and quarantine facilities. The risk of escape of these organisms from infected plants is quite high although if frozen and stored in sealed containers the risk is minimal. It is therefore useful to be able to preserve some of these fungi away from the host even if it is only for the short term.

9.06 Cryoinjury and cryoprotection

The damage to the fungal cells associated with freezing is usually due to membrane lesions. Mazur (1966) did extensive work on yeast cells and suggested that cell injury was due to intracellular ice crystal formation. Membrane damage can also occur before freezing and may be a result of cold osmotic shock. Leakage of some proteins involved in the transport of tryptophan (Wiley, 1970), glucosamine and aminoisobutyrate (Patching & Rose, 1971) has been reported from cold shocked cells. Although fungi may survive freezing without being damaged crystalization of ice occurring during warming from low temperatures subsequently results in membrane damage.

The effect of cooling at different rates on fungal hyphae was not known and therefore a cryomicroscopic study was initiated. Observations during the cooling of hyphae of Penicillium expansum in growth medium showed that intracellular ice formation occurred at temperatures between -11 and -14 ^{o}C at cooling rates of 15 ^{o}C min^{-1} or faster. At slower rates the cell lost water at a sufficiently rapid rate to maintain equilibrium with the extracellular solution and intracellular ice was not formed. If the cooling rate is too rapid water does not leave at the required rate and intracellular ice nucleation results. Yeast cells have been shown to lose 90% of their intracellular water after cooling at 1 ^{o}C min^{-1} to -15 ^{o}C and since the remainder does not freeze the cells contained no intracellular ice (Mazur, 1968). The hyphae of Penicillium expansum shrank to less than 50% of their diameter at -30 ^{o}C and as the septa remained the same distance apart, it was calculated that they lost 75% of their

volume. When the hyphae of P. expansum shrank to 60% of their original diameter in NaCl solutions at room temperature and were re-expanded in isotonic medium no loss in viability occurred. The viability of colonies was reduced when cooled at rates that induce intracellular ice formation. However, after cooling at rates greater than $50^{o}C\ min^{-1}$, where intracellular ice formed in all cells, over 60% of colonies remained viable. The addition of glycerol to the suspending medium reduced the effect of shrinkage during slow rates of cooling and increased the cooling rate necessary to induce intracellular ice formation. As a result higher viabilities of P. expansum were obtained. The recrystalization of ice, the formation of gas bubbles and the rupture of septa and cell walls were seen in some hyphae during thawing of intracellular ice. These events were probably the cause of the loss in viability of some colonies of this fungus at fast rates of cooling.

In contrast, Phytophthora nicotianae did not survive at any of the cooling rates unless pretreated and frozen in the presence of 10% (v/v) glycerol. Shrinkage was extensive at all rates of cooling without glycerol and at cooling rates faster than $11^{o}C\ min^{-1}$ in its presence. A loss in viability in the presence of glycerol correlated with an increase in the extent of shrinkage. When the hyphae were placed in 2.75M sodium chloride at $+20^{o}C$ they shrank to 60% of their original diameter and after re-expansion the viability was reduced to 19%. There was very little intracellular ice formation at any cooling rate and this was unaffected by the addition of glycerol.

Penicillium expansum was extremely resistant to both shrinkage at normal temperatures and cooling at all rates. However, the viability of Phytophthora nicotianae colonies was low when shrunken without cooling and were not viable after cooling when extensive shrinkage occurred. Phytophthora citrophthora was even more sensitive to shrinkage during cooling as it did not survive despite the presence of glycerol. The death of these three fungi is not due to intracellular ice formation but to freeze induced shrinkage or lethal events during the thawing of intracellular ice. This finding is contradictory to the hypothesis of cryoinjury by Mazur (1968) in which he states that intracellular ice formation is a lethal event.

In this study 10% (v/v) glycerol has proved to be an effective cryoprotectant. It is able to penetrate the cell (Calcott, 1978) and come into equilibrium with the concentration in the suspending medium. This reduces the freezing point of both the cell solution and the suspending medium. During the formation of extracellular ice at slow rates of cooling glycerol continues to penetrate the cell as water passes out reducing the amount of shrinkage. At faster rates the exchange cannot be balanced and shrinkage occurs to a greater extent in the concentrated glycerol and growth medium than it did in the growth medium alone. At rates $>35\ ^{o}C\ min^{-1}$ water does not leave the hyphae quickly enough and equilibrium with the external medium is achieved by intracellular ice nucleation.

PVP, a cryoprotectant that does not penetrate the cell, normally protects by reducing the rate of ice formation outside the cell thereby reducing the increase in extracellular

concentration and secondly by altering the permeability of the cell to ions. However this additive is unable to protect some fungi. Phytophthora palmivora IMI 202528 failed to survive cooling in PVP but survived in 10% (v/v) glyerol and DMSO mixtures. Other members of the Mastigomycotina, Pythium sylvaticum IMI 248394 and 248395, also failed in PVP but survived in some of the smaller molecular weight glycerol - DMSO mixtures (Tables, 5.031 and 5.032). As discussed earlier rapid warming rates can not be achieved through the PVP and death of these fungi may not be occurring during cooling but during the slow thawing when crystalization of extracellular ice and localised melting may expose the hyphae to concentrated solutions and irreversibly damage their membranes.

The ability of the fungal cell in these cases to absorb the cryoprotectant could be critical for the survival of the organism. The selection of a cryoprotectant that had the correct mode of action could improve preservation of many fungi.

The evaluation of cryoprotectants showed that different compounds and concentrations are benificial with particular fungi. However, it was also seen that some fungi remained viable without a cryoprotective additive. Aspergillus carbonarius survived all treatments (100% of samples) whereas the survival of other fungi depended entirely upon the cryoprotectant. Pythium sylvaticum, for example, survived only in the 8% glucose-10% DMSO mixture and a total of only 4% of samples survived all treatments. Glycerol was found not to be as effective as the glucose-DMSO mixture.

It appears that there is no universal cryoprotectant available at present for the successful freezing and storage of all fungi. Some of the cryoprotectants that allowed the freezing and storage of the test fungi (Tables, 5.031 and 5.032) were not successful with Bremia lactucae. The mixture of 8% glucose and 10% DMSO and the single cryoprotectant 10% glycerol did not protect this fungus from freezing damage. An infectivity of 35% was achieved without a cryoprotectant and 37% with distilled water, totally unexpected results.

The technique must be carefully investigated for each isolate that does not survive the routine method and the finer points of the procedure must be optimized for each isolate. Further cryomicroscope studies of examples of fungi representing the full taxonomic range will help in the understanding of the mechanisms of their cryoinjury and enable improved methods of low temperature storage to be developed.

9.07 Freeze drying

Investigation of the centrifugal freeze drying technique revealed that many isolates of fungi failed to survive evaporative freezing. A method was developed using freezing rates similar to those used prior to liquid nitrogen storage. The isolates tested survived this stage of the method but many of them lost viability during the subsequent vacuum drying stages. Thus only limited improvement was made to the range of fungi surviving freeze drying.

It was confirmed that the residual water content of between 1 and 2%, which proved most suitable for other microorganisms, gave equally good results for fungi.

Freeze dried fungi do not have a long shelf life if dried to water contents above 2%. Armillariella mellea died during the first year of storage when dried to residual water contents of 5.3% and above or less than 1%. Viability was retained at residual water contents of 1 and 2%. In samples of Aspergillus niger and Penicillium ochrochloron freeze dried to residual water contents of above 2% and below 1% viability decreased during the first year of storage.

It was important that the residual water content was reduced significantly (<5%) before allowing the temperature of the cells to rise above -15^{o}C to get best revivals. When the residual water was above 24% when the temperature rose above -15^{o}C fewer isolates survived. However, some isolates still failed to survive the following dehydration treatment. One reason why some organisms fail centrifugal freeze drying is that the lowest temperature reached with evaporative cooling in some apparatus is -12^{o}C (Haskins, 1957).

Freshly isolated cultures of fungi were more likely to survive the shelf freeze drying technique than old isolates (Table, 8.076). It is therefore essential that freshly isolated healthy cultures should be prepared for preservation as soon as possible, normally between 2 and 6 weeks.

Having established that the improved shelf technique which enables prefreezing of cultures at different rates of cooling and drying at varied warming rates retained viability of some isolates of fungi that failed centrifugal freeze drying in the short term, 1 year in many cases, it is necessary to determine

whether the fungi will survive longer periods. Fungi have survived 18 years storage at CMI when freeze dried by the centrifugal technique and 20 years elsewhere (Rhoades, 1970). Loss of viability and the deterioration of properties occurs after 30 years storage by some freeze drying techniques (Jong, Levy & Stevenson, 1984). However, the rubber bung to glass seal in vials used on the shelf freeze drier may allow leakage of gases or moisture during extended storage. This may inevitably lead to more rapid deterioration. Work carried out on the storage of such vials has shown little leakage at temperatures above zero but at temperatures of liquid nitrogen some leakage has been encountered (Barbaree and Smith, 1981). Methods of predicting shelf life of microorganisms have been used for bacteria (Damjanovic, & Radulovic, 1968) and viruses (Greiff & Rightsel, 1965). These techniques have been used for fungi (Rogan & Terry, 1973). They rely on storage at high temperatures to determine the shelf life and predicting the time the organism would survive at normal storage temperatures. Such tests could be performed to predict ultimate storage periods.

9.08 Methods of preservation recommended for particular groups of fungi

The Mastigomycotina are best stored in liquid nitrogen although there are isolates that do not survive its freezing stages. In these cases they can be kept under mineral oil for periods up to 6 months at which time they should be transferred to fresh media and re-immersed in oil. Alternatively, cultures can be kept viable in water storage and transferred every 2 years. The latter two techniques are not so successful at

retaining particular properties of the fungi but can be used as a last resort or as a back up to liquid nitrogen storage. The silica gel technique cannot be used to preserve this group successfully and at present freeze drying techniques do not retain viability in the long term.

Cultures belonging to the Zygomycotina are again preserved best in liquid nitrogen. The viability of most isolates is retained by both centrifugal and shelf freeze drying. However, not all fungi belonging to this group survive dehydration particularly in silica gel. The genera Coemansia and Martensiomyces of the Kickxellales and Conidiobolus, Entomophthora and Piptocephalis of the Entomopthorales do not survive silica gel storage. Of these groups only isolates of Piptocephalis and Coemansia could be preserved successfully by centrifugal freeze drying although one isolate of Martensiomyces survived but grew abnormally. This latter isolate failed to survive the improved shelf freeze drying technique in its deteriorated state. Only isolates belonging to the genera Coemansia and Piptocephalis from the Kickxellales and Entomophthorales have survived the shelf freeze drying process. However, isolates representing all above genera have been succesfully preserved in liquid nitrogen.

Syzygites was the only isolate of the Mucorales that failed to survive the initial dehydration of silica gel storage and another isolate of this genus failed centrifugal freeze drying. However, isolates survived storage in liquid nitrogen and by the improved shelf freeze drying technique.

As discussed ealier (1.038) some Ascomycetes do not survive silica gel as well as centrifugal freeze drying or liquid nitrogen storage. It is therefore recommended that the majority of this group be freeze dried and/or stored in liquid nitrogen. However, many examples of some orders in this group do not survive centrifugal freeze drying for example only 17% of the Clavicipitales, 57% of the Diaporthales, 44% of the Pezizales, 31% of the Sphaeriales, generally those that do not sporulate well in culture, survive. Only one isolate of the Elaphomycetales was frozen and stored in liquid nitrogen this isolate failed to recover, no examples were processed by any other technique. However, over 85% of isolates belonging to most orders have survived storage in nitrogen and therefore it is the best technique available for storage of this group of fungi.

The Basidiomycetes generally grow vegetatively in culture and therefore present problems in preservation. Usually such fungi can only be preserved by serial transfer on agar with and without oil or stored in liquid nitrogen. Those fungi producing thick walled hyphae can be freeze dried but their viabilities are usually low. The Uredinomycetes fail to survive but 19 (79%) of those tested belonging to the Ustilaginomycetes survived.

The Deuteromycotina are relatively easy to preserve though silica gel storage does kill up to 25% of Coelomycetes and 19% of the Hyphomycetes tested. The soil storage technique is a method that has been used extensively for the storage of representatives of the Deuteromycotina particularly the genus Fusarium. However, this genus shows variation in storage but the method can be used for many examples of fungi that are inherently less variable.

Although shelf freeze drying can be used to preserve many isolates of Fusarium results can be unpredictable for some strains. High viabilities are usually obtained but on occasion microconidia do not survive whereas the chlamydospores of the strain remain viable. However, the microconidia survive centrifugal freeze drying. It is recommended that centrifugal or shelf freeze drying and liquid nitrogen storage should be used for the preservation of this group.

9.09 Summary

Freezing and storage in liquid nitrogen is the best preservation technique available for the filamentous fungi. The handling techniques, freezing protocol, cryoprotection and thawing rates can be optimized for difficult fungi. Techniques can thus be designed for specific organisms to get maximum survival. Once the organism has been successfully frozen and stored in liquid nitrogen, assuming that the ultralow temperatures are retained, the storage periods are presumed to be exceptionally long as no chemical and very few physical changes can occur at such low temperatures. However, there will still remain the problem of ice crystal growth during thawing albeit small at rapid rates of warming.

Despite liquid nitrogen storage being the most successful (Table, 9.091) and one of the most adaptable preservation methods available for fungi it is necessary for culture collections to have alternative storage techniques available for their isolates as a safeguard. The liquid nitrogen storage technique relies upon a constant supply of liquid nitrogen and double jacketed

vacuum sealed storage vessels which could break down. In such cases uncontrolled thawing can result in loss of viability.

It has been shown in this study that other techniques are less suitable for the preservation of filamentous fungi Table 9.091 shows the relative costs of the methods and the longevity and stability of the fungi stored. The prime concern of a culture collection is the stability of the cultures held. The serial transfer methods give poor to moderate stability. The methods that should be used to retain stability are silica gel, freeze drying and liquid nitrogen storage. The cost of labour and materials is another major consideration and simple methods that may be inexpensive in materials are usually high in labour costs. Both freeze drying and liquid nitrogen storage are much more costly than all others in terms of materials and equipment. However, the silica gel technique which is inexpensive cannot be used for the preservation of all fungi.

Future work should include the development of techniques that can offer a suitable alternative to freeze drying and liquid nitrogen. The freezing and storage at ultralow temperatures is possibly the best available avenue to follow. At present a chest freezer has been developed by Queue Systems, USA, (distributed in the UK by CAMLAB Ltd.) that can maintain temperatures below $-140\,^{o}C$. If this equipment can be shown to be reliable for long term use then this may prove to be an ideal alternative.

There is still a requirement for further work in the area of preservation of fungi. It is necessary to ensure that not only viability is retained by the techniques but also that the organisms remain stable. Work has been initiated at the CMI on

Table 9.091 A comparison of methods of preservation

Method of Preservation	Cost		Longevity	Genetic Stability
	Material	Labour		
Serial transfer on agar				
(i) Storage at room temperature	low	high	1-6months	variable
(ii) Storage in the refrigerator	*medium	high	6-12months	variable
(iii) Storage under oil	low	low/medium	1-32years	poor
(iv) Storage in water	low	low/medium	2-5years	moderate
(v) Storage in the deep freeze	*medium	low/medium	4-5years	moderate
Drying				
In soil	low	medium	5-20years	moderate to low
Silica gel	low	medium	5-11years	good
Freeze drying	high	**initially medium	4-40years	good
Freezing				
Liquid nitrogen Storage	high	low	infinite 14 years to date at CMI	good

*Refrigerator or deep freeze costs included
**Initial processing is costly depending on the method, maintenance is negligible

the metabolic and physiological characterization of fungi. The isolates characterized will be preserved by the techniques of freeze drying and liquid nitrogen storage and re-characterized to assess the retention of stability during storage.

In conclusion it has been found that the success of liquid nitrogen storage for the preservation of fungi cannot be matched by other techniques including freeze drying. The freeze drying technique has been improved but there are fungi that will not survive the dehydration stages of the technique. The liquid nitrogen storage method has been shown to be adaptable for the preservation of recalcitrant and obligate plant pathogenic fungi.

APPENDICES

APPENDIX I Publications

The following are papers published after completing the analysis of the CMI preservation results and after two preliminary experiments.

1. Smith, D. (1982). Liquid nitrogen storage of fungi. Transactions of the British Mycological Society 79, 415-421.
2. Smith, D. (1983a). A two stage centrifugal freeze drying method for the preservation of fungi. Transactions of the British Mycological Society 80, 333-337.
3. Smith, D. (1983b). Cryoprotectants and the cryopreservation of fungi. Transactions of the British Mycological Society 80, 360-363.
4. Smith, D.; Onions, A.H.S. (1983). A comparison of some preservation techniques for fungi. Transactions of the British Mycological Society 81, 535-540.

Trans. Br. mycol. Soc. 79 (3) 415–421 (1982) *Printed in Great Britain*

LIQUID NITROGEN STORAGE OF FUNGI

BY D. SMITH
Commonwealth Mycological Institute, Kew, Surrey

A method is described by which over 3000 isolates of microfungi have been successfully frozen and stored in liquid nitrogen for up to 13 years. The majority of the isolates that failed to survive were from the Mastigomycotina. Results of successful and unsuccessful storage of *Phytophthora* and *Pythium* species and lower Phycomycetes are presented. Out of 189 isolates of *Phytophthora* 108 were successful, though out of 58 isolates of *Pythium* only 27 survived.

Liquid nitrogen storage of fungi began at the Commonwealth Mycological Institute (CMI) in 1968 though successful storage by this method had already been reported by Hwang of the American Type Culture Collection (Hwang, 1960). Hwang (1968) described the successful storage of 104 strains of fungi in liquid nitrogen. The first method to be adopted by CMI was described by Onions (1971). Butterfield, Jong & Alexander (1974) detailed a technique for the preservation of living fungi pathogenic for man and animals. This is very similar to that used at CMI today.

At present over 3000 isolates are stored at these ultra-low temperatures in the Culture Collection. One of the main advantages of this technique is that the cultures are inactivated during storage. This allows the maintenance of living stocks of fungi that are unable to grow on culture media and also fungi that undergo rapid change or deterioration when kept alive by frequent-transfer methods.

METHODS

The fungi were grown on slopes of suitable agar in 1 oz. universal bottles. When the culture had reached maturity 10 ml of 10% glycerol was added and the mycelium and/or spores were brought into suspension by gentle agitation and scraping of the fungal colony. A small portion (0·5 ml) of this suspension was added to 1·0 ml borosilicate glass ampoules, which were then heat sealed. The seals were tested by immersing the ampoules in an erythrocin B dye bath at 4–7 °C, pre-cooling the fungal suspensions. These were frozen at 1°/min to −35° by suspending in the vapour phase of a liquid nitrogen refrigerator at −35° for 40–45 min; this was followed by rapid cooling to −196° by immersing the ampoules in liquid nitrogen. The ampoules were stored clipped to aluminium canes in boxes in a Union Carbide 250 l refrigerator or in the drawer rack system of a Union Carbide 320 l refrigerator. For thawing and checking viabilities the ampoules were removed from the liquid nitrogen refrigerator and immersed in a water bath at 37°. The ampoules were opened and the contents streaked on to suitable medium. Success was evaluated by growth of the cultures when revived after 4 days.

Some fungi were damaged by excessive manipulation so minor adjustments were made to this technique for their reprocessing. The organism was placed in the ampoule on small slivers of agar to avoid mechanical damage that may have occurred during preparation of suspensions. If this still proved unsuccessful the organism was grown in the ampoule itself on a small amount of suitable agar medium. If the culture did not survive using glycerol as the cryoprotectant, 10% dimethyl sulphoxide (DMSO) or a mixture of 5% DMSO and 8% glucose was used.

Cold-hardening, now used for all cultures prior to freezing, was achieved by pre-growth at temperatures between 4 and 7° in a refrigerator.

Alternative methods used for preservation of fungi have been water storage and storage under mineral oil. The water storage technique involved the cutting of 6 mm blocks of agar from a Petri dish culture and placing these in sterile distilled water in a McCartney bottle (Boesewinkel, 1976). The lids of the bottles were tightly screwed down and the cultures stored at temperatures of 15–20°.

For the mineral oil storage method cultures were grown on shallow slopes of suitable agar in 1 oz. universal bottles. The mature cultures were covered by 1 cm of mineral oil (liquid paraffin or medicinal paraffin, specific gravity 0·830–0·890 g) and stored at temperatures of 15–20° (Onions, 1971).

RESULTS

A taxonomic list of the isolates based on Ainsworth & Bisby (1971) supplemented by the names published in the *Index of Fungi* (1971–1981) is

Table 1. *Viability of isolates stored in liquid nitrogen for 2–13 years*

	Number of genera	Number of species	Number of isolates		Percentag success
			Tested	Viable	
MYXOMYCOTA					
Acrasiomycetes	1	2	3	3	100
Myxomycetes	0	0	2	0	0
EUMYCOTA					
MASTIGOMYCOTINA					
Chytridiomycetes	4	7	56	9	16
Hyphochytriomycetes	1	1	5	3	60
Oomycetes	9	54	348	172	50
ZYCOMYCOTINA					
Zygomycetes	43	176	267	254	95
ASCOMYCOTINA		7 of uncertain classification (15 isolates)			
Hemiascomycetes	11	21	30	27	90
Plectomycetes	40	89	103	102	99
Pyrenomycetes	83	274	388	381	98
Discomycetes	23	39	72	65	90
Loculoascomycetes	4	9	18	10	55
BASIDIOMYCOTINA					
Teliomycetes	7	10	17	16	94
Hymenomycetes					
(*a*) Phragmobasidiomycetidae	6	6	12	11	92
(*b*) Holobasidiomycetidae	32	58	154	134	87
Gasteromycetes	3	1	7	6	86
DEUTEROMYCOTINA					
Blastomycetes	2	2	2	2	100
Hyphomycetes	257	1210	1541	1463	95
Coelomycetes	67	163	238	224	94
ACTINOMYCETES	4	19	26	19	73
LICHENS	2	2	2	2	100

given in Table 1. These comprise 3004 different isolates belonging to 2134 species and 615 genera. The Deutermycotina, the Zygomycotina, excluding the Trichomycetes, and the Ascomycotina, excluding the Laboulbeniomycetes and Loculoascomycetes, gave successful storage; over 90% of the isolates tested survived. No isolates representing the Trichomycetes or the Laboulbeniomycetes were tested. Only 55% of the isolates belonging to the Loculoascomycetes survived. The isolates of Basidiomycotina tested survived the method of liquid nitrogen storage, giving a success rate 86–92%.

Table 2 shows general figures for successf storage for classes within the Mastigomycotii The rate of success varies between 16% in t Chytridiomycetes and 50% in the Oomycetes.

Table 3 shows the results of attempts made store particular oomycetes in liquid nitrogen. T Saprolegniales have not been tested extensive the results give percentage success ranging from to 20. The Peronosporales, represented by *Phytop*

Table 2. *Viability of the Mastigomycotina stored in liquid nitrogen for 1–12 years*

	Number of species			Number of isolates		
	Tested	Viable	Percentage success	Tested	Viable	Percentag success
Chytridiomycetes	27	7	26	56	9	16
Hyphochytriomycetes	1	1	100	5	3	60
Oomycetes	73	45	62	348	172	50

Table 3. *Viability of some Oomycetes stored in liquid nitrogen 1–12 years*

	Number of species			Number of isolates		
	Tested	Viable	Percentage success	Tested	Viable	Percentage success
PERONOSPORALES						
Phytophthora	26	18	69	189	108	57
Pythium	27	17	63	56	27	48
Sclerospora	1	1	100	9	9	100
SAPROLEGNIALES						
Achlya	6	3	50	42	6	14
Aphanomyces	2	2	100	10	2	20
Dictyuchus	2	0	0	2	0	0
Isoachlya	1	0	0	1	0	0
Protoachlya	1	0	0	3	0	0
Saprolegnia	5	3	60	15	3	20
Thraustotheca	1	1	100	5	1	20

Table 4. *Viability of* Phytophthora *species stored in liquid nitrogen using 10% glycerol as cryoprotectant except where indicated*

Species	No. of isolates tested	No. of isolates viable	Percentage success	No. of isolates re-processed (included)	Survival period (years) (up to 1982)
P. arecae (Coleman) Pethybr.	1	1	100	0	4
P. boehmeriae Saw.	2	2	100	0	5
P. botryosa Chee	4	3	75	1*	4–8
P. cactorum (Lebert & Cohn) Schroeter	4	4	100	0*	8–10
P. cambivora (Petri) Buisman	6	5	83	1*	6–9
P capsici Leonian	6	2	33	2*	4–5
P cinnamomi Rands	12	7	58	0	10
P. citricola Saw.	4	3	75	0	5–9
P. citrophthora (Smith & Smith) Leonian	2	0	0	0	< 4 days
P. cryptogea Pethybr. & Lafferty	6	4	66	1*	4–10
P. drechsleri Tucker	3	2	66	1*	9–10
P. erythroseptica Pethybr.	12	7	58	1*	6–11
P. fragariae Hickman	5	0	0	0	< 4 days
P. heveae Thompson	7	4	57	1*	4–8
P. infestans (Mont.) de Bary	8	3	38	1*	8–9
P iranica Ershad	1	0	0	0	< 4 days
P. lateralis Tucker & Milbrath	1	0	0	0	< 4 days
P meadii McRae	1	0	0	0	< 4 days
P megasperma Hildebrand	19	8	42	4*	6–10
P mexicana Hotson & Hartse	1	0	0	0	< 4 days
P nicotianae Van Breda de Haan	16	16	100	0	5–12
P palmivora (Butler) Butler	80	39	49	4†*	5–12
P porri Foister	5	1	20	0	9
P megasperma v. *sojae* Drechsler	1	0	0	0	< 4 days
P. syringae (Kleb.) Kleb.	1	0	0	0	< 4 days
P. sp.	1	1	100	0	< 4 days

* Re-processed by cold-hardening and avoiding excessive manipulation.
† One re-processed using 5% DMSO + 8% glucose.

Table 5. *Viability of* Pythium *species stored in liquid nitrogen using 10% glycerol as cryoprotectant*

Species	No. of isolates tested	No. of isolates viable	Percentage success	Survival period (years)
P. acanthicum Drechsler	1	0	0	< 4 days
P. aphanidermatum (Edson) Fitzp.	2	2*	100	8
P. aristosporum Vanterpool	4	0	0	< 4 days
P. arrhenomanes Drechsler	1	1*	100	7
P. artotrogus de Bary	1	0	0	< 4 days
P. butleri Subram.	3	2	66	7, 9
P. coloratum Vaartaja	1	1	100	5
P. debaryanum Hesse	3	3	100	6–8
P. deliense Meurs	2	1	50	9
P. flevoense van der Plaats-Niterink	4	4	100	5
P. fluminum D. Park	4	0	0	< 4 days
P. graminicola Subram.	2	0	0	< 4 days
P. helicoides Drechsler	3	1	33	5
P. hydnosporum (Mont.) Schroeter	1	0	0	< 4 days
P. irregulare Buisman	3	2	66	5, 7
P. iwayamae S. Ito	4	0	0	< 4 days
P. middletoni Sparrow	2	2	100	4, 8
P. myriotylum Drechsler	3	3	100	4–8
P. oligandrum Drechsler	2	0	0	< 4 days
P. periplocum Drechsler	1	0	0	< 4 days
P. polymorphon Sideris	1	1	100	7
P. spinosum Saw.	1	1	100	8
P. splendens Braun	1	1	100	9
P. sulcatum Pratt & Mitchell	2	0	0	< 4 days
P. tracheiphilum Matta	1	1	100	2
P. undulatum Peterson	1	1	100	8
P. vexans de Bary	2	1	50	5

* These isolates required re-processing, achieved by cold-hardening of fresh cultures.

thora, *Pythium* and *Sclerospora*, have been dealt with in detail and gave rates of success of 48% with the genus *Pythium* to 100% success with the one species of *Sclerospora* tested (nine isolates).

Table 4 gives details of the *Phytophthora* species tested. Seventeen isolates of 10 species that did not survive the process immediately required further attempts to achieve successful storage. Cold-hardening, and the avoidance of mechanical damage that may be incurred during excessive manipulation in the preparation of fungal suspensions, enabled the preservation of 16 of these isolates. One isolate of *P. palmivora* required the use of 5% DMSO and 8% glucose as cryoprotectant before it survived. The survival period of all the species of *Phytophthora* is given as the number of years that the isolates have remained viable prior to 1981, and to date none has died. The years of successful storage are therefore not limiting. Some isolates of *P. nicotianae* and *P. palmivora* have remained viable for 12 years. The percentage successes of these two species were 100 and 49 (16 out of 16 and only 39 out of 80 isolates survive respectively).

Table 5 lists the 27 species of *Pythium* tested an indicates the species which were successfull processed; however, only 56 isolates were tried, 2 of which survived. Although many attempts wer made to re-process the failures very little succes was achieved. *P. aphanidermatum* (two isolates) an *P. arrhenomanes* (one isolate) required freshl grown cultures that had received cold hardenin (1–2 weeks growth at 4–7°) before success wa achieved. The range of success was quite wide from 0% with *P. aristosporum* and *P. iwayama* (four isolates of each), to 100% with *P. flevoens* (four isolates).

Table 6 shows the lack of success achieved witl some of the lower phycomycetes tested. All 1 isolates of *Achlya bisexualis* tested failed and onl three isolates of *A. ambisexualis* remained viabl from the 13 tried. Percentage successes wer omitted from the table because of the low number involved.

Table 6. *Viability of some lower Phycomycetes stored in liquid nitrogen using 10% glycerol as cryoprotectant*

	No of isolates tested	No. of isolates viable	Failures re-processed successfully	Survival period (years)
Achlya ambisexualis J. R. Raper	13	3	3*	1–5
A. americana Humphrey	2	1	1*	4
A. bisexualis Coker	10	0	0	< 4 days
A. debaryana Humphrey	3	0	0	< 4 days
A. racemosa Hildebrand	7	3	2*	4–8
A. radiosa Maurizio	4	0	0	< 4 days
Allomyces anomalus Emers.	2	2	0	3, 8
A. cystogenus Emers.	1	1	0	4
A. javanicus Kniep	3	2	0	4, 5
Aphanomyces brassicae S. L. Singh & M. S. Pavgi	2	1	1*	4
A. euteiches Drechsler	12	5	3*	4–7
Chytridium olla Braun	2	0	0	< 4 days
C. ottariense Roane	1	0	0	< 4 days
Dictyuchus sterilis Coker	1	0	0	< 4 days
Dictychus sp.	1	0	0	< 4 days
Entophlyctis confervaeglomerata (Cienkowski) Sparrow	2	2	0	4, 8
Hyphochytrium catenoides Karling	7	5	0	4–10
Phlyctochytrium acuminatum D. J. S. Barr	4	2	0	4, 10
P. arcticum D. J. S. Barr	2	1	1*	4
P. californicum D. J. S. Barr	3	1	0	3
P. plurigibbosum D. J. S. Barr	2	0	0	< 4 days
P. reinboldtae D. J. S. Barr	1	0	0	< 4 days
P. sp.	1	0	0	< 4 days
Protoachyla paradoxa Coker	3	0	0	< 4 days
Rhizoclosmatium globosum H. E. Petersen	3	0	0	< 4 days
Rhizophydium bisporum (Couch) D. J. S. Barr	2	1*	1*	4
R. capillaceum D. J. S. Barr	3	0	0	< 4 days
R. chlorogonii (Serbinov) Jacz.	1	0	0	< 4 days
R. granulosporum Scherffel	3	0	0	< 4 days
R. haynaldii (Schaarschmidt) Fischer	3	0	0	< 4 days
R. karlingii Sparrow	1	0	0	< 4 days
R. patellarium Scholz	3	0	0	< 4 days
R. sphaerocarpum (Zopf) Fischer	2	0	0	< 4 days
R sphaerotheca Zopf	7	0	0	< 4 days
R. sp.	1	0	0	< 4 days
Saprolegnia ferax (Gruith) Thuret	4	1	0	4
S. glomerata (Tiesenhausen) A. Lund	1	0	0	< 4 days
S. litoralis Coker	3	0	0	< 4 days
S. megasperma Coker	2	2	0	4, 6
S. parasitica Coker	6	1	1*	1
Thraustotheca clavata (de Bary) Humphrey	5	1	1*	4

* Re-processed by cold-hardening and avoidance of excessive manipulation.

DISCUSSION

During a period of 13 years over 3004 different isolates have been processed. Of these 543 species failed, though on re-processing 359 of these were successful, leaving in total 184 not surviving. Those fungi that survived the process represent 615 genera and over 2000 different species (see Table 1).

The species of *Phytophthora* that have been stored without failures using this method of liquid nitrogen storage are *P. arecae* (1 isolate), *P. boehmeriae* (2 isolates), *P. cactorum* (4 isolates) and *P. nicotianae* (16 isolates). The remaining species gave varying degrees of success and this apparently depended on the strength of growth in culture. Those isolates that only gave poor restricted growth on suitable agar media, Lima bean agar, oat agar or corn meal agar, tended not to survive the process.

Cold-hardening achieved by pre-growth at temperatures between 4–7° gave improved survival with some of the initial failures. These were *P. botryosa* (1 isolate), *P. cambivora* (1 isolate), *P. capsici* (2 isolates), *P. megasperma* (2 isolates) and *P. palmivora* (2 isolates). There were eight species involving only 13 isolates (see table 4) which completely failed. Neither cold-hardening, ensuring no mechanical damage by growth in ampoules to be frozen, nor the use of alternative cryoprotectants improved the initial attempts to preserve these isolates. Attempts will be made to preserve these species with their host tissue, which is a method that has proved successful with downy mildews at CMI.

The genus *Pythium* was a little more difficult to preserve in liquid nitrogen and gave a failure rate of over 50%. There is extensive work still to be carried out on perfecting the general method for this genus. The majority of the isolates are being kept under mineral oil and in sterile water; this is also the case with many of the *Phytophthora* species tested. These two alternative storage methods are not as desirable as liquid nitrogen for they allow growth to occur under adverse conditions and can be selective. Water storage has given survival periods of between 2 and 3 years for *Phytophthora* and *Pythium* species. It is also successful with many other genera, for work by Boesewinkel (1976) showed successful storage of 650 plant pathogens for longer periods than 2–3 years. The mineral oil storage techniques (Fennell, 1960; Onions, 1971) will only give storage periods of 6–12 months in some instances, though some isolates of these two genera have survived longer periods of 2 years. Experience with this technique at CMI has shown that many fungi become unstable in their vegetative growth (i.e. sectoring is more frequent), lose their ability to sporulate and often fail to survive the technique at all, inasmuch as they are unable to grow out from the oil medium.

The Mastigomycotina represent less than 4% of the CMI liquid nitrogen collection. Storage of the isolates in this subdivision was difficult and the percentage success ranged from only 16 to 50. The majority of these failures were due to physical damage, either incurred during preparation of the suspension prior to freezing by excessive manipulation, or due to ice formation during the freezing process. The damage to the fungal cells associated with freezing is usually shown by membrane lesions. Mazur (1966) did extensive work on yeast cells and suggested that cell injury was due to intracellular ice crystal formation. Membrane damage can also occur before freezing and may be a result of cold osmotic shock. Leakage of some proteins involved in the transport of tryptophan (Wiley, 1970), glucosamine and amino-isobutry. (Patching & Rose, 1971) has been reported fr cold-shocked cells. If the fungi survive freezi without being damaged recrystallization of ice m occur during storage at low temperatures and res in membrane damage.

The failure to store some fungi by this meth may therefore be due to freezing damage, to wh these strains or isolates may have been mc susceptible. The lack of cross-septa in t Mastigomycotina and the fact that some isolates a difficult to grow in culture and give colonies th tend to be weak and non-sporulating may ha contributed to the susceptibility to freezi damage. The cryoprotectant mixture of DMS and glucose was not used with some of the fun and no attempt was made to cut down mechanic damage by growing colonies in the stora ampoules. These two minor changes may impro the results obtained with these cultures.

Survival periods at the ultra-low temperatur range between 2 and 13 years. The maximu survival periods were noted for the sporulati Eumycota excluding the Mastigomycotina. No sporulating cultures producing sclerotia or ster ascocarps survive similar periods. Shorter successf storage periods were encountered with some of t Acrasiomycetes and some Mastigomycotina. great advantage of this method is that no sporulating cultures will also survive if minim damage is inflicted during suspension cryoprotectants.

Success has also been achieved with the obliga plant pathogen *Sclerospora graminicola* by freezir the fungus in the infected host tissue both with an without cryoprotectant. Successful storage of th genus in liquid nitrogen has also been carried o by Gale, Schmitt & Broomfield (1975) and Lon Woods & Schmitt (1978).

The author wishes to thank Dr B. C. Sutton an Dr C. Booth of CMI and Dr J. Gay of Imperi College London for their support and encourage ment and Mr S. M. Ward for his valuable technic assistance. Acknowledgement is also given to th CMI for access to results prior to the author employment there in 1974.

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A TWO STAGE CENTRIFUGAL FREEZE DRYING METHOD FOR THE PRESERVATION OF FUNGI

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A method is described by which over 7400 different isolates of fungi have been successfully stored by freeze drying. A list of the fungi tested, showing both successes and failures, is included.

Pre-freezing in liquid nitrogen prior to vacuum drying gave improved results. An isolate of *Phytophthora palmivora* survived 3 months storage in unfavourable conditions where previously it had not survived freeze drying.

Many methods have been used to preserve fungi and these have been reviewed by Fennell (1960) and Heckly (1978). One of the most useful of these is freeze drying. Harris & Shackell (1911) described a method by which rabies virus was successfully freeze dried; as methods improved, more sophisticated machinery was developed to carry out the process (Rowe, 1970, 1971).

Freeze drying provides a very stable storage technique, cells remain dormant and undergo no metabolic activity whilst in a dry condition (Heckly, 1978). The sealed ampoule removes any possibility of contamination during storage and is also ideal for the distribution of the organism. Living cultures are susceptible to contamination and, if delay occurs during transit, may suffer deterioration or death. Both problems of viability and contamination are overcome by using freeze dried ampoules. At the Commonwealth Mycological Institute (CMI) freeze drying was first used to store fungi in 1966. There are now over 7400 different species freeze dried and over 120000 ampoules containing suspended fungi stored in the collection

METHODS

Fungi were grown in 1 oz universal bottles on a slope or plate of suitable agar medium to give maximum sporulation. Some fungi required special treatment to induce sporulation; for example, the addition of plant material or filter paper to media (Booth, 1971) or stimulation by near ultra-violet light (Leach, 1971)

Spore suspensions were prepared by adding approximately 10 ml of a sterile mixture of skimmed milk and inositol (10 g of Sainsbury's skimmed milk powder, 5 g of inositol and 100 ml of distilled water) to each culture and gently scraping the fungal colony. Aliquots (0·2–0·5 ml) of the suspension were placed in sterile 0·5 ml neutral glass ampoules (Anchor Glass, London) and covered with a sterile lint cap prepared by folding and stapling the edges of a rectangle of lint 100 mm × 120 mm; 15 replicates of each fungus tested were prepared. Alternatively, 2 mm squares were cut from a culture and placed into each of 15 ampoules and covered with a lint cap. The ampoules were centrifuged at 1425 r.p.m in an Edwards EF6 primary freeze dryer, and the chamber evacuated. After 15 min when the suspensions were frozen, as indicated by a deflection in the Pirani vacuum gauge, the centrifuge was switched off. After a further 3 h drying period the chamber was brought to atmospheric pressure, the ampoules removed and plugged with sterile cotton wool. This time interval during which the freeze dried material is exposed to oxygen and water vapour in the atmosphere was kept to a minimum as over exposure can cause deterioration (Rey, 1977). The plugs were compressed to 1 cm in depth and pushed down the ampoule just above the slope of the dried suspension. The ampoules were constricted using an air/gas torch about 1 cm above the top of the cotton wool plug, attached to the manifold of an Edwards 30S2 secondary freeze drying machine and evacuated over phosphorus pentoxide desiccant. This secondary drying stage was carried out overnight for a period of 17 h and reduced the residual moisture content to between 1 and 2%. While still evacuated the ampoules were sealed at the constriction and stored in an air-conditioned room maintained between 15 and 20 °C. After 3–4 days storage a test ampoule was opened and the contents reconstituted by adding 3–4 drops of sterile distilled water and allowing 15–20 min for absorption of the moisture. The contents of the ampoules were then streaked onto a suitable agar medium and incubated at a temperature appropriate to the organism for

periods of up to 14 days or longer. Viability was determined by the resumption of normal growth

An alternative method using 2 stage cooling and vacuum drying was used in some tests. The spore suspension was cooled to $-35°$ (determined by a resistance thermometer) in the vapour phase of a liquid nitrogen refrigerator (LR256 Union Carbide) at a rate of 1°/min and then plunged into liquid nitrogen at $-196°$. Prefrozen samples were then transferred to the centrifuge racks and into the chamber of the Edwards EF6 freeze dryer, evacuated and dried as described above.

RESULTS

A list with a taxonomic breakdown of the isolates maintained in the CMI collection with their percentage success is given in Table 1. It shows that many Zygomycotina, Ascomycotina and Deuteromycotina survive this storage technique. Zygomycetes and Hemiascomycetes showed survivals of over 90% whereas Pyrenomycetes did not survive as well. The proportion of isolates in the Basidiomycotina survived even less well (40–79%) and no survival was achieved with the small number of isolates from the Mastigomycotina tested.

Table 2 summarizes the numbers of genera, species and isolates that have been tested between 1966 and 1981 From the 8198 isolates that were freeze dried 7479 survived, a percentage success of 91.

Table 2. *Summary of isolates processed by centrifugal freeze drying*

No. of	Tested	Viable	Viability (%)
Genera	783	717	92
Species	3187	2891	91
Isolates	8198	7479	91

Table 3 shows a small sample of cultures of Mastigomycotina, Zygomycotina, Ascomycotina and Deuteromycotina, which were treated by 2 stage cooling before vacuum drying. Most gave improved results with this new technique. Some isolates were chosen to represent cultures that normally survive centrifugal freeze drying, e.g. *Cunninghamella*, *Chaetomium* and *Coprinus*, and some to represent those that had proved difficult or impossible to process. Although isolates of *Hypoxylon* have survived the centrifugal method, *Hypoxylon fragiforme* (IMI 192581) gave poor sporulation after treatment, but this was improved by the 2 stage cooling technique. *Cercospora* spp. and *Ceratocystis* spp. had only survived after special treatments to ensure good sporulation prior to processing and several attempts were made before

Table 1. *Viability of isolates after centrifugal freeze drying*

	No of genera	No of species	No of isolates		
			Tested	Viable	Viability (%)
MYXOMYCOTA					
Acrasiomycetes	1	2	4	4	100
Myxomycetes	0	0	1	0	0
EUMYCOTA					
Mastigomycotina					
Chytridiomycetes	0	0	6	0	0
Oomycetes	0	0	29	0	0
Zygomycotina					
Zygomycetes	48	222	821	754	92
Ascomycotina					
Hemiascomycetes	19	41	68	64	94
Plectomycetes	51	162	277	255	92
Pyrenomycetes	93	413	904	761	84
Discomycetes	32	58	136	116	85
Loculoascomycetes	8	18	78	32	41
Basidiomycotina					
Teliomycetes	7	14	24	19	79
Hymenomycetes					
(*a*) Phragmobasidiomycetidae	2	7	13	8	62
(*b*) Holobasidiomycetidae	17	24	79	33	42
Gasteromycetes	2	2	5	2	40
Deuteromycotina					
Hyphomycetes	320	1565	5091	4822	95
Coelomycetes	106	302	576	526	91

Table 3 *Isolates frozen by 2 stage cooling (1°/min to −35° and then plunged into liquid nitrogen at −196°C) before vacuum drying*

	IMI number	Class	No of tests	Survival periods: 1 week	3 months	6 months
Basidiobolus ranarum	89715	Zygomycetes	4	+ + + −	+ + + −	+ + + −
Ceratocystis paradoxa	77668	Pyrenomycetes	2	+ +	+ +	+ +
Cercospora xanthosomatis	179717	Hyphomycetes	3	+ + +	+ + +	+ + +
Chaetomium globosum	16203	Pyrenomycetes	3	+ + +	+ + +	+ + +
Coniophora puteana	79127	Holobasidiomycetes	2	sss	sss	sss
Coprinus hexagonosporus	161417	Holobasidiomycetes	3	+ + +	+ + +	+ + +
Cunninghamella elegans	200332	Zygomycetes	3	+ + +	+ + +	+ + +
Hypoxylon fragiforme	192581	Pyrenomycetes	2	+ +	+ +	+ +
Macrophomina phaseolina	179649	Coelomycetes	3	+ + +	+ + +	+ + +
Phytophthora palmivora	202528	Oomycetes	3	+ + −	+ + −	− − −
Pythium sylvaticum	248394	Oomycetes	3	− − −	− − −	− − −
P sylvaticum	248395	Oomycetes	3	− − −	− − −	− − −
Saprolegnia parasitica	169621	Oomycetes	3	− − −	− − −	− − −

+ −, Grew and sporulated well, 2 ampoules were opened for each recorded result
s−, Grew well but no sporulation as in the original culture from which it was freeze dried
− −, Did not survive If a negative result occurred all ampoules were opened and later test dates were recorded as negative, 10 ampoules were opened in each case
A symbol is given for each test result after revivals for the 3 periods of storage

Table 4 *Survival periods of isolates centrifugally freeze dried on agar disks without suspending medium*

Isolate	IMI no	Date freeze dried	Last date tested	Maximum survival period (yr)
Acremonium sp	55286	8. 1 67	4 1 82	14
Ascocoryne sarcoides	68130	9 8 67.	5 11 81	14
Aspergillus amstelodami	71295	18 10 67	17 4 75	8*
A candidus	73074	18 10 67	4 1 82	14
A carneus	73777	19 10 67	4 1 82	14
A nidulans v *echinulatus*	614541i	28 6 67	22 8 81	14
A niger	753531i	5 9 67	5 11 81	14
A quadrilineatus	72733	12 10 67	4 1 82	14
A ostianus	93445	23 10 67	4 1 82	14
Chaetomium abuense	114513	19 9 67	5 11 81	14
Curvularia trifolii f sp *gladioli*	75377	16 1 68	4 1 82	13
Cylindrocarpon congoense	69504	6 9 67	5 11. 81	14
Embellisia chlamydospora	67737	25 7 67.	30 10. 81	14
Fusarium graminearum	69695	6 9 67	5 11 81	14
Nectria gliocladioides	71095	19 10 67	4 1 82	14
Paecilomyces dactylethromorphus	65752	25 7 67	30 10 81	14
Penicillum cyclopium v *echinulatum*	68236	26 7 67.	30 10 81	14
P. nigricans	96660	5 9 67	5. 11 81	14
P paraherquei	68220	26 7 67	30 10 81	14
P raperi	71625	18 10 67	4 12 80	13+
P roquefortii	129207	28 9. 67.	7 6 78	14
P spinuloramigenum	68617	26 7 67	29 10 81.	14
P steckii	72029	4. 10 67	4 1 82	14
Pestalotiopsis gracilis	69749	6 9 67	5 11 81	14
Phaeotrichoconis crotalariae	69755	6 9 67	5 11 81	14
Phialomyces macrosporus	110130	20 9 67	4 1 82	14
Phomopsis oncostoma	68344	9 8 67.	5 11 81	14
Pycnoporus sanguineus	75002	27 11 73	4 1 82	9
Sagenomella griseoviridis	113160	26 9 67	12 2 80	13+
Scopulariopsis carbonaria	86941	6 9 67	5 11 81	14
Sporidesmium flexum	246524	6 3 81	4 1 82	1

* Died after 8 years storage

Table 5. *Genera failing the centrifugal freeze drying technique*

Genus	No isolates tested	Genus	No isolates tested	Genus	No isolates tested
Achlya	3	*Herpotrichia*	1	**Pythium*	16
Allomyces	3	*Kretzschmaria*	1	*Quaternaria*	1
Areolospora	2	*Lacellinopsis*	1	**Saprolegnia*	2
Armillaria	3	*Lasiobolidium*	1	*Searchomyces*	2
Arthrocladium	1	*Lentinus*	1	*Selenosporella*	1
Ascocalvatia	1	*Lenzites*	1	*Selinia*	1
Balansia	2	*Leptoporus*	1	*Sigmoidea*	1
Battaraea	1	*Lomachashaka*	1	*Sphaerobolus*	2
Biscogniauxia	1	*Marasmius*	1	*Sphaerostilbe*	2
Blastocladiella	1	*Melanconis*	2	*Spondylocladiopsis*	1
Calospora	1	*Monotosporella*	1	*Stereum*	2
Camposporium	5	*Nummularia*	3	*Sympodiella*	2
Chytridium	1	*Panus*	1	*Syzygites*	1
Cladobotryum	1	*Penicillifer*	2	*Tetracladium*	1
Coriolus	1	*Phaeoisariopsis*	2	*Tetranacrium*	1
Dactuliophora	1	*Phyllosticta*	1	*Umbelopsis*	1
Eleutherascus	1	*Physarum*	2	*Urohendersonia*	1
Entomophthora	2	**Phytophthora*	28	*Ustilaginoidea*	1
Eremomyces	1	*Piedraia*	1	*Ustulina*	6
Fomes	2	*Platystomum*	1	*Volvariella*	2
Ganoderma	2	*Puccinia*	3		

* Genera tested by the 2 stage cooling-vacuum drying method.

success was achieved. It was possible to freeze dry poor sporulating isolates by the 2 stage cooling method. Isolates of *Basidiobolus* and *Coniophora* were usually difficult to freeze dry and success depended upon the condition of the isolate in culture; good growth and sporulation normally gave successful results. The isolates chosen had given poor results when freeze dried by the centrifugal technique but survivals were improved by the 2 stage cooling method *Phytophthora palmivora* had not previously survived freeze drying, but when it was processed by the 2 stage cooling technique it survived for 3 months despite its storage in vials in the presence of oxygen and water vapour. These are amongst the most reactive and dangerous contaminants (Rey, 1977) and normally cause very rapid deterioration. *Pythium sylvaticum* and *Saprolegnia parasitica* did not survive either freeze drying technique.

Table 4 lists the isolates that survived freeze drying without a suspending medium. They represent the Hyphomycetes, Ascomycetes and Coelomycetes and have all survived the process with only the isolate of *Aspergillus amstelodami* dying during storage.

Only 62 of 783 genera freeze dried by the centrifugal method failed to provide any successful isolates. A list of these genera has been given in Table 5 with the number of isolates tested in each. Not all genera were tested extensively. For 37 of those listed only one representative was tested, although 28 isolates of *Phytophthora* and 16 of *Pythium* were attempted. Of those that failed, each isolate was tested at least twice (20–30 ampoules)

DISCUSSION

Freeze drying has proved to be a valuable preservation method for sporulating fungi; of the major subdivisions only the representatives of the Mastigomycotina tested did not survive at all. Also some of the non-sporulating cultures belonging to the Basidiomycetes show quite good viabilities after processing Unfortunately due to the large numbers of fungi involved only a summary of the results in the form of a taxonomic list (Table 1) could be included here although a full list of the species tested is available from CMI (Smith, 1982*b*)

Spores, sclerotia and resting mycelium were probably the parts of the fungus that survived the process. These have lower moisture contents than the mycelia. Cells with higher water contents tend to be more susceptible to freezing damage (Meryman, 1966). Many attempts have been made to improve the method by changing the freezing procedure of the freeze drying process and these have often proved successful with the fungi that do not survive the evaporative freezing of the centrifugal method. For example, some fungi have survived the liquid nitrogen freezing methods described by Clark & Loegering (1967), Heckly (1978) and Smith (1982*a*). Although repeated

attempts were made to freeze dry some of the recalcitrant cultures the centrifugal method did not always prove successful. Staffeldt & Sharp (1954) also modified the method for freeze drying *Pythium*, pre-freezing infected plant material to −45° and then vacuum drying at room temperature. The pre-freezing and rehydration, which was carried out in a controlled environment, improved the viabilities of *P. acanthicum* and *P. irregulare*. Successful storage of *Puccinia* urediniospores also necessitated freezing to between −45° and −50° before drying under vacuum at −10° (Sharp & Smith, 1952).

At CMI a 2 stage cooling, vacuum drying technique was developed for the preservation of those fungi that failed the centrifugal method, a similar technique which proved successful as a preliminary to liquid nitrogen storage preservation (Smith, 1982*a*). Only one of the Oomycetes subjected to the method survived, though failure may have been due to the adverse storage conditions. It is likely that the successful freeze drying of some fungi will depend upon the production of suitable freezing steps prior to the drying under vacuum.

In contrast some fungi will survive freeze drying almost regardless of the technique. Most certainly they do so without suspending media that may offer protection during the process. Two genera that belong to this category are *Penicillium* and *Aspergillus*. In conclusion it can be seen that freeze drying is a most useful long term storage technique that keeps fungi stable, a most important factor when maintaining any collection of micro-organisms.

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Table 1 *Extracellular enzymes, associated with bacterial breakdown, assayed in the culture supernatants of fungi growing on killed* B subtilis *in Treschow basal salts medium*

Organism	Incubation (days)	Final supernatant pH[a]	Total dry weight loss in cultures(%)	βNAGase (activity[b] units ml^{-1} of culture supernatant)	βNAGALase (activity[c] units ml^{-1} of culture supernatant)	Protease activity[b] (ml^{-1} of culture supernatant)		
						Acid (pH 3 8)	Neutral (pH 7 0)	Alkaline (pH 9 1)
			Ascomycetes					
Chaetomium globosum Kunze	21	8 9	29 71	16 14	1 62	0 46	0 30	1 06
Diehliomyces microsporus (Diehl & Lambert) Gilkey	40	4 7	3 50	0	0	—*	—	—
Neurospora tetrasperma Shear & Dodge	8	8 5	58 37	11 06	6 11	3 33	52 16	56 33
Sordaria fimicola (Rob.) Ces & de Not	30	8 8	31 24	14 73	2 27	0	0	0
			Basidiomycetes					
Agaricus bisporus (Lange) Imbach D621	44	7 6	51 26	14 70	—	10 0	37 0	13 0
Agaricus macrosporus (Møll & Schaff.) Pilat	40	4 6	11 95	—	0	0	0	0
Favolus arcularius (Fr) Ames	32	5 2	62 38	3 86	2 00	7 19	16 29	1 69
Flammulina velutipes (Curt · Fr) Karst	30	7 8	71 57	7 52	12 83	7 09	17 29	15 02
Lentinus lepideus (Fr. Fr) Fr	40	4 4	23 94	—	—	4 15	0 15	0
Pleurotus ostreatus (Jacq. Fr) Kummer	21	8 3	74 75	9 27	16 44	13 31	15 83	13 09
Schizophyllum commune Fr	21	7 0	49 56	0 41	0	8 82	7 80	2 83
Stropharia merdaria Fr	30	8 7	64 02	0 70	0 54	0	3 76	1 06
Volvariella volvacea (Bull. Fr) Sing	32	6 1	24 29	0 02	0	0	7 06	5 10
			Fungi imperfecti					
Aspergillus niger van Tieghem	27	7 5	35 00	41 40	25 57	4 68	0 72	0
Penicillium sp.	27	6 7	57 18	37 82	18 79	4 69	6 77	5 20
			Phycomycetes					
Mucor hiemalis Wehmer	10	8 6	55 90	1 74	1 50	0 85	11 06	21 65
Phycomyces blakesleanus Burgeff, (+) IMI 118491	21	8 4	42 70	2 31	1 68	1 00	2 01	0 92

* No observation made

[a, b, c] Average of 4, 3 and 2 replicates, respectively

attack the different fractions of such a complex substrate It is probable that the fungi produce other enzymes capable of degrading bacteria but they have not yet been sought. It must be mentioned here that the observed fungal growth was entirely dependent on insoluble bacterial polymers. The whole bacteria had been killed, washed and extracted with ether to remove the soluble cell contents (which would have been a readily available food source for fungal mycelium), and to prevent bacterial autolysis.

It is thought that this is the first report of βNAGase and βNAGALase having been obtained from several of the fungi screened here, particularly the Basidiomycetes. Intracellular βNAGase and βNAGALase were first shown in extracts from two fungi, *Aspergillus oryzae* (Ahlburg) Cohn and *Lycoperdon giganteum* Pers (Woollen, Walker & Heyworth, 1961). Otakara (1964) isolated an extracellular βNAGase from *A. niger* and Bahl & Agrawal (1969) purified and characterized βNAGase from the same species. Eriquez & Pisano (1979) detected both βNAGase and βNAGALase activity in culture supernatants of *Paecilomyces persicinus* Bain., after growth on soybean meal-corn meal medium. Several species of Fungi Imperfecti, *Aposphaeria*, *Aspergillus*, *Aureobasidium*, *Penicillium* and *Scopulariopsis* grown on synthetic media containing soy flour, were found to produce βNAGase activity (Ulezlo, Zaprometova, Ozerskaya & Bezborodov, 1980). In none of these studies has βNAGase been associated with breakdown of bacteria. It is not known whether βNAGase activity of the fungi in the present experiments was induced by the presence of bacteria, though such an induction has been shown for *A. bisporus* (Fermor & Wood, 1981). βNAGase and βNAGALase activities may be closely related, since both may reside in the same protein (Enriquez & Pisano, 1979). Woollen *et al.* (1961) concluded that where βNAGase was present it was accompanied by βNAGALase but the ratios of the two activities covered a wide range; this was precisely the situation found here

As would be expected fungi have been shown to be capable of degrading artificially killed bacteria It is difficult however, to relate the results of *in vitro* experiments to field conditions though the degradation of bacteria by fungi in soil has been observed (Bohlool & Schmidt, 1973) By using immunofluorescence techniques Bohlool and Schmidt demonstrated the degradation of *Rhizobium japonicum* by an unidentified soil fungus Both immunofluorescence and radiolabelling are being used in our current experiments to determine the fate of bacteria in composted wheat straw.

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CRYOPROTECTANTS AND THE CRYOPRESERVATION OF FUNGI

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The freezing and storage of fungi in or above liquid nitrogen is now a well-established preservation technique Though different cooling protocols have been used (Heckly, 1978) it is common practice to suspend the fungi in a cryoprotective solution to improve their viability after cryopreservation. The

Table 1. *Comparison of cryoprotectants used in the freezing and liquid nitrogen storage for 6 months of 16 test fungi*

(Results are expressed as the number of ampoules giving good recoveries over the total number opened for each isolate)

	10% PVP	5% Glu	10% Glu	5% DMSO	10% DMSO	5% Glu +10% DMSO	8% Glu +10% DMSO	10% Glu +10% DMSO	10% Glu +5% DMSO	10% Glu +8% DMSO	10% Glu +15% DMSO	10% Gly	None
Aspergillus carbonarius (186307)	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
Cercospora xanthosomatis (179717)	4/4C	0/8	0/8	0/8	3/4	4/4	4/4	4/4S	4/4S	4/4	4/5S	3/3	0/8
Corynespora cassiicola (56007)	0/6	o	o	o	3/4	3/4	4/4	4/4	4/4	o	0/6	4/4	0/6
Cunninghamella elegans (200332)	5/5C	0/7	3/4	4/5	4/4	4/5	4/5	4/4	4/4	3/4	0/8	4/4	4/4
Diplosclerophoma ceratoniae (180575)	0/5	o	0/6	o	4/5	o	4/4	o	o	o	o	4/5	0/6
Drechslera sp (181085)	4/4	o	3/3	o	4/4	o	4/4	o	o	o	o	4/4	0/8
Emericellopsis terricola (68332)	3/4	0/5	0/5	5/5S	3/5	4/4	4/4	4/4	3/4	5/5	0/5	4/4	0/6
Entomophthora exitialis (155908)	o	0/8	0/8	0/7	0/7	0/6	3/4	3/5	3/4	1/8	0/8	0/7	0/8
Myxotrichum thaxteri (83470)	0/6	o	0/6	o	2/3	o	4/4	o	o	o	o	4/4	0/7
Penicillopsis clavariiformis (60372)	5/5C	o	4/6	o	4/4	4/4S	4/4	4/4	5/5S	0/5	0/6	4/4	0/5
Phycomyces blakesleeanus (118496)	6/6C	o	4/4	o	4/4	o	4/4	o	o	o	o	4/4	0/8
Phytophthora palmivora (202528)	0/6	0/6	0/6	0/5	0/6	0/6	4/6W	o	4/5W	1/6	0/6	4/5	0/5
Pseudophaeolus baudonii (ODA 641)	o	o	0/5	o	0/4	0/5	3/6	3/6	0/5	0/6	0/5	0/4	0/5
Pythium sylvaticum (248394)	0/5	0/6	0/6	0/6	0/6	3/4W	4/4	4/4	0/6	0/6	0/6	4/4	0/6
P sylvaticum (248395)	0/6	0/6	0/6	0/6	0/6	0/5	3/6	0/6	0/6	0/6	0/6	0/6	0/6
Wallemia sebi (86292)	7/7*	4/4	3/3	4/4	4/4	4/4	4/4	4/4	4/4	4/4	0/6	4/4	1/6
Fraction of isolates surviving	7/14	2/9	6/15	4/9	11/16	8/12	16/16	10/11	9/12	5/11	2/12	13/16	2/16
Fraction of ampoules giving positive results	38/73	8/54	21/80	17/50	59/74	30/55	61/71	38/49	35/55	22/58	8/71	51/70	9/94

o, Not tested.
C, Normal growth but contaminated.
W, Weak growth – sparse growth of mycelium
S, Grew but had lost the ability to sporulate
* The fungus was present on each test but was overgrown by a contaminant

mechanisms by which these cryoprotectants offer protection have been reviewed by Meryman (1966), Calcott (1978) and Heckly (1978). It is thought that the cell membrane is the primary site of injury (Mazur, 1970) due to solution concentration and ice crystal effects occuring during cooling

Many cryoprotectants have been used for the cryopreservation of living cells including dimethyl sulphoxide (DMSO) (Hwang & Howells, 1968, Barnhart & Terry, 1971), polyvinyl-pyrrolidone (PVP) and dextran (Ashwood-Smith & Warby, 1971), ethylene glycol, propylene glycol (Lovelock, 1954), acetamide and urea (Keane, 1953). Solutions of glycerol are more commonly used; a 10% concentration has proved most useful at the Commonwealth Mycological Institute (CMI) for the cryopreservation of fungi (Onions, 1971; Smith, 1982). Mixtures of cryoprotectants have also been used, for example, Daily & Higgens (1973) used 10% glycerol and 5% of either, lactose, maltose or raffinose for some bacteria and algae, and Finkle & Ulrich (1979) used DMSO and glucose for the storage of sugarcane cells. Following the latter success, it was decided to evaluate cryoprotectant mixtures in the freezing of fungi.

This article reports on the viability of some test fungi when preserved in liquid nitrogen using some of these cryoprotectants. Suspensions of the test fungi (Table 1) were made by adding 10 ml of the cryoprotectant to a culture growing in a universal bottle and bringing the mycelium or spores into suspension by gentle agitation and scraping of the fungal colony. Aliquots (0 5 ml) of this suspension were added to each 1 ml borosilicate glass ampoule which was then heat sealed. Controls were prepared by sealing fungi, on agar blocks (2 mm square), or spores or mycelia scraped from cultures, in ampoules without any suspending media All seals were tested by immersing the ampoules in an erythrocin B dye bath at 4–7 °C to precool the fungal suspensions. The fungi were subsequently cooled at 1°/min to −35°, plunged into liquid nitrogen at −196° and stored in the liquid/vapour phase of a Union Carbide LR320 refrigerator (Smith, 1982).

Test ampoules were removed from the liquid nitrogen refrigerator and thawed by immersion in a water bath at 37°. They were opened and the viability checked by streaking the contents onto a suitable agar medium. If the cultures were revived after both 4 days and 6 months storage the procedure was considered successful.

To determine if the mixture of 8% glucose and 10% DMSO had any toxic effects on the growth of the some of the test fungi they were left in contact with the cryoprotectant mixture for a period of 24 h. No loss in viability was observed when subcultures were taken from the suspension and regrown on fresh media. It was therefore considered unnecessary to wash away or dilute the cryoprotectant after thawing prior to growing the fungi for viability tests.

Twelve cryoprotectants or cryoprotectant mixtures were used as suspending media in the preparation for freezing 16 test organisms (Table 1). Similar results were obtained for each fungus after both 4 days and 6 months. *Aspergillus carbonarius* and *Cunninghamella elegans* alone survived without any suspending medium *A carbonarius* also survived all other treatments whereas *C. elegans* did not survive when 5% glucose or a mixture of 10% glucose and 15% DMSO were used as the cryoprotectants. The cryoprotectant allowing the survival of most species was the mixture of 8% glucose and 10% DMSO, both higher and lower concentrations of the glucose and the DMSO in the mixture gave fewer survivors. The mixture of 10% glucose and 15% DMSO was the least successful of all treatments.

Although the mixture of 8% glucose and 10% DMSO gave better survival of the test organisms than 10% glycerol it is not used as the routine cryoprotectant for liquid nitrogen storage at CMI However, it is now common practice to use the mixture if the fungus fails to survive the routine method The reason for this choice is that DMSO is a harmful substance when it comes in contact with the skin or eyes, or if taken internally.

Hwang & Howells (1968) have shown that DMSO is a better cryoprotectant than glycerol with some fungi. Experiments reported here have shown that 10% DMSO was more successful than 5% DMSO but that 10% glycerol allowed more species to survive. However, the viabilities, as expressed by the fraction of positive ampoules from those opened, were greater for the 10% DMSO than for the 10% glycerol, suggesting it to be more

Table 2. *Viability of isolates suspended in 8% glucose/10% DMSO*

		Period of treatment			
	IMI no	0	1 h	5 h	24 h
Entomophthora exitialis	155908	+	+	+	+
Pythium sylvaticum	248394	+	+	+	+
P sylvaticum	248395	+	+	+	+

Viability was assessed by incubation on agar media for 7–14 days at room temperature

† Resumption of growth after inoculation onto fresh media

consistant in its protection. Mixtures of glucose and DMSO were shown by Finkle & Ulrich (1979) to be effective as cryoprotectants for sugar cane cells and have been shown here as useful in the storage and protection of fungi.

Use of 8% glucose/10% DMSO as a cryoprotectant has facilitated the preservation of some species of *Entomophthora* and *Pythium* that would otherwise not have survived the CMI liquid nitrogen storage technique.

Protection of cells during freezing seems to be enhanced by holding at subzero temperatures. This appears to cause shrinkage that reduces the probability of intracellular ice formation on further cooling to −196° (Morris, 1978) This, and the effect of the cryoprotectant on the size and structure of ice crystals, may be involved in the protection from cryo-injury of the fungi tested; however, the holding period at subzero temperatures here was very short. The time lapse between the fungi reaching −35° and plunging into liquid nitrogen was, in all cases, less than 15 minutes.

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HOMING ON BASIDIOSPORES AND PRODUCTION OF OIDIA IN *TUBARIA FURFURACEA*

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In connexion with an interest in winter fungi, I had occasion to look at spore germination in *Tubaria furfuracea* (Pers.: Fr.) Gillet Sporophores were collected near Benson in February 1982. Light spore deposits were allowed to form on 0 2% malt agar in open Petri dishes from pilei placed a few centimetres overhead for about 20 min. Each deposit, which covered an area of 1–2 cm², contained many thousands of spores In making observations, a square of agar with the spore deposit was cut out, placed on a slide and, without added water, covered with a cover-slip The undisturbed deposit could then be examined microscopically.

After several days at room temperature less than 0 1% of the spores had germinated, and, although during the next 2 weeks a few more produced germ-tubes, the percentage never rose above 0 1%. At first only unclamped mycelium was formed from the germination of individual spores, but soon, no doubt following mating between compatible monokaryons, a faster-growing mycelium was produced with clamp-connexions and a different pattern of branching.

A few of the hyphae of both the clamped and the unclamped mycelium approached, contacted and fused with ungerminated basidiospores (Fig. 1). Some of the leading hyphae of the clamped mycelium appeared to have departed markedly from their straight, forward growth to fuse with spores. However, at the end of 2 weeks the tapped spores greatly exceeded the number of germinated

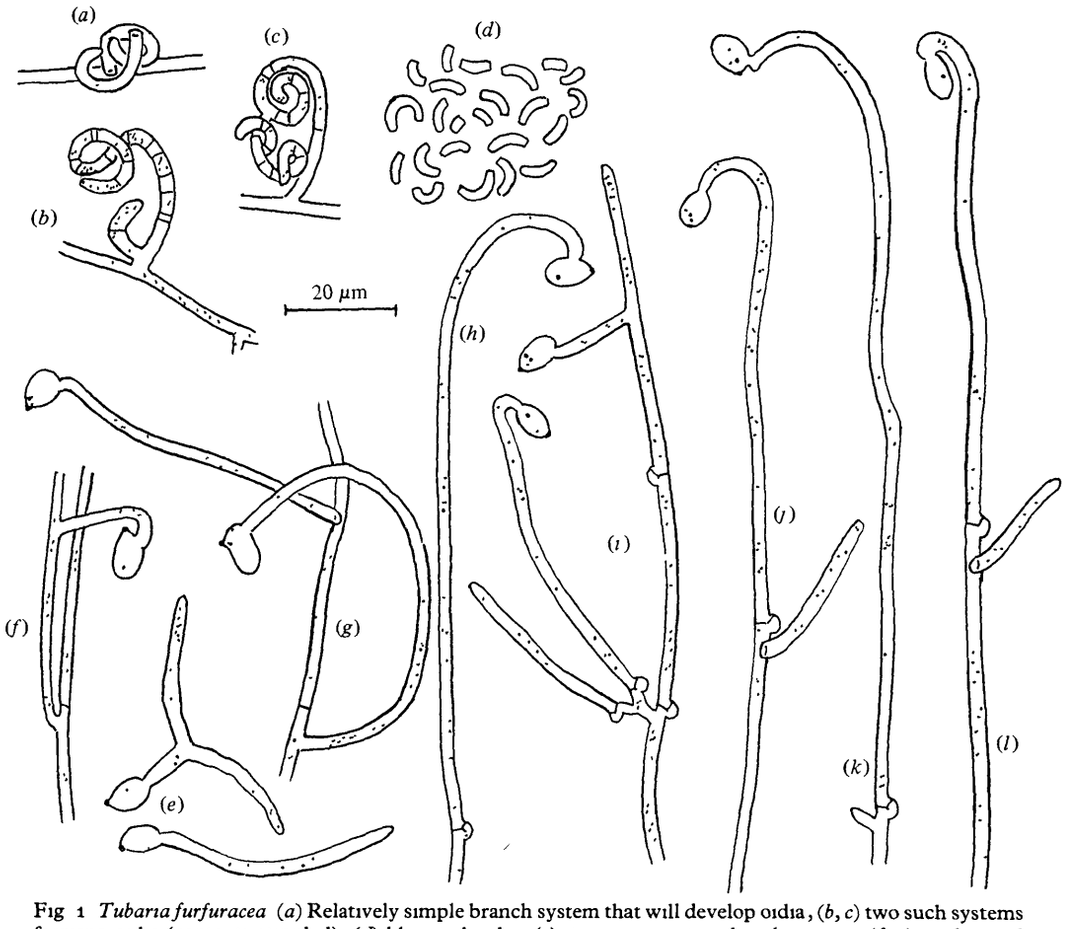

Fig 1 *Tubaria furfuracea* (*a*) Relatively simple branch system that will develop oidia, (*b, c*) two such systems forming oidia (contents stippled), (*d*) liberated oidia, (*e*), two germinating basidiospores, (*f, g*) unclamped mycelium tapping basidiospores; (*h–l*) clamped mycelium tapping basidiospores.

spores, amounting to over 5% of the total. Following contact and fusion, a basidiospore changed in appearance by losing most of its contents, although a few small oil drops usually remained.

The contacting of basidiospores by hyphae in *T. furfuracea* and the frequent curvature of the hypha around a spore before fusion, strongly recalls the homing of hyphae on oidia (Kemp, 1970). Again there is a striking similarity with the situation reported by Fries (1981) in which hyphae of *Leccinum aurantiacum* S F. Gray home on the germination vesicles produced by basidiospores of the same and related species However, in *T. furfuracea* homing is on the ungerminated basidiospore

By the end of two weeks, oidia (arthroconidia) were formed in dense, sessile clusters on the unclamped mycelium They tended to be C-shaped (Fig. 1*d*) due to having developed by the fragmentation of rather elaborate branch systems in which the individual hyphae were in the form of tight helices or solenoids. Relatively simple examples of this branching are illustrated in Fig. 1*a–c*. However, most were much more complex.

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A COMPARISON OF SOME PRESERVATION TECHNIQUES FOR FUNGI

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Of 58 fungi covered in mineral oil in 1950, 47 were viable after 32 years storage and 7 of those not surviving the full period remained viable for 20 years or more. Oil storage remains a useful alternative to liquid-nitrogen storage for isolates of *Phytophthora*, *Pythium*, basidiomycetes and mycelial forms which do not readily survive freeze-drying.

Storage in anhydrous silica gel was compared with oil, freeze-drying and liquid-nitrogen storage techniques as a cheap alternative. More stable and consistent growth was achieved after storage in silica gel than under oil though the range of fungi surviving was much narrower. Although some of the plectomycetes, hyphomycetes and zygomycetes tested survived 11 years in silica gel in good condition, the technique was not as successful as freeze-drying or liquid-nitrogen storage but was considered to be a useful alternative for laboratories with limited resources or those requiring a simple technique.

The main methods of culture storage of fungi at the Commonwealth Mycological Institute (CMI) are now freeze-drying and storage at the ultra low temperatures of liquid nitrogen. Thousands of isolates are preserved by these methods and reports on viability have been given by Smith (1982, 1983). Originally cultures were maintained by frequent transfer but after a preliminary trial in 1950 the collection was transferred to oil storage in 1955. The method was used as the only extended storage method until the introduction of freeze-drying in 1966 and liquid-nitrogen storage in 1968.

Keeping fungal cultures under a layer of mineral oil has been the method used for many years to reduce growth and extend the time interval between transfers to fresh media (Buell & Weston, 1947; Fennell, 1960). Problems of such continuous growth techniques have been discussed by Onions (1971) and Heckly (1978). The dehydration caused by silica-gel storage, freeze-drying and the ultra low temperatures of liquid nitrogen overcomes some of these difficulties by suspending metabolism (Onions, 1983). The costs of the latter two techniques are often prohibitive, making preservation using silica gel as a desiccant more attractive. In 1971 an investigation of a silica-gel storage technique as a simple and inexpensive method of preservation was started.

At the Second International Conference on Culture Collections in 1973, and published as part of the proceedings, Onions (1976) reported on the survival of the fungal cultures preserved at the CMI in mineral oil in 1950, and also gave a preliminary report on preservation by means of silica gel. The results were compared with the survival of the same isolates preserved by means of freeze-drying and liquid-nitrogen storage.

This paper describes the methods of storage for fungal cultures at the CMI and the results of a further ten years' storage by those methods, and compares:

(I) the results from storage of the original cultures placed in oil in 1950,

(II) the results from the cultures stored in silica gel between 1971 and 1972 (10–11 years),

(III) the results from storage of isolates using additional material preserved in silica gel between 1972 and 1982, and compares the viabilities of the same isolates preserved by other means including oil storage, freeze-drying and liquid-nitrogen storage.

METHODS

Growth of cultures

The fungi were grown on slopes of suitable agar in 1 oz universal bottles and incubated at optimum growth temperatures. Some fungi required special treatment to induce sporulation. The addition of plant material or filter paper to media (Booth, 1971) was required by some species and stimulation by near ultraviolet light (Leach, 1971) by others.

Oil storage

Twice-autoclaved (121 °C for 15 min) mineral oil (paraffin specific gravity 0·830–0·890) was added to mature healthy cultures grown in universal bottles without cap liners.

The culture and agar were completely covered to

a depth of 1 cm above the highest point to avoid the possibility of evaporation of moisture resulting in the culture drying out. The cultures under oil have been stored in an air-conditioned room at 15° for the last 10 years, but were previously held at room temperature.

Revival of the oiled cultures was achieved by removal of a small amount of fungal colony on a mounted needle and draining away as much oil as possible on the neck of the culture bottle The fungus was then streaked on to suitable agar medium and incubated at its optimum growth temperature.

Silica gel storage

The method used was similar to that of Ogata (1962) and Perkins (1962). As silica gel gives off heat on the addition of water the bottles, silica gel, suspending media and cultures were precooled to ensure that the temperature did not rise to a damaging level. Glass universal bottles were quarter-filled with non-indicating silica gel, 6–22 mesh, that had been heated to 180° for 3 h for sterilizing. The bottles were placed in a tray of water and frozen in a deep freeze (−20°). Precooled 5% skimmed milk was added to mature sporulating cultures on precooled (4–7°) agar plates or slants in universal bottles. The spores were released and brought into suspension by gently scraping the colony. The tray of frozen water containing the silica gel in universal bottles was removed from the deep-freeze 20–30 minutes before required. This allowed the ice to melt slightly around the bottles and facilitated their removal, whilst being a convenient method of dissipating the heat evolved. The silica gel was three-quarters wetted with the cooled spore suspension and agitated to ensure even distribution of the spores. The bottles were incubated at 25° with the lids loose for 10–14 days and when the particles of silica gel readily separated the lids were screwed down tightly. The bottles were then placed in air-tight plastic containers with an open bottle of indicator gel to absorb any free moisture, and stored at 4°.

Revival was achieved by sprinkling a few particles on to suitable agar medium and incubation at the optimum growth temperature for the fungus.

Liquid-nitrogen storage

When the culture had reached maturity, 10 ml of 10% glycerol was added and the mycelium and/or spores were brought into suspension by gentle agitation and scraping of the fungal colony. A small portion (0·5 ml) of this suspension was added to sterile 1 ml borosilicate glass ampoules which were then heat-sealed. The seals were tested by immersing the ampoules in an erythrocin-B dye bath at 4–7°, which also pre-cooled the fungal suspensions. These were frozen at approximately 1°/min by suspending in the vapour phase of a liquid-nitrogen refrigerator at −35° for 40–45 min; this was followed by rapid cooling to −196° by immersing the ampoules in liquid nitrogen. The ampoules were stored clipped to aluminium canes in boxes in a Union Carbide 250 l refrigerator or in the drawer rack system of a Union Carbide 320 l refrigerator.

For thawing and checking viabilities the ampoules were removed from the liquid-nitrogen refrigerator and immersed in a water bath at 37°. The ampoules were opened and the contents streaked on to a suitable medium. Viability was evaluated by growth of the cultures when revived after 4 days.

Freeze-drying

A spore suspension was prepared by adding 10 ml of either 10% skimmed milk or a 10% skimmed milk and 5% inositol mixture to a pure culture and gently scraping the fungal colony. Approximately 0·5 ml of this suspension was added to a sterile 0·5 ml neutral glass ampoule by means of a sterile pasteur pipette. Batches of 15 replicates were prepared for each fungus freeze-dried. Contamination whilst transferring the ampoules from a sterile filling area to the freeze-drying machine was prevented by covering the batches with sterile lint caps. The ampoules were placed in the centrifuge racks of an Edwards EF6 primary freeze-dryer, though the earliest (to 1971) freeze-drying at the CMI was carried out in an Edwards 5PS freeze-dryer. They were centrifuged at 1425 rev./min and the chamber evacuated. After approximately 15 min the suspensions were frozen by loss of heat during evaporation, indicated by a deflexion of the vacuum gauge as less vapour was evolved after freezing, and the centrifuge was switched off. The suspensions were dried for a further 3 h before the chamber was brought to atmospheric pressure, and the ampoules removed and plugged with sterile cotton wool. The plugs were compressed to 1 cm in depth and pushed down the ampoule to stop above the slope of the dried suspension. The ampoules were constricted about 1 cm above the top of the cotton-wool plug by using an air/gas torch. This stage was completed as quickly as possible because the period the freeze-dried material is exposed to oxygen and water vapour in the atmosphere must be kept to a minimum. Over-exposure can cause deterioration (Rey, 1977). The ampoules were then attached to the secondary stage. Those primary dried on the 5PS machine

underwent their secondary drying stage on the same machine with a secondary drying manifold. The secondary drying stage of the samples freeze-dried by using the EF6 was carried out on the manifold of an Edwards 30S2 secondary freeze-drying machine and continued for a further 17 h, which left a residual moisture content of the dried suspension of 1–2%. The ampoules were then sealed by means of an air/gas torch, and after 4 days storage an ampoule was opened and reconstituted by adding 3–4 drops of sterile distilled water from a Pasteur pipette and allowing 15–20 min for absorption of the moisture by the spores. The contents of the ampoule were streaked on to a suitable agar medium and the degree of growth evaluated.

RESULTS

Cultures examined after 32 years in mineral oil

Fifty-eight isolates were preserved under mineral oil in 1950 When tested in 1971, 51 of the isolates were reported to have survived 21 years storage and 7 had died during this period When they were tested in June 1982 a further 4 isolates had died but 47 isolates were still viable. Some isolates did not grow on the first streaking or gave poor growth so more than one attempt was necessary to obtain a healthy culture. The genera that survived 32 years without morphological change were *Aspergillus* (2 isolates), *Botryosphaeria* (2 isolates), *Ceratocystis* (3 isolates), *Chlamydomyces* (1 isolate), *Corticium* (2 isolates), *Drechslera* (1 isolate), *Eleutherascus* (1 isolate), *Helicodendron* (1 isolate), *Helminthosporium* (1 isolate), *Humicola* (1 isolate), *Nectria* (1 isolate), *Penicillium* (14 isolates), *Petriella* (1 isolate), *Phytophthora* (2 isolates), *Podospora* (3 isolates), *Rhizoctonia* (2 isolates), *Sclerotium* (1 isolate), *Scopulariopsis* (2 isolates), *Stephanosporium* (1 isolate), *Torula* (2 isolates), *Ustilago* (1 isolate), *Verticillium* (1 isolate) and *Volutella* (1 isolate). Two isolates of *Aspergillus*, and one isolate of *Corticium*, one of *Mucor* and three of *Penicillium* survived similarly for 20 years or more. Of those that died during storage, *Sporendonema casei* survived 17 years, *Mortierella alpina* and *Thielaviopsis basicola* 12 and an isolate of *Podospora fimbriata* 10 years (Onions & Smith, 1983) Of the 58 isolates only 4 were processed in silica gel, and one of these died after 4 years Only 9 were processed into liquid nitrogen and still survive. Thirty-six of the isolates were freeze-dried, of which 33 have survived well, 1 died after 10 years' storage, and 2 failed to survive Five of those not processed were of species (mostly Mastigomycotina and sterile mycelium) which do not normally survive freeze-drying (Onions & Smith, 1983)

Survival of isolates placed in storage in silica gel in the years 1971 and 1972 which survived the initial processing compared with survivals of the same cultures stored by other means

Table 1 lists 87 of the 88 isolates successfully processed in silica gel during 1971 and 1972. The remaining isolate successfully processed in 1972, *Fusarium oxysporum*, was an atypical strain and was discarded from the collection. Of the 87 isolates, 24 died during storage, 12 within 2 years of processing, and 12 after 4 years or more. Onions (1976) reported that 31 isolates out of 119 failed to survive the initial processing, a success rate of 74%.

Among the 24 original species that died between 1971 and 1982, 11 had other isolates processed between 1973 and 1981 that survived silica gel storage (Onions & Smith, 1983). The ability to survive does not appear to be species-limited. A comparison of the isolate's condition after recovery from the preservation techniques was made for the 87 isolates stored in silica gel from 1971 and 1972. Forty-two isolates were retrieved from silica gel in better condition than they were from storage under mineral oil, whereas 29 of the isolates gave poorer growth (Table 1) When recoveries from silica-gel storage were compared with those from freeze-dried specimens 9 isolates were in better condition whereas 35 were in poorer condition. Fifteen of the 32 isolates that were frozen and stored in liquid nitrogen were in better condition after recovery than they were after storage in silica gel, though 3 isolates survived storage in silica gel more successfully than in liquid nitrogen.

Survival of all isolates placed in storage in silica gel between 1971 and 1982 compared with survival under other methods of preservation

After initial success with silica-gel storage, more isolates were processed. By 1982, 426 had been processed, 118 failed or died during storage, and 308 survived (72% success). Eighty-five per cent of the plectomycetes (11 isolates), 81% of the hyphomycetes (202 isolates), 75% of the coelomycetes (21 isolates), 63% of the pyrenomycetes (35 isolates), 50% of the zygomycetes (20 isolates) and 52% of the Holobasidiomycetidae (10 isolates) survived the storage method (Onions & Smith, 1983) Of the 118 isolates which failed or died during silica-gel storage, 100 were freeze-dried, of which 11 failed and 89 survived, though 4 of these died during storage.

Only 41 of the isolates failing to survive silica-gel storage were frozen in liquid nitrogen. Thirty-nine of these have survived 2 or more years and are still viable. The two isolates that failed were *Chytri-*

Table 1. *Survival of isolates placed in storage in silica gel (SG) between the years 1971 and 1972 and tested in March 1982, together with survival periods when stored in mineral oil, by freeze-drying (FD) or in liquid nitrogen (LN)*

		Survival period (years)*			
	IMI no	SG	Oil	FD	LN
Allomyces arbuscula	152201	5D	7D	NT	11S
Alternaria alternata	89343	11S	20F	15G	NT
A. brassicae	151659	10F	8F	11P	NT
A. chlamydosporum	156426	10S	11S	10S	NT
Ascobolus viridulus	85125	10S	6D	7S	NT
Aspergillus avenaceus	16140	11G	10G	13G	NT
A candidus	127260	11S	11P	15S	NT
A citrisporus	25285	4D	11P	13G	NT
A giganteus	112341	10G	17G	10G	12G
A. nidulans	134679	11G	22G	15G	13G
A. nidulans v *echinulatus*	141181	11G	13F	14G	NT
A. niger	17454	11G	24G	14G	NT
A. ochraceus	162471II	2D	11G	11P	NT
A sejunctus	168779	10G	10F	9D	NT
A. wentii	162039	4D	11P	10G	NT
Aureobasidium pullulans	45533	11G	19F	15G	NT
Beltraniella humicola	155820	10G	11F	10G	9G
Botryotrichum piluliferum	49832	11G	24F	13S	NT
Byssochlamys fulva	163641	10G	10S	10G	10G
B nivea	163642	10G	10F	10G	10G
Ceratocystis ulmi	147188	2D	9P	1S	NT
Chaetomium cuniculorum	155487	10F	11F	10S	9F
C elatum	17424a	10S	20P	11G	NT
C globosum	16203	11S	11P	14G	NT
Coemansia pectinata	142377	2D	10P	9F	10G
Colletotrichum dematium	80025	10G	21S	4P	7G
C dematium f sp *spinaciae*	156656	10F	11G	12P	10G
C. gossypii	82269	2D	5ST	12S	12F
C musae	83256	10G	10P	10G	NT
C. tabacum	54048	4D	10S	NT	NT
C. truncatum	86431	1D	21S	5F	NT
C. typhae	86896b	10G	10S	10P	NT
Coprinus hexagonosporus	161417	10S	10ST	10G	10S
C. utrifer	161422	10P	11D	10S	10ST
Curvularia lunata v *aeria*	96846	10F	13F	11P	11F
Endothia parasitica	59815	10F	28P	15S	NT
Eremascus fertilis	86727	11G	20F	5G	NT
Fusarium solani	68412	11G	25S	15G	NT
F. solani	76761	1D	23D	15G	NT
Gelasinospora cerealis	76253a	10G	23ST	9G	NT
Gliocladium roseum	101020h	10P	19S	12G	NT
Heterocephalum aurantiacum	131684	8D	12P	12G	14G
Humicola grisea v *thermoidea*	126329	10G	15D	15P	13G
Isaria cretacea	159339	10G	10F	10G	2G
Metarhizium anisopliae	98375	< 1D	13P	10G	NT
Micromonospora vulgaris	126892	5D	15P	11S	NT
Mucor hiemalis (+)	21216	11G	19F	15G	NT
M. hiemalis (−)	21217	11G	20G	13G	NT
Mycelophthora thermophila	158756	11G	1D	11G	NT
Neurospora crassa	19419	5D	12S	6S	NT
Paecilomyces variotii	108007	10G	18S	12G	NT
Penicillium baarnense	40590	11P	25ST	14S	12F
P brefeldianum	153725	10P	11S	3S	10S
P. chrysogenum	26210	11G	25F	4G	NT
P claviforme	44744	11G	25ST	15G	NT

Table 1. (*cont.*)

		Survival period (years)*			
	IMI no.	SG	Oil	FD	LN
P. corylophilum	101082	2D	19S	11P	NT
P. lavendulum	40570	1D	27F	15G	NT
P. luteum	95152	11S	20S	10S	10F
P notatum	15378	11G	24S‡	6G	11S
P. wortmanni	40047	10S	15ST	10S	10G
Pestalozziella parva	124039	10F	10G	10G	10S
Peziza ostracoderma	60977	10S	25D	10G	NT
Phoma herbarum v *medicaginis*	156652	10S	10D	5G	9G
P violacea	499481	11G	15S	14G	3G
Phycomyces blakesleeanus (+)	118496	5D	12P	8G	NT
P. blakesleeanus (−)	118497	11D	11P	12G	NT
Piptocephalis virginiana	709101	11G	9S	7G	12S
P xenophila	156650	4D	10P§	5P	11G
Polystictus sanguineus	75002	11G	18F	12G	NT
Pyrenophora graminea	129760	< 1D	15ST	10D	NT
Rhizoctonia solani	20697	1D	32P	NT	5S
Rhizopus homothallicus	89714	11G	20G	11G	10G
Rhodotorula rubra	38784	1D	12G	5G	NT
Saccharomyces cerevisiae	140023	11P	13P	13G	NT
Sarcina lutea	44915	10F	8P	15G	NT
Schizophyllum commune	892951	10F	13P	7P	8G
S radiatum	90347	11S	20G	7S	NT
Seiridium sp	151978	8D	11F	10P	NT
Sordaria fimicola (+)	105390	11F	17G	15F	12G
S. fimicola (−)	105391	11G	11G	6G	NT
Sporormiella intermedia	148830	11G	12S	12F	NT
Stachybotrys atra	82021	11S	22F	13F	NT
Thermoascus crustaceus	158741	11G	1D	11G	NT
Thielavia albomyces	126326	10G	15S	10G	NT
T. terricola	104951	11GST	18F	4S	8G
Trichoderma viride	57421	5D	25F	4S	NT
Verticillium dahliae	81822	11F	22S	8S	NT

Key. D, Died after the given successful storage period; F, fair, growth and sporulation restricted; G, good growth and sporulation, NT, not tested, P, poor, deteriorated, very little growth, S, satisfactory growth and sporulation, ST, sterile, no sporulation but satisfactory to good growth

* The fungi that have been tested were processed on different occasions and consequently longevities cannot be compared directly for the isolates that are still viable Different methods of preservation have been introduced at CMI over the years and the maximum storage period for oil is 32 years, for freeze-drying 15 years, for liquid-nitrogen storage 12 years and for silica-gel storage 11 years

‡ Lost the ability to produce pigment

§ The host grew well but the myco-parasite barely grew, though it sporulated

dium olla (IMI 86666) and *Rhizophydium sphaerotheca*, both notoriously difficult to keep *C. olla* was discarded from the collection

Of the isolates stored under mineral oil, 77 survived 10 years or more, and 20 of these survived 20 years. Seven died during storage, only one of which died within one year, and 14 were poor or non-sporulating on revival The majority of the poor cultures were over 10 years old when examined Only two *Fusarium* cultures, which notoriously deteriorate in oil in a short time were included; one was viable for 9 years and the other died after 21 years. However, *Chytridium olla* survived 7 years, the three isolates of *Pythium* for 31, 28 and 8 years, and *Phytophthora* for 32 years. Some fungi which do not freeze-dry showed good viability after storage under mineral oil. *Armillaria* (27 years), *Basidiobolus* (12 years), *Corticium* (22 years) and *Rhizoctonia* (10 years) have all remained viable.

DISCUSSION

In this paper it has been shown that some cultures have survived up to 32 years in oil storage and many more for 10–20 years In particular these have included isolates which do not freeze-dry, so, although the method is suspect because of the deterioration of some isolates, it still remains useful. It is also of value for the storage of fungi that fail to survive in silica gel and as a back-up to liquid-nitrogen storage or in its place when liquid-nitrogen storage is too expensive. Freeze-drying (Fennell, 1960; Mazur 1968) and liquid-nitrogen storage (Hwang, 1966, 1968, Hwang, Kwolek & Haynes 1976) have been established for some time and have proved successful with fungi (Heckly, 1978; Alexander *et al.*, 1980). Silica-gel storage is an alternative technique that has proved capable of maintaining genetic stability in fungi (Ogata, 1962; Perkins, 1962). The results at CMI indicate that both liquid-nitrogen storage and freeze-drying can be more successful than silica-gel storage, though they are much more expensive to achieve. The silica-gel technique was found to be successful for the sporulating hyphomycetes, the coelomycetes and the ascomycetes with the exception of the discomycetes. It is apparent that this technique is valuable for these groups, especially for maintaining fungi in laboratories with limited resources.

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APPENDIX II List of suppliers

1. Adelphi (Tubes) Manufacturing Ltd., Duncan Terrace, London.

2. Anchor Glass Co., Brent Cross Works, North Circular Road, London.

3. Baird & Tatlock (London) Ltd., P.O. Box 1, Romford, Essex.

4. British Drug Houses Ltd., Laboratory Chemicals Division, Poole, Dorset.

5. Buck and Hickman, Sterling Industrial Estate, Rainham Road South, Dagenham, Essex.

6. CAMLAB Ltd., Nuffield Road, Cambridge CB4 1TH.

7. Denley Instruments Ltd., Daux Road, Billinghurst, Sussex.

8. Digitron Instrumentation Ltd., Merchant Drive, Mead Lane Industrial Estate, Hertford.

9. Edwards High Vacuum, Manor Royal, Crawley, West Sussex.

10. Edme Ltd., Mistley, Manningtree, Essex.

11. Imperial Chemical Industries Ltd., Mond Division Sales, Star House, Clarendon Road, Watford.

12. Jencons (Scientific) Ltd., Leighton Buzzard, Bedfordshire.

13. Mi-dox Ltd., Smarden, Kent.

14. Payne Scientific Ltd., 31, Hillside, Slough, Berkshire.

15. Queue Systems (Distributers, CAMLAB Ltd. above).

16. R. & L. Slaughter Ltd., Balgores Lane, Gidea Park, Romford, Essex.

17. Sterilin (Distributors, R. & L. Slaughter Ltd.,above).

18. Union Carbide (Distributors, Jencons (Scientific) Ltd., above)

19. T.W. Wingent, 115-150 Cambridge Road, Milton, Cambridge.

APPENDIX III The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Absidia spinosa	68077	-	-	-	-	+	+	-	PDA	23	DL
Achaetomium globosum	82626	-	-	-	-	+	-	+	OA+LUP	23	DL
A. globosum	82626ii	-	-	-	-	+	-	+	OA+LUP	23	DL
Achlya ambisexualis	93805	-	-	-	+	+	+	-	ONION	23	DL
A. ambisexualis	93806	-	-	-	-	+	-	-	ONION	23	DL
A. ambisexualis	143524	-	-	-	-	-	-	+	ONION	23	DL
A. ambisexualis	143525	-	-	-	-	-	-	+	ONION	23	DL
A. americana	191108	-	-	-	-	-	-	+	ONION	23	DL
A. bisexualis	141473	-	-	-	-	-	-	+	ONION	23	DL
A. bisexualis	141474	-	-	-	-	-	-	+	ONION	23	DL
A. bisexualis	146646	-	-	-	-	-	-	+	ONION	23	DL
A. bisexualis	146647	-	-	-	-	-	-	+	ONION	23	DL
A. debaryana	161801	-	-	-	-	-	-	+	ONION	23	DL
A. racemosa	110079	-	-	-	-	-	-	+	ONION	23	DL
A. racemosa	137396	-	-	-	-	-	-	+	ONION	23	DL
A. radiosa	137645	-	-	-	-	-	-	+	ONION	23	DL
A. radiosa	137966	-	-	-	-	-	-	+	ONION	23	DL
Acremonium alternatum	166204	-	-	-	+	+	+	-	PCA	23	DL
A. chrysogenum	91579	-	-	-	-	+	-	-	PCA	23	DL
A. furcatum	57398	-	-	-	-	+	-	-	PCA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Acremonium recifei	277456	-	-	-	-	-	+	-	PCA	23	DL
A. strictum	178506	-	-	-	-	+	+	-	PCA	23	DL
A. strictum	276794	-	-	-	-	+	+	-	PCA	23	DL
A. padwickii	136436a	-	-	-	-	+	+	-	PCA	23	DL
Acrospeira mirabilis	278058	-	-	-	-	-	+	-	PCA	23	DL
Actinomucor elegans	56159	-	-	-	-	+	-	-	PCA	23	DL
Akenomyces costatus	279296	-	-	-	-	+	+	-	PCA	23	DL
Allomyces arbuscula	129543	-	-	+	+	+	-	+	ONION	23	DL
A. arbuscula	152201	-	-	+	+	-	-	+	ONION	23	DL
A. cystogenus	93807	-	-	-	-	+	-	-	ONION	23	DL
A. javanicus	86906	-	-	-	-	+	-	-	ONION	23	DL
A. javanicus	144364	-	-	+	+	-	-	-	ONION	23	DL
Alternaria alternata	89343	-	-	+	+	+	-	-	TWA+W	23	BL
A. alternata	89345	-	-	-	-	+	-	-	PCA	23	DL
A. brassicae	156655	-	-	-	-	+	-	-	PCA+FP	23	DL
A. brassicae	151659	-	-	+	+	+	-	-	TWA+W	23	BL
A. chlamydospora	156426	-	-	+	+	+	-	-	TWA+W	23	BL
A. dianthi	280151	-	-	-	-	-	+	-	PCA	23	BL
A. poonensis	278420	-	-	-	-	-	+	-	PCA	23	BL
A. radicina	279636	-	-	-	-	-	+	-	PCA	23	BL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Alternaria solani	129087	-	-	-	-	+	-	-	PCA	23	DL
A. zinnae	278126d	-	-	-	-	-	+	-	PCA	23	BL
Anixiella endodonta	148368	-	-	-	-	-	-	+	YPSS	23	DL
A. endodonta	148369	-	-	-	-	-	-	+	YPSS	23	DL
Anthostomella spartii	185019	-	-	-	+	+	+	+	OA	23	DL
Aphanomyces brassicae	188398	-	-	-	-	-	-	+	ONION	23	DL
A. euteiches	155842	-	-	-	-	-	-	+	ONION	23	DL
Apiocrea chrysosperma	109891	-	-	-	-	+	-	-	PDA	23	DL
Apiosordaria verruculosa	51496	-	-	-	-	+	-	-	PCA	23	DL
Arachnomyces nitidus	147447	-	-	-	+	+	+	+	PCA	23	DL
Areolospora bosensis	184595	-	-	-	-	+	-	+	MA	23	DL
Armillariella mellea	61755	-	-	+	+	-	-	-	MA	23	DL
A. mellea	158162	-	-	-	+	+	+	-	MA	23	DL
Arnium arizonense	169785	-	-	+	+	+	-	+	RDA	23	DL
Arthrobotrys arthrobotryoides	96726	-	-	-	-	-	-	+	OA	23	DL
A. cylindrospora	140015	-	-	-	-	+	-	-	PDA	23	DL
A. oligospora	102121	-	-	+	+	+	-	-	CMA	23	BL
A. scaphoides	50675	-	-	-	-	+	-	-	OA	23	DL
Arthrocladium caudatum	201693	-	-	-	-	+	-	-	PCA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Arthrocristula hyphenata	272976	-	-	-	-	+	+	-	PCA	23	DL
Arthroderma lenticularum	113772	-	-	-	-	-	+	-	MA	23	DL
Arxiella terrestris	176392	-	-	-	-	-	-	+	OA	23	BL
Ascobolus viridulus	85125	-	-	+	+	+	-	-	RDA	23	BL
A. winteri	145962	-	-	-	-	-	-	+	RDA	23	DL
Ascocalvatia alveolata	151071	-	-	-	-	+	-	+	PCA	23	DL
Ascochyta fabae	135517	-	-	+	+	+	-	-	OA	23	BL
A. fabae	156657	-	-	-	-	+	+	-	PDA	23	DL
A. fimbriata	87300	-	-	-	-	+	-	-	OA	23	BL
A. pisi	141220	-	-	-	-	+	+	-	PCA	23	DL
Ascochytula obiones	282137	-	-	-	-	-	+	-	PCA	23	DL
Ascocoryne sarcoides	68130	-	-	-	-	+	-	-	OA	23	DL
Ascosphaera major	160841	-	-	-	-	-	-	+	MA	23	DL
A. osmophila	277755	-	-	-	-	-	+	-	M/20	23	DL
Ascotricha lusitanica	147693	-	-	+	+	+	-	+	PCA	23	DL
Aspergillus alliaceus	275535	-	-	-	-	-	+	-	MCZ	23	DL
A. amstelodami	17455	-	-	-	-	+	-	-	M/20	23	DL
A. amstelodami	71295	-	-	-	-	+	-	-	M/20	23	DL
A. amstelodami	140508	-	-	-	-	+	-	-	M/20	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Aspergillus amylovorus	129961	-	-	-	-	+	-	-	MCZ	23	DL
A. arenarius	556321i	-	-	-	-	+	-	-	MCZ	23	DL
A. asperescens	46813	-	-	-	-	+	-	-	MCZ	23	DL
A. athecius	32048	-	-	-	-	+	-	-	M/20	23	DL
A. avenaceus	16140	-	-	+	+	+	-	-	MCZ	23	BL
A. caespitosus	16034	-	-	-	-	+	-	-	MCZ	23	DL
A. candidus	73074	-	-	-	-	+	-	-	MCZ	23	DL
A. candidus	127259	-	-	-	-	-	+	-	PDA	23	DL
A. candidus	127260	-	-	+	+	+	-	-	MCZ	23	DL
A. candidus	130667	-	-	+	+	+	-	-	MCZ	23	DL
A. carbonarius	186307	-	-	-	-	-	-	+	MCZ	23	DL
A. carneus	73777	-	-	-	-	+	-	-	MCZ	23	DL
A. citrisporus	25285	-	-	+	+	+	-	-	M/20	23	DL
A. crystallinus	139270	-	-	-	-	+	-	-	MCZ	23	DL
A. flavus	91019b	-	-	-	-	+	+	-	MCZ	23	DL
A. flavus	91019bii	-	-	-	-	+	+	-	MCZ	23	DL
A. flavus	91456	-	-	-	-	+	+	-	MCZ	23	DL
A. flavus	277248	-	-	-	-	-	+	-	MCZ	23	DL
A. fumigatus	174456	-	-	-	-	+	+	-	MCZ	30	DL
A. giganteus	112341	-	-	+	+	+	-	+	MCZ	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Aspergillus glaucus	53242	-	-	-	-	+	-	-	MCZ	23	DL
A. itaconicus	16119	-	-	-	-	+	-	-	MCZ	23	DL
A. nidulans	61454	-	-	-	-	+	-	-	MCZ	23	DL
A. nidulans	61454ii	-	-	-	-	+	-	-	MCZ	23	DL
A. nidulans	134679	-	-	+	+	+	-	+	MCZ	23	DL
A. nidulans	141181	-	-	+	+	+	-	-	MCZ	23	DL
A. niger	17454	-	-	+	+	+	-	-	MCZ	23	DL
A. niger	75353ii	-	-	-	-	+	+	-	MCZ	23	DL
A. niger	91855ii	-	-	-	-	+	+	-	MCZ	23	DL
A. niger	149007	-	-	-	-	+	+	-	MCZ	23	DL
A. ochraceus	16247ii	-	-	+	+	+	-	-	M/20	23	DL
A. ochraceus	225094	-	-	-	+	-	+	-	MCZ	23	DL
A. ostianus	93445	-	-	-	-	+	-	-	MCZ	23	DL
A. parasiticus	15957ii	-	-	-	-	+	-	-	MCZ	23	DL
A. penicillioides	144121	-	-	-	-	+	-	-	MCZ	23	DL
A. penicillioides	274334	-	-	-	-	-	+	-	MCZ	23	DL
A. peyronellii	139272	-	-	-	-	+	-	-	MCZ	23	DL
A. pulverulentus	91886	-	-	-	-	+	-	-	MCZ	23	DL
A. quadrilineatus	72733	-	-	-	-	+	-	-	MCZ	23	DL
A. quercinus	95251	-	-	-	-	+	+	-	MCZ	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Aspergillus restrictus	127782	-	-	+	+	+	-	-	MCZ	23	DL
A. restrictus	140815	-	-	-	-	+	-	-	M/20	23	DL
A. sclerotiorum	67759b	-	-	-	-	+	+	-	MCZ	23	DL
A. sclerotiorum	112328	-	-	-	-	+	+	-	MCZ	23	DL
A. sclerotiorum	191603	-	-	-	-	+	+	-	MCZ	23	DL
A. sejunctus	91862	-	-	-	-	+	-	-	M/20	23	DL
A. sejunctus	168779	-	-	+	+	+	-	-	MCZ	23	DL
A. sejunctus	274335	-	-	-	-	-	+	-	M/20	23	DL
A. stellatus	136778	-	-	-	-	+	+	-	MCZ	23	DL
A. terreus	442431i	-	-	-	-	-	+	-	PCA	23	DL
A. ustus	100391	-	-	+	+	+	-	-	MCZ	23	DL
A. wentii	162039	-	-	+	+	+	-	-	MCZ	23	DL
Aureobasidium microstictum	275632	-	-	-	-	-	+	-	MA	23	DL
A. pullulans	45533	-	-	+	+	+	-	-	MA	23	DL
A. pullulans	70103	-	-	-	-	+	+	-	MA	23	DL
A. pullulans	278350	-	-	-	-	-	+	-	MA	23	DL
Auxarthron zuffianum	76603	-	-	-	-	+	+	-	PCA	23	DL
Balansia sclerotica	138634	-	-	-	-	+	-	+	PCA	23	DL
Bartalinia bischofiae	81612	-	-	-	-	+	-	-	PCA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Basidiobolus haptosporus	108126	-	-	-	+	+	+	+	PDA	23	DL
B. haptosporus	113159	-	-	-	-	+	-	-	PDA	23	DL
B. meristosporus	108476	-	-	+	+	+	-	-	PDA	23	DL
B. microsporus	93345	-	-	-	-	+	-	+	PDA	23	DL
B. ranarum	89715	-	-	-	-	+*	-	-	PDA	23	DL
Battarraea phalloides	151693	-	-	-	-	+	-	+	MA	23	DL
Beauveria alba	278647	-	-	-	-	-	+	-	MA	23	DL
B. bassiana	262947	-	-	-	-	-	+	-	PCA	23	DL
B. bassiana	282533	-	-	-	-	-	+	-	PCA	23	DL
Beltrania africana	149586	-	-	-	-	+	-	+	OA	23	DL
Beltraniella humicola	155820	-	-	+	+	+	-	+	PCA+FP	23	BL
B. portoricensis	909921i	-	-	-	-	-	-	+	OA	23	DL
Bipolaris australis	261917	-	-	-	-	-	+	-	PCA	23	BL
B. crustacea	276037	-	-	-	-	-	+	-	PCA	23	BL
B. indica	164633	-	-	-	-	+	+	-	TWA+W	23	DL
B. multiformis	281320	-	-	-	-	-	+	-	TWA+W	23	BL
B. nicotiae	202589	-	-	-	-	+	+	-	TWA+W	23	DL
B. ovariicola	261919	-	-	-	-	-	+	-	PCA	23	BL
Biscogniauxia simplicior	245191	-	-	-	+	+	+	+	MA	23	BL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested; *, 2 stage cooling, vacuum drying technique;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Blakeslea trispora	283659	-	-	-	-	-	+	-	RDA	23	DL
Blastocladiella emersonii	136816	-	-	-	-	+	-	-	MA	23	BL
Botryodiplodia ricinicola	145809	-	-	-	-	+	-	-	PCA	23	BL
B. theobromae	125847	-	-	+	+	+	-	-	PCA	23	DL
Botryotrichum piluliferum	49832	-	-	+	+	+	-	-	OA	23	DL
Botrytis cinerea	100465	-	-	-	-	+	+	-	PDA	23	DL
Byssochlamys fulva	163641	-	-	+	+	+	-	+	PDA	37	NL
B. nivea	163642	-	-	+	+	+	-	+	PDA	37	NL
Calcarisporium thermophilum	144750	-	-	-	-	+	-	-	MA	35	NL
Calonectria diploa	156281	-	-	-	-	+	-	-	PSA	23	BL
C. diploa	278994	-	-	-	-	-	+	+	PSA	23	BL
C. ilicicola	237462	-	-	-	-	+	-	-	PSA	23	BL
C. ilicicola	237463	-	-	-	-	+	-	-	PSA	23	BL
C. quinquiseptata	136139	-	-	+	+	+	-	+	PSA	23	BL
C. rigidiuscula	77037a	-	-	-	-	+	-	-	PSA	23	BL
C. rigidiuscula	84749	-	-	-	+	+	+	+	PSA	23	BL
C. rigiduscula	174223	-	-	-	-	+	-	-	PSA	23	BL
Caloscypha fulgens	144877	-	-	-	-	+	-	+	PCA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Calospora arausiaca	80737c	-	-	-	-	+	-	-	OA	23	DL
Calyptella campanula	262248	-	-	-	-	-	+	-	PDA	23	DL
Camarops lutea	146521	-	-	-	-	+	-	-	MA	23	BL
Candida lipolytica	93743	-	-	+	+	+	-	-	MA	23	DL
Catenaria anguillulae	175996	-	-	-	-	-	-	+	PDA	23	DL
Ceratocystis autographa	173177	-	-	-	-	-	+	-	PCA	23	DL
C. cainii	176523	-	-	-	-	+	-	-	PCA	23	DL
C. doluminuta	176538	-	-	-	-	+	-	-	PCA	23	DL
C. falcata	274631	-	-	-	-	-	+	-	PCA	23	DL
C. fimbriata	80787	-	-	-	-	+	-	-	CMA	23	DL
C. fimbriata	80795	-	-	-	+	+	+	-	CMA	23	DL
C. fimbriata	123637	-	-	-	-	+	-	-	CMA	23	DL
C. fimbriata	123638	-	-	-	-	+	-	+	CMA	23	DL
C. fimbriata	123677	-	-	-	-	+	-	-	CMA	23	DL
C. paradoxa	77668	-	-	-	-	+*	-	-	CMA	23	DL
C. pilifera	274632	-	-	-	-	-	+	-	PCA	23	DL
C. ulmi	147188	-	-	+	+	+	-	-	TWA+W	23	BL
C. ulmi	173135	-	-	+	+	+	-	-	PCA	23	BL
C. ulmi	173136	-	-	+	+	+	-	-	PCA	23	BL
Cercospora beticola	77043	-	-	+	+	-	-	-	V8	23	BL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested; *, 2 stage cooling, vacuum drying technique;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Cercospora beticola	198685	-	-	-	-	-	-	+	V8	23	BL
C. bizzozzeriana	161110	-	-	-	-	-	-	+	PCA	4	NL
C. dulcamarae	161112	-	-	-	-	+	-	+	PCA	4	NL
C. erysimi	161113	-	-	-	-	-	-	+	PCA	4	NL
C. fusimaculans	166241	-	-	-	-	+	-	+	PCA	23	DL
C. fusimaculans	167426	-	-	-	-	+	-	-	PCA	23	DL
C. malvicola	161115	-	-	-	-	-	-	+	PCA	4	NL
C. olivascens	124975	-	-	-	-	+	-	-	MA	23	DL
C. plumbaginea	161116	-	-	-	-	+	-	+	PCA	4	NL
C. rautensis	161117	-	-	-	-	+	-	+	PCA	4	NL
C. sesami	111779	-	-	+	+	+	-	+	PCA	23	DL
C. xanthosomatis	179717	-	-	-	-	+*	-	+	V8	23	BL
Cercosporidium bolleana	161111	-	-	-	-	+	+	+	PCA	23	DL
Ceuthospora innumera	106837	-	-	-	-	-	-	+	MA	23	BL
Chaetocladium jonesii	190954	-	-	-	-	+	-	-	MA	23	DL
Chaetomium abuense	114513	-	-	-	-	+	-	-	PCA+FP	23	DL
C. amberpetense	144976	-	-	-	-	+	-	-	PCA+FP	23	BL
C. anguipilium	281165	-	-	-	-	-	+	-	OA	23	DL
C. bostrychodes	139638	-	-	-	-	+	+	-	PCA+FP	23	DL
C. cochliodes	279189	-	-	-	-	-	+	-	PCA+FP	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested; *, 2 stage cooling, vacuum drying technique;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Chaetomium cuniculorum	155487	-	-	+	+	+	-	+	PCA+FP	23	BL
C. cupreum	279208	-	-	-	-	-	+	-	PCA+FP	23	DL
C. dreyfussii	281164	-	-	-	-	-	+	-	PCA+FP	23	DL
C. elatum	17424a	-	-	+	+	+	-	-	PCA+FP	23	BL
C. elatum	280816	-	-	-	-	-	+	-	PCA+FP	23	DL
C. globosum	16203	-	-	+	+	+**	+	-	PCA+FP	23	BL
C. gracile	84227	-	-	-	-	+	+	-	PCA+FP	23	DL
C. jodhpurense	276987	-	-	-	-	-	+	-	PCA+FP	23	DL
C. mollicellum	276168	-	-	-	-	-	+	-	PCA+FP	23	DL
C. murorum	279726	-	-	-	-	-	+	-	PCA+FP	23	DL
C. quizotiae	279573	-	-	-	-	-	+	-	PCA+FP	23	DL
C. reflexum	279252	-	-	-	-	-	+	-	PCA+FP	23	DL
C. robustum	281166	-	-	-	-	-	+	-	PCA+FP	23	DL
C. spinigerum	73515	-	-	-	-	+	-	-	PCA+FP	23	BL
C. tenuissimum	81769	-	-	-	-	+	-	-	PCA+FP	23	DL
Chaetomium sp.	260186	-	-	-	-	-	+	-	PCA+FP	23	DL
Chlamydomyces palmarum	130823	-	-	-	-	-	-	+	MCZ	23	DL
C. palmarum	278355	-	-	-	-	-	+	-	PCA	23	DL
Choanephora cucurbitarum	121212	-	-	-	-	+	-	-	PCA	23	DL
C. cucurbitarum	164967	-	-	-	-	+	+	-	PCA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested; **, 2 stage cooling, vacuum drying and CFD;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Choanephora cucurbitarum	265041	-	-	-	-	-	+	-	PCA+FP	23	DL
C. cucurbitarum	276406	-	-	-	-	-	+	-	PCA	23	DL
Chrysosporium synchronum	282433	-	-	-	-	-	+	-	PDA	23	DL
Chytridium olla	86666	-	-	+	+	+	-	+	ONION	23	DL
C. ottariense	194741	-	-	-	-	-	-	+	ONION	23	BL
Circinella mucoroides	276533	-	-	-	-	-	+	-	PDA	23	DL
C. simplex	101093	-	-	-	-	+	-	-	MA	23	DL
Cladobotryum chlamydosporum	98099	-	-	-	-	+	-	-	MA	23	DL
Cladosporium allii-cepae	275849	-	-	-	-	-	+	-	PCA	15	DL
C. allii-cepae	275851	-	-	-	-	-	+	-	MA	15	NL
C. allii-cepae	275852	-	-	-	-	-	+	-	MA	15	NL
C. cladosporioides	45534	-	-	-	-	+	+	-	PDA	23	DL
C. cucumerinum	249540	-	-	-	-	+	+	-	PDA	23	DL
C. herbarum	49627	-	-	-	-	+	+	-	PCA	23	DL
Clathrospora diplospora	68086	-	-	-	-	-	-	+	OA	23	DL
Claviceps paspali	82999	-	-	-	-	-	-	+	OA	23	DL
C. purpurea	126133	-	-	-	-	-	+	-	MA	23	DL
Cochliobolus dactyloctenii	276040	-	-	-	-	-	+	-	PCA+W	23	BL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Cochliobolus dactyloctenii	276041	-	-	-	-	-	+	-	PCA+W	23	BL
C. peregianensis	264355	-	-	-	-	-	+	-	PCA+W	23	BL
C. peregianensis	276042	-	-	-	-	-	+	-	PCA+W	23	BL
C. peregianensis	276043	-	-	-	-	-	+	-	PCA+W	23	BL
C. perotidis	264356	-	-	-	-	-	+	-	PCA+W	23	BL
C. sativus	166172	-	-	+	+	+	-	+	TWA+W	23	DL
C. sativus	166173	-	-	+	+	+	-	+	PCA	23	DL
C. tuberculatus	99410	-	-	-	-	+	+	-	PCA	23	DL
Coemansia erecta	279145	-	-	-	-	-	+	-	PCA	23	DL
C. formosensis	170166	-	-	+	+	+	-	+	PDA	23	DL
C. mojavensis	140079	-	-	+	-	-	-	-	PDA	23	DL
C. pectinata	142377	-	-	+	+	+	+	+	MA	23	DL
Colletotrichum ampelinum	82267	-	-	-	-	-	-	+	CMA	23	DL
C. dematium	80025	-	-	+	+	+	-	+	CMA	23	DL
C. dematium	156656	-	-	+	+	+	-	+	CMA	23	DL
C. gossypii	82269	-	-	+	+	+	-	+	CMA	23	BL
C. musae	83256	-	-	+	+	+	-	-	CMA	23	DL
C. sublineolum	275716	-	-	-	-	-	+	-	PDA	23	BL
C. sublineolum	275718	-	-	-	-	+	+	-	CMA	23	BL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Colletotrichum sublineolum	275720	-	-	-	-	-	+	-	PDA	23	BL
C. sublineolum	279189	-	-	-	-	-	+	-	PDA	23	BL
C. tabacum	54048	-	-	+	+	-	-	-	PCA	23	DL
C. trichellum	82378	-	-	+	+	+	-	-	OA	23	DL
C. trichellum	279999	-	-	-	-	-	+	-	OA	23	BL
C. truncatum	86431	-	-	+	+	+	-	-	CMA	23	DL
C. typhae	86896b	-	-	+	+	+	-	-	CMA	23	DL
Conidiobolus bangalorensis	118284	-	-	-	-	+	-	-	MA	23	DL
C. coronatus	68174	-	-	+	+	+	-	-	PDA	23	DL
C. coronatus	145949	-	-	-	-	+	-	-	PDA	23	DL
C. couchii	128727	-	-	-	+	+	+	+	MA	23	DL
C. heterosporus	102043	-	-	+	+	+	-	+	MA	23	DL
C. humicola	92300	-	-	-	-	+	-	+	MA	23	DL
C. khandalensis	102045	-	-	-	-	+	-	+	MA	23	DL
C. lichenicola	113700	-	-	-	-	+	-	+	MA	23	DL
C. lobatus	138635	-	-	+	+	+	-	-	PDA	23	DL
C. mycophagus	113701	-	-	+	+	+	-	+	PDA	23	DL
C. mycophilus	113698	-	-	-	-	+	-	-	MA	23	DL
C. nodosus	118285	-	-	-	-	+	-	-	MA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Conidiobolus stromatoideus	92298ii	-	-	-	-	-	-	+	MA	23	DL
Coniochaeta leucoplaca	144079	-	-	-	-	+	-	-	OA	23	DL
Coniophora puteana	79127	-	-	-	-	+*	+	-	MA	23	DL
Coniothyrium minitans	134523ii	-	-	-	-	+	+	-	PDA	23	DL
Coprinus alkalinus	133856	-	-	-	-	+	-	+	RDA	23	DL
C. amphibius	133855	-	-	-	+	+	+	+	RDA	23	DL
C. atramentarius	132648	-	-	-	-	+	-	+	RDA	23	DL
C. hexagonosporus	161417	-	-	+	+	+*	-	+	RDA	23	DL
C. luteocephalus	161421	-	-	+	-	+	-	+	RDA	23	DL
C. macrocephalus	182008	-	-	-	-	+	-	+	RDA	23	DL
C. semitalis	161423	-	-	+	-	+	-	-	RDA	23	DL
C. utrifer	161422	-	-	+	+	+	-	+	RDA	23	DL
Coriolus versicolor	79126	-	-	-	-	+	-	+	MA	23	DL
Corticium rolfsii	77445a	-	-	+	+	+	-	-	RDA	23	DL
Corynespora cassiicola	56007	-	-	-	-	+	-	-	PCA	23	BL
Cryphonectria cubensis	279614	-	-	-	-	-	+	-	PSA	23	DL
C. eugeniae	56425a	-	-	-	+	+	+	-	CMA	23	DL
C. eugeniae	279035	-	-	-	-	-	+	-	CMA	23	DL
C. eugeniae	279036	-	-	-	-	-	+	-	CMA	23	DL
C. eugeniae	279618	-	-	-	-	-	+	-	CMA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested; *, 2 stage cooling, vacuum drying technique;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Cryphonectria parasitica	59815	-	-	+	+	+	-	-	CMA	23	DL
Cryptocline cinerescens	275743	-	-	-	-	-	+	-	MA	23	BL
Cryptospora suffusa	173497	-	-	+	+	+	-	+	MA	23	DL
Culicinomyces clavosporus	177011	-	-	+	+	+	-	-	PDA	23	DL
Culcitalna achraspora	132773	-	-	-	-	+	-	-	MA	23	DL
Cunninghamella blakesleeana	53586	-	-	-	-	+	-	-	PDA	23	DL
C. echinulata	199844	-	-	-	-	+	+	-	OA	23	DL
C. elegans	188670	-	-	-	-	+	+	-	PDA	23	DL
C. elegans	200332	-	-	-	-	+*	-	+	PDA	23	DL
C. vesiculosa	93346	-	-	-	-	+	-	+	PDA	23	DL
C. vesiculosa	93346ii	-	-	-	-	+	-	-	MA	23	DL
C. vesiculosa	130775	-	-	-	-	+	-	-	MA	23	DL
Curvularia andropogonis	192062	-	-	-	-	-	-	+	PCA	23	DL
C. borreriae	155733	-	-	-	-	+	+	-	TWA+W	23	DL
C. crepinii	605	-	-	-	-	+	+	-	PCA	23	DL
C. deightonii	148188	-	-	-	-	+	+	-	TWA+W	23	DL
C. fallax	79737	-	-	-	-	+	-	-	PCA+FP	23	DL
C. lunata	96846	-	-	+	+	+	-	+	PCA+FP	23	DL
C. trifolii	75377	-	-	-	-	+	-	-	PCA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested; *, 2 stage cooling, vacuum drying technique;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Cylindrocarpon congoense	69504	-	-	-	-	+	-	-	PSA	23	BL
C. obtusisporum	96731	-	-	-	-	+	-	-	CMA	23	DL
C. olidum	182099	-	-	-	-	+	-	-	CMA	23	DL
C. pseudocandidum	171060	-	-	-	-	+	-	-	CMA	23	DL
Cylindrocladium oumaiensis	167983	-	-	-	-	+	-	-	CMA	23	DL
C. quinqueseptatum	78332	-	-	-	-	+	-	-	CMA	23	DL
C. quinqueseptatum	136139	-	-	-	-	+	-	-	CMA	23	DL
Cytospora eriobotryae	136523	-	-	-	-	+	-	+	PDA	23	DL
Dactuliophora tarrii	102794	-	-	-	-	+	-	+	PCA	23	DL
Dactylaria juncic	131540	-	-	-	-	+	-	-	PDA	23	DL
Dendryphiella infuscans	144005	-	-	-	-	-	-	+	TWA+W	23	BL
Diaporthe phaseolorum	158864	-	-	-	-	+	+	-	OA	23	DL
Dictyoarthrinium rabaulense	51264	-	-	-	-	+	-	-	OA	23	DL
Dictyostelium discoideum	69094	-	-	-	-	+	-	-	RDA	23	DL
D. discoideum	69094ii	-	-	+	+	+	-	+	RDA	23	DL
Dictyuchus sterile	182416	-	-	-	-	-	-	+	ONION	23	DL
Didymella bryoniae	280801	-	-	-	-	-	+	-	OA	23	BL
D. excitialis	183209	-	-	-	-	+	-	+	OA	23	BL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Didymella excitialis	183210	-	-	-	-	-	-	+	OA	23	BL
Didymosphaeria donacina	187053	-	-	-	-	+	-	-	OA	23	BL
Dimargaris bacillospora	130774	-	-	-	-	+	-	-	YPSS	23	DL
D. verticillata	278511	-	-	-	-	-	+	-	YPSS	23	DL
D. xerosporica	113067	-	-	-	-	+	-	-	YPSS	23	DL
Diplosclerophoma ceratoniae	180575	-	-	-	-	-	-	+	PCA	23	DL
Dipodascopsis uninucleata	86676	-	-	-	-	+	-	-	MA	23	DL
Dispira cornuta	775901i	-	-	-	-	-	-	+	MA	23	DL
Doratomyces purpureofuscus	137993	-	-	-	-	+	-	-	PCA	23	DL
D. stemonitis	43604	-	-	-	-	+	-	-	PCA	23	DL
Drechslera brizae	276557	-	-	-	-	-	+	-	PCA	23	BL
D. heveae	163331	-	-	-	-	+	+	-	TWA+W	23	DL
Echinopodospora jamaicensis	135507	-	-	-	-	+	-	-	OA	23	DL
E. sacchari	141542	-	-	-	-	+	-	+	OA	23	DL
Elaphomyces muricatus	213783	-	-	-	-	-	-	+	MA	23	DL
Eleutherascus tuberculatus	45134ii	-	-	-	-	+	-	-	YPSS	23	DL
Embellisia abundans	279172	-	-	-	-	-	+	-	PCA	23	DL
E. abundans	279181	-	-	-	-	-	+	-	PCA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Embellisia chlamydospora	67737	-	-	-	-	+	-	-	PCA	23	DL
E. hyacinthi	279179	-	-	-	-	-	+	-	PCA	23	DL
E. indefessa	279175	-	-	-	-	-	+	-	PCA	23	DL
E. planifunda	115034	-	-	-	-	-	+	-	PCA	23	DL
E. planifunda	279178	-	-	-	-	-	+	-	PCA	23	DL
E. telluster	279180	-	-	-	-	-	+	-	PCA	23	DL
E. turnida	279176	-	-	-	-	-	+	-	PCA	23	DL
Ellisiella caudata	176619	-	-	-	-	+	-	-	OA	23	DL
Emericellopsis minima	69015	-	-	-	-	+	-	-	PCA	23	DL
E. terricola	68332	-	-	-	-	-	-	+	PCA	23	DL
Entomophthora oligolophi	225446	-	-	-	-	-	-	+	EGG	23	DL
Entosordaria perfidiosa	185020	-	-	-	-	-	-	+	RDA	23	DL
Epicoccum purpurascens	79496	-	-	-	-	+	-	-	PCA	23	DL
Eremascus albus	100446a	-	-	-	-	+	+	-	M/40	23	DL
E. fertilis	86727	-	-	+	+	+	-	-	M/40	23	DL
Eremomyces bilateralis	151076	-	-	-	-	+	-	+	M/20	23	DL
Erynia excitalis	155908	-	-	-	-	+	+	+	EGG	23	DL
Exobasidiellum culmigenum	136517	-	-	+	+	+	-	-	CMA	23	DL
Exophiala jeanselmei	279566	-	-	-	-	-	+	-	PCA	23	BL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Flammulina velutipes	144603	-	-	-	-	+	-	-	MA	23	DL
Fomes fraxineus	81803	-	-	-	-	+	-	-	MA	23	DL
F. ulmarius	82772	-	-	-	-	+	-	+	MA	23	DL
F. ulmarius	82773	-	-	-	-	+	-	-	MA	23	DL
F. ulmarius	86444	-	-	-	-	+	-	-	MA	23	DL
Fulvia fulva	54976	-	-	-	-	+	+	-	V8	23	DL
Fusariella concinna	277515	-	-	-	-	-	+	-	TWA+W	23	DL
Fusarium acuminatum	106901	-	+	-	-	-	-	-	PSA	23	BL
F. acuminatum	129863	-	+	-	-	-	-	-	PSA	23	BL
F. acuminatum	136675	-	+	-	-	+	-	-	PSA	23	BL
F. arthrosporioides	163864	-	+	-	-	-	-	-	PSA	23	BL
F. avenaceum	85564	-	+	-	-	-	-	-	PSA	23	BL
F. avenaceum	103230b	-	+	-	-	-	-	-	PSA	23	BL
F. avenaceum	103227	-	+	-	-	-	-	-	PSA	23	BL
F. avenaceum	175486	-	+	-	-	-	-	-	PSA	23	BL
F. avenaceum	272884	-	-	-	-	+	+	-	PSA	23	BL
F. avenaceum	272887	-	-	-	-	-	+	-	PSA	23	BL
F. avenaceum	273040	-	-	-	-	-	+	-	PSA	23	BL
F. avenaceum	273492	-	-	-	-	-	+	-	PSA	23	BL
F. concolor	136902	-	+	-	-	-	-	-	PSA	23	BL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Fusarium culmorum	14764	-	-	-	-	+	+	-	PSA	23	BL
F. culmorum	135794	-	+	-	-	-	-	-	PSA	23	BL
F. culmorum	135667	-	+	-	-	-	-	-	PSA	23	BL
F. culmorum	149131	-	+	-	-	-	-	-	PSA	23	BL
F. culmorum	164746	-	+	-	-	-	-	-	PSA	23	BL
F. culmorum	175485	-	-	+	+	+	-	-	PSA	23	BL
F. culmorum	272853	-	-	-	-	+	+	-	PSA	23	BL
F. culmorum	273711	-	-	-	-	-	+	-	PSA	23	BL
F. crookwellense	281151	-	-	-	-	-	+	-	PSA	23	BL
F. equiseti	111911	-	+	-	-	-	-	-	PSA	23	BL
F. equiseti	127561	-	+	-	-	-	-	-	PSA	23	BL
F. flocciferum	131515	-	+	-	-	+	-	-	PSA	23	BL
F. fujikuroi	202879	-	-	-	-	+	+	-	PSA	23	BL
F. graminearum	69695	-	-	-	-	+	-	-	PSA	23	BL
F. graminearum	105494	-	+	-	-	-	-	-	PSA	23	BL
F. graminearum	140790b	-	+	-	-	-	-	-	PSA	23	BL
F. graminearum	155426	-	+	-	-	+	-	-	PSA	23	BL
F. graminearum	160243	-	-	-	-	+	+	-	PSA	23	BL
F. heterosporum	100469a	-	+	-	-	-	-	-	PSA	23	BL
F. heterosporum	124108	-	+	-	-	-	-	-	PSA	23	BL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Fusarium heterosporum	169066	-	+	-	-	-	-	-	PSA	23	BL
F. lateritium	129623	-	+	-	-	-	-	-	PSA	23	BL
F. lateritium	134593	-	+	-	-	-	-	-	PSA	23	BL
F. merismoides	101143	-	+	-	-	-	-	-	PSA	23	BL
F. merismoides	105043	-	+	-	-	+	-	-	PSA	23	BL
F. merismoides	279297	-	-	-	-	-	+	-	PSA	23	BL
F. merismoides	280230	-	-	-	-	-	+	-	PSA	23	BL
F. moniliforme	113173	-	+	-	-	-	-	-	PSA	23	BL
F. moniliforme	151906	-	+	-	-	-	-	-	PSA	23	BL
F. moniliforme	152300	-	+	-	-	-	-	-	PSA	23	BL
F. moniliforme	158047	-	+	-	-	+	-	-	PSA	23	BL
F. oxysporum	136160	-	+	-	-	-	-	-	PSA	23	BL
F. oxysporum	138619	-	+	-	-	-	-	-	PSA	23	BL
F. oxysporum	141140	-	-	-	-	+	-	-	PSA	23	BL
F. oxysporum	159029	-	+	-	-	+	+	-	PSA	23	BL
F. oxysporum	264170	-	-	-	-	-	+	-	PSA	23	BL
F. poae	128054	-	+	-	-	-	-	-	PSA	23	BL
F. poae	272890	-	-	-	-	+	+	-	PSA	23	BL
F. poae	273481	-	-	-	-	-	+	-	PSA	23	BL
F. sambucinum	111826	-	+	-	-	-	-	-	PSA	23	BL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Fusarium sambucinum	135683	-	+	-	-	-	-	-	PSA	23	BL
F. sambucinum	136929	-	+	-	-	-	-	-	PSA	23	BL
F. sambucinum	155388	-	+	-	-	-	-	-	PSA	23	BL
F. sambucinum	155389	-	+	-	-	-	-	-	PSA	23	BL
F. sambucinum	155390	-	+	-	-	-	-	-	PSA	23	BL
F. sambucinum	160850	-	+	-	-	-	-	-	PSA	23	BL
F. scirpi	52129	-	+	-	-	-	-	-	PSA	23	BL
F. semitectum	135410	-	-	-	-	+	-	-	PSA	23	BL
F. semitectum	157845b	-	+	-	-	-	-	-	PSA	23	BL
F. solani	63862	-	-	-	-	-	+	-	PSA	23	BL
F. solani	68412	-	-	+	+	+	+	-	PSA	23	BL
F. solani	76761	-	-	+	+	+	-	-	PSA	23	BL
F. solani	172507	-	-	+	-	+	-	-	PSA	23	BL
F. sporotrichioides	281904	-	-	-	-	-	+	-	PSA	23	BL
F. stilboides	276798	-	-	-	-	-	+	-	PSA	23	BL
F. tricinctum	273620	-	-	-	-	-	+	-	PSA	23	BL
F. udum	275452	-	-	-	-	-	+	-	PSA	23	BL
Gabarnaudia betae	72913	-	-	-	-	+	-	-	PDA	23	DL
Gaeumannomyces graminis	160145	-	-	+	+	+	-	+	PDA	23	BL
G. graminis	187782	-	-	-	-	+	-	-	PCA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Gaeumannomyces graminis	189119	-	-	-	-	+	-	-	PCA	23	DL
G. graminis	272213	-	-	-	-	+	+	-	TWA+W	23	DL
G. graminis	280418	-	-	-	-	-	+	-	TWA+W	23	DL
Ganoderma applanatum	157816	-	-	-	+	+	+	-	MA	23	DL
G. applanatum	157818	-	-	-	-	+	+	+	MA	23	DL
G. philippii	108700	-	-	-	-	+	-	+	MA	23	DL
Gelasinospora cerealis	45147	-	-	-	-	+	-	-	OA	23	DL
G. cerealis	76253a	-	-	+	+	+	-	-	OA	23	DL
G. kobi	281199	-	-	-	-	-	+	-	OA	23	DL
G. longispora	130041	-	-	-	-	+	-	+	MA	23	DL
Genicularia bogoriensis	109554	-	-	-	-	+	-	-	CMA	23	DL
Geosmithia lavendula	40570	-	-	+	+	+	-	-	CZ	23	DL
G. namyslowskii	40033	-	-	-	-	+	-	-	MA	23	DL
G. swiftii	40045	-	-	-	-	+	-	-	MA	23	DL
Geotrichum amycelicum	96824	-	-	-	-	+	-	-	MA	23	DL
Gilbertella persicaria	280841	-	-	-	-	-	+	-	RDA	23	DL
Gliocladium roseum	101020h	-	-	+	+	+	-	-	PDA	23	DL
G. roseum	278745	-	-	-	-	-	+	-	MA	23	DL
G. vermoeseni	279785	-	-	-	-	-	+	-	PCA	23	DL
Gloeosporium kaki	86556	-	-	-	-	+	-	-	CMA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Glomerella cingulata	280101	-	-	-	-	-	+	-	OA	23	DL
G. tucumanensis	78362	-	-	-	-	-	-	+	CMA	23	BL
G. tucumanensis	88875	-	-	-	-	-	-	+	CMA	23	BL
Gnomonia fructicola	164147a	-	-	+	+	+	-	-	MA	23	BL
G. leptostyla	77378	-	-	-	-	+	-	-	MA	23	BL
Goidanichiella scopula	278477	-	-	-	-	-	+	-	PCA	23	DL
Gonytrichum macrocladium	278340	-	-	-	-	-	+	-	PCA	23	DL
Guignardia cocoicola	280132	-	-	-	-	-	+	-	CMA	23	BL
Gymnoascus petalosporus	183752	-	-	-	-	-	-	+	RDA	23	DL
Hansfordia pulvinata	20743	-	-	-	-	+	-	-	OA	23	DL
Harposporium helicoides	87013	-	-	-	-	+	-	-	YPSS	23	DL
Helicodendron tubulosum	92743	-	-	+	+	+	-	+	PCA	23	DL
Helicosporina veronae	114458	-	-	+	+	+	-	+	PDA	23	DL
Heliscus submersus	82609	-	-	+	+	+	-	+	PDA	23	DL
Heterocephalum aurantiacum	131684	-	-	+	+	+	-	+	PDA	23	DL
H. aurantiacum	276848a	-	-	-	-	-	+	-	PDA	23	DL
Humicola grisea	126329	-	-	+	+	+	-	+	YPSS	42	NL
H. grisea	149015	-	-	-	-	+	+	-	MA	23	DL
H. insolens	126330	-	-	-	-	+	+	-	YPSS	37	NL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Hymenula cerealis	80179	-	-	-	-	-	-	+	MA	23	DL
H. cerealis	135525	-	-	-	-	+	-	-	OA	23	BL
Hypoxylon argillaceum	146527	-	-	-	-	+	-	+	MA	23	DL
H. cohaerans	146054	-	-	-	-	+	-	+	OA	23	DL
H. cohaerans	198632	-	-	-	-	+	-	+	OA	23	DL
H. confluens	93350	-	-	-	-	-	-	+	MA	23	BL
H. confluens	146053	-	-	-	+	+	+	+	MA	23	BL
H. confluens	193350	-	-	-	-	-	-	+	MA	23	BL
H. fragiforme	192581	-	-	-	-	+*	-	-	MA	23	DL
H. fuscum	146525	-	-	-	-	+	-	+	MA	23	DL
H. howeianum	192590	-	-	-	-	-	-	+	MA	23	BL
H. mediterraneum	75991	-	-	+	+	-	-	-	MA	23	BL
H. multiforme	146526	-	-	-	-	+	-	+	MA	23	DL
H. multiforme	198631	-	-	-	-	+	-	-	MA	23	DL
H. nummularium	146051	-	-	+	+	+	-	-	MA	23	BL
H. nummularium	198629	-	-	-	-	+	-	+	MA	23	DL
H. nummularium	198630	-	-	-	-	+	-	+	MA	23	DL
H. rutilum	198628	-	-	-	-	-	-	+	MA	23	BL
H. udum	198633	-	-	-	-	+	-	-	MA	23	DL
Idriella australiensis	149915	-	-	-	-	-	-	+	OA	23	BL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested; *, 2 stage cooling, vacuum drying technique;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Infundibura adhaerens	274154	-	-	-	-	+	+	-	MA	23	BL
Ingoldiella fibulata	177453	-	-	-	-	+	-	-	PDA	23	DL
Iodophanus carneus	70912ii	-	-	-	-	+	-	-	MA	23	DL
Isaria felina	159339	-	-	+	+	+	-	+	PCA	23	DL
Isoachlya eccentrica	146648	-	-	-	-	-	-	+	ONION	23	DL
Isthmotricladia gombakiensis	184588	-	-	-	-	-	-	+	MA	23	BL
Karlingia rosea	158999	-	-	-	-	-	-	+	ONION	23	DL
Kickxella alabastrina	139630	-	-	-	-	+	-	-	MA	15	NL
Kretzschmaria clavus	245190	-	-	-	-	+	+	-	MA	23	BL
Lacellinopsis sacchari	143987	-	-	-	-	+	-	+	TWA+W	23	BL
Lasiobolidium spirale	151083	-	-	-	-	+	-	+	MA	23	DL
Lentinus degener	110525	-	-	-	+	+	+	+	MA	23	DL
Lenzites betulina	144608	-	-	-	-	+	-	-	MA	23	DL
Leptodothiorella sp.	258560b	-	-	-	-	-	+	-	OA	23	BL
Leptoporus albellus	144609	-	-	-	-	+	-	+	MA	23	DL
Leptosphaeria avenaria	190917	-	-	-	-	-	-	+	MA	23	BL
L. doliolum	199777	-	-	+	+	-	-	+	OA	23	BL
L. nodorum	86734	-	-	-	-	+	-	-	OA	23	BL
L. nodorum	190918a	-	-	-	-	+	-	-	OA	23	BL
L. taiwanensis	202008	-	-	-	-	-	-	+	MA	23	BL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Lophiostoma angustilabrum	143287	-	-	-	-	+	-	-	CMA	23	BL
Macrophoma mangiferae	278638	-	-	-	-	-	+	-	MA	23	BL
Macrophomina phaseolina	147229	-	-	-	-	+	-	-	TWA+W	23	BL
M. phaseolina	179649	-	-	-	-	+*	-	-	OA	23	BL
Malbranchea sulfurea	126327	-	-	-	-	+	-	-	YPSS	45	NL
Marasmius palmivorus	123937	-	-	-	-	+	-	+	MA	23	DL
Marssonina brunnea	211287	-	-	-	-	-	-	+	YPSS	23	DL
Martensiomyces pterosporus	60573	-	-	+	+	+	+	+	RDA	24	DL
Massariothea attenuata	280903	-	-	-	-	-	+	-	PCA	23	BL
Melanconis modonia	80245	-	-	-	-	+	-	-	PDA	23	DL
M. modonia	80246	-	-	-	-	+	-	-	PDA	23	DL
Melanospora zamiae	68202	-	-	-	-	+	-	-	MA	23	DL
Metarhizium anisopliae	98375	-	-	+	+	+	-	-	OA	23	DL
M. anisopliae	129065	-	-	-	-	+	-	-	PCA	23	DL
M. anisopliae	152223	-	-	-	-	+	+	-	OA	23	DL
M. anisopliae	170138	-	-	-	-	+	-	-	PCA	23	DL
M. anisopliae	177416	-	-	-	-	+	+	-	PCA	23	DL
M. anisopliae	274954ii	-	-	-	-	-	+	-	PCA	23	DL
Microdochium bolleyi	277137	-	-	-	-	-	+	-	PDA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested; *, 2 stage cooling, vacuum drying technique;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Microdochium bolleyi	278995	-	-	-	-	-	+	-	PDA	23	DL
M. dimerum	109832	-	+	-	-	-	-	-	PSA	23	BL
M. dimerum	121317	-	-	-	-	+	-	-	PSA	23	DL
Micromonospora vulgaris	126892	-	-	+	+	+	-	-	MA	45	NL
Monacrosporium gephyrophagum	143688	-	-	-	-	-	-	+	PDA	23	DL
M. mutabile	138221	-	-	-	-	+	+	-	PCA	23	DL
M. oxysporum	78728	-	-	-	-	+	-	-	OA	23	DL
M. rutgeriensis	129960	-	-	-	-	+	-	+	PCA	23	DL
M. salinum	109555	-	-	+	+	+	-	+	CMA	23	DL
Monascus purpureus	123954	-	-	-	-	+	-	-	PCA	23	DL
Monilinia fructigena	103791	-	-	-	-	+	-	-	MA	23	DL
M. fructigena	143628	-	-	-	-	+	+	-	MA	23	DL
M. fructigena	162408	-	-	-	-	+	-	-	MA	23	DL
M. laxa	133266	-	-	-	-	+	-	-	OA	23	DL
Monosporascus eutypoides	226000	-	-	-	-	+	-	-	PDA	23	DL
Monotosporella setosa	139138	-	-	-	-	+	-	+	PCA	23	BL
Mortierella ambigua	149024	-	-	-	-	+	-	+	PDA	23	DL
M. bainieri	167609	-	-	+	+	+	-	-	PDA	23	DL
M. minutissima	146672	-	-	-	-	+	-	-	PDA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Mortierella nantahalensis	158113	-	-	-	-	+	-	-	CMA	23	DL
M. polycephala	144610	-	-	-	-	+	-	-	MA	23	DL
M. ramanniana	144619	-	-	-	-	+	+	-	PDA	23	DL
M. vesiculosa	140982	-	-	-	-	+	-	-	PDA	23	DL
M. vesiculosa	140983	-	-	-	-	+	-	-	PDA	23	DL
Mucor azygospora	101213	-	-	-	-	+	-	+	PDA	23	DL
Mucor flavus	280011	-	-	-	-	-	+	-	PDA	23	DL
M. hiemalis (+)	21216	-	-	+	+	+	+	-	PDA	23	DL
M. hiemalis (-)	21217	-	-	+	+	+	+	-	PDA	23	DL
M. hiemalis	276667	-	-	-	-	-	+	-	PDA	23	DL
M. mucedo	133298	-	-	-	-	+	-	-	MA	23	DL
M. mucedo	184726	-	-	-	-	+	+	-	PDA	23	DL
M. piriformis	276599	-	-	-	-	-	+	-	PDA	23	DL
M. racemosus	17364	-	-	-	-	+	+	-	PDA	23	DL
Myceliophthora thermophila	158756	-	-	+	+	+	-	-	MA	37	NL
Mycocentrospora acerina	128980	-	-	-	-	+	-	-	PCA	23	DL
M. acerina	142050	-	-	-	-	+	-	-	V8	23	DL
Mycoleptodiscus sphaericus	159038	-	-	-	-	+	-	+	PDA	23	DL

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Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Mycosphaerella deightonii	119431	-	-	+	+	+	-	-	PDA	23	DL
M. fijiensis	162753	-	-	-	-	+	-	-	PDA	23	DL
M. mori	281787	-	-	-	-	-	+	-	PDA	23	BL
Mycotypha microspora	282443	-	-	-	-	-	+	-	PDA	23	DL
Mycovellosiella ferruginea	124973	-	-	+	+	+	-	-	OA	23	DL
M. vaginae	107865	-	-	-	-	+	-	-	CMA	23	DL
Myriodontium keratinophilum	273431	-	-	-	-	-	+	-	MA	23	BL
Myrothecium cinctum	45148	-	-	-	-	+	-	-	OA	23	DL
M. lachastrae	273160	-	-	-	+	+	+	-	PDA	23	DL
Myrothecium leucotrichum	152595	-	-	-	-	+	-	-	PCA	23	DL
Myxotrichum deflexum	109888	-	-	-	-	+	+	-	MA	23	DL
M. thaxteri	83470	-	-	-	-	-	-	+	PCA	23	DL
Nectria dealbata	53165	-	-	-	-	+	-	-	PSA	23	BL
N. episphaeria	86977	-	+	-	-	-	-	-	PSA	23	BL
N. freycinetiae	208153	-	-	-	-	+	-	+	PSA	23	BL
N. fuckeliana	277828	-	-	-	-	-	+	-	PSA	23	BL
N. gliocladioides	71095	-	-	-	-	+	-	-	PSA	23	BL
N. mammoidea	120337	-	-	-	-	+	+	-	PSA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Nectria peristomialis	208155	-	-	-	-	+	-	+	PSA	23	BL
N. pertusa	208156	-	-	-	-	+	-	-	PSA	23	BL
N. phormiicola	208158	-	-	-	-	-	-	+	PSA	23	BL
Nematoctonus haptocladus	129984	-	-	-	-	-	-	+	PCA	23	DL
Neocosmospora vasinfecta	277708	-	-	-	-	-	+	-	PDA	23	BL
Neurospora crassa	19419	-	-	+	+	+	-	-	CMA	23	DL
N. crassa	53239	-	-	-	-	+	-	-	CMA	23	DL
N. crassa	68614ii	-	-	+	+	+	-	+	CMA	23	DL
N. crassa	147001	-	-	+	+	+	-	-	OA	23	DL
N. sitophila	60354	-	-	-	-	-	-	+	CMA	23	DL
Nigrospora sphaerica	46685	-	-	-	+	+	+	-	PCA	23	DL
Nodulisporium argillaceum	107226	-	-	-	-	+	-	-	CMA	23	DL
Nomuraea atypicola	186963	-	-	+	+	+	-	-	OA	23	DL
Nummularia dennisii	245192	-	-	-	-	+	-	+	MA	23	BL
N. discreta	233095	-	-	-	+	+	+	+	MA	23	BL
Oidiodendron chlamydosporicum	131498	-	-	-	-	+	-	-	MA	23	BL
O. flavum	184623	-	-	-	-	+	+	-	MA	23	DL
O. periconioides	131497	-	-	-	-	+	+	-	PDA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Olpitrichum tenellum	89327	-	-	-	-	+	-	+	PCA	23	DL
Onychophora coprophila	275663	-	-	-	-	-	+	-	RDA	23	DL
Ophiobolus oryzinus	88663	-	-	-	-	+	-	-	TWA+W	23	DL
Ophionectria trichospora	166077	-	-	-	-	+	-	+	PDA	23	DL
Otthia lisae	122089	-	-	-	-	+	-	-	CMA	23	DL
Paecilomyces dactylethromorphus	65752	-	-	-	-	+	-	-	PDA	23	DL
P. farinosus	276202	-	-	-	-	-	+	-	PDA	23	DL
P. marquandii	39815	-	-	-	-	-	+	-	MCZ	23	DL
P. niphetodes	136368	-	-	-	-	+	-	-	MA	23	DL
P. variotii	108007	-	-	+	+	+	-	-	PDA	23	DL
P. variottii	276211	-	-	-	-	-	+	-	MCZ	23	DL
Panus stypticus	144612	-	-	-	-	+	-	-	MA	23	DL
Papulaspora byssina	275864	-	-	-	-	-	+	-	MA	23	BL
Penicillifer pulcher	134023	-	-	-	-	+	-	-	MCZ	23	DL
P. pulcher	162653	-	-	-	-	+	-	+	MCZ	23	DL
Penicilliopsis clavariaeformis	60372	-	-	-	-	-	-	+	MCZ	23	DL
Penicillium aculeatum	133243	-	-	-	-	+	+	-	CZ	23	DL
P. aculeatum	186297	-	-	-	-	+	+	-	CZ	23	DL
P. aromaticum	129964	-	-	-	-	+	+	-	CZ	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Penicillium asperosporum	80450ii	-	-	-	-	+	-	-	MCZ	23	DL
P. baarnense	40590	-	-	+	+	+	-	-	MA	23	DL
P. brefeldianum	153725	-	-	+	+	+	-	+	MCZ	23	DL
P. brevicompactum	17456	-	-	+	+	+	-	-	CZ	23	DL
P. canescens	149218	-	-	+	+	+	-	-	CZ	23	DL
P. capsulatum	272959	-	-	-	-	-	+	-	CZ	23	DL
P. capsulatum	274311	-	-	-	-	-	+	-	CZ	23	DL
P. chrysogenum	26210	-	-	+	+	+	-	-	CZ	23	DL
P. chrysogenum	416061i	-	-	-	-	+	+	-	CZ	23	DL
P. chrysogenum	92241	-	-	-	-	+	+	-	CZ	23	DL
P. chrysogenum	277731	-	-	-	-	-	+	-	CZ	23	DL
P. citrinum (white mutant)	274774	-	-	-	-	-	+	-	CZ	23	DL
P. claviforme	44744	-	-	+	+	+	-	-	PDA	23	DL
P. corymbiferum	68414	-	-	+	+	+	-	-	CZ	23	DL
P. coryophilum	101082	-	-	+	+	+	-	-	CZ	23	DL
P. coryophilum	273248	-	-	-	-	-	+	-	CZ	23	DL
P. cyclopium	19759	-	-	+	+	+	-	-	CZ	23	DL
P. cyclopium	68236	-	-	-	-	+	-	-	CZ	23	DL
P. cyclopium	276203	-	-	-	-	-	+	-	CZ	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Penicillium daleae	89338	-	-	-	-	+	+	-	CZ	23	DL
P. digitatum	91956	-	-	+	+	+	-	-	CZ	23	DL
P. digitatum	92217	-	-	+	+	+	+	-	CZ	23	DL
P. dimorphosporum	149680	-	-	-	-	-	-	+	CZ	23	DL
P. expansum	39761	-	-	-	-	+	+	-	CZ	23	DL
P. expansum	191205	-	-	+	+	+	-	-	CZ	23	DL
P. fellutanum	68224	-	-	-	-	+	-	-	MCZ	23	DL
P. funiculosum	114933	-	-	-	-	+	+	-	CZ	23	DL
P. helicum	197479	-	-	+	+	+	-	+	CZ	23	DL
P. idahoense	148393	-	-	+	+	+	-	-	CZ	23	DL
P. implicatum	99927	-	-	-	-	-	+	-	CZ	23	DL
P. isariforme	205074	-	-	-	-	+	+	-	CZ	23	DL
P. italicum	181050	-	-	-	-	+	+	-	CZ	23	DL
P. janthinellum	75589	-	-	-	-	-	+	-	CZ	23	DL
P. janthinellum	108033	-	-	+	+	+	-	-	CZ	23	DL
P. luteum	95152	-	-	+	+	+	-	+	CZ	23	DL
P. luteum	112513	-	-	+	+	+	-	-	CZ	23	DL
P. melinii	119893	-	-	-	-	-	+	-	CZ	23	DL
P. nigricans	96660	-	-	-	-	+	-	-	CZ	23	DL
P. notatum	15378	-	-	+	+	+	-	+	CZ	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Penicillium notatum	39759	-	-	-	-	+	+	-	CZ	23	DL
P. ochrochloron	61271	-	-	-	-	+	+	-	CZ	23	DL
P. olsonii	274654	-	-	-	-	-	+	-	CZ	23	DL
P. oxalicum	39750	-	-	-	-	+	+	-	CZ	23	DL
P. paraherquei	68220	-	-	-	-	+	-	-	CZ	23	DL
P. purpurogenum	90178	-	-	-	-	+	+	-	CZ	23	DL
P. raperi	71625	-	-	-	-	+	-	-	CZ	23	DL
P. roquefortii	129207	-	-	-	-	+	-	-	CZ	23	DL
P. rugulosum	40041	-	-	-	-	+	+	-	CZ	23	DL
P. spinuloramigenum	68617	-	-	-	-	+	-	-	CZ	23	DL
P. stecki	72029	-	-	-	-	+	-	-	CZ	23	DL
P. stolkiae	136210	-	-	-	-	+	+	-	CZ	23	DL
P. stoloniferum	143520	-	-	-	-	+	+	-	CZ	23	DL
P. viridicatum	200310	-	-	-	-	+	+	-	CZ	23	DL
P. wortmannii	40047	-	-	+	+	+	-	+	CZ	23	DL
Pestalotiopsis gracilis	69749	-	-	-	-	+	-	-	PCA	23	BL
P. sydowiana	82405a	-	-	-	-	+	-	-	PCA	23	BL
Pestalozziella parva	124039	-	-	+	+	+	-	+	MCZ	23	DL
Pezicula alba	68994	-	-	-	-	+	-	+	CMA	23	DL
P. cinnamomea	280102	-	-	-	-	-	+	-	PDA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Petriellidium fusoidem	271728	-	-	-	-	-	+	-	PCA	23	DL
Peziza brunneoatra	137181	-	-	-	-	+	-	-	RDA	23	DL
P. ostracoderma	60977	-	-	+	+	+	-	-	RDA	15	NL
P.ostracoderma	61802	-	-	-	-	+	-	-	RDA	23	DL
P. trachycarpa	59800	-	-	-	-	-	-	+	RDA	23	DL
Phacidiopsis tuberivora	79162	-	-	-	-	+	-	-	OA	23	DL
Phaeodactylium alpiniae	276708	-	-	-	-	-	+	-	PCA	23	DL
Phaeoisariopsis griseola	137804	-	-	-	-	+	-	-	MA	23	DL
P. griseola	144489	-	-	-	-	+	-	-	MA	23	DL
Phaeoseptoria musae	187050	-	-	-	-	+	-	-	OA	23	DL
Phaeotrichoconis crotalariae	69755	-	-	-	-	+	-	-	PCA	23	DL
Phialomyces macrosporus	110130	-	-	-	-	+	-	-	MA	23	DL
Phialophora lignicola	96746	-	-	-	-	+	-	-	PCA	23	DL
Phlyctochytrium arcticum	143636	-	-	-	-	-	-	+	ONION	23	DL
P. plurigibbosum	143638	-	-	-	-	-	-	+	ONION	23	DL
P. plurigibbosum	143639	-	-	-	-	-	-	+	ONION	23	DL
P. reinboldtae	143635	-	-	-	-	-	-	+	ONION	23	DL
Phoma destructiva	188639	-	-	-	-	+	+	-	OA	23	DL
P. epicoccina	164070	-	-	+	+	+	-	+	OA	23	BL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Phoma eupyrena	282253	-	-	-	-	-	+	-	OA	23	BL
P. glomerata	202773	-	-	-	-	-	-	+	OA	23	BL
P. herbarum	156652	-	-	+	+	+	-	+	PCA+FP	23	BL
P. herbarum	276968	-	-	-	-	-	+	-	OA	23	BL
P. nebulosa	282252	-	-	-	-	-	+	-	OA	23	BL
P. violacea	499481i	-	-	+	+	+	-	+	OA	23	DL
Phomopsis castanea	278057	-	-	-	-	-	+	-	PSA	23	BL
P. cocoina	277002	-	-	-	-	-	+	-	OA	23	BL
P. cocoina	280133	-	-	-	-	-	+	-	OA	23	BL
P. coffeae	277260	-	-	-	-	-	+	-	OA	23	BL
P. folliculicola	279970	-	-	-	-	-	+	-	OA	23	BL
P. malvacearum	279619	-	-	-	-	-	+	-	OA	23	BL
P. mangiferae	280033	-	-	-	-	-	+	-	OA	23	BL
P. oncostoma	68344	-	-	-	-	+	-	-	OA	23	DL
P. sclerotioides	151823	-	-	-	-	+	-	+	OA	23	BL
Phycomyces blakesleeanus	63219	-	-	-	-	+	+	-	PDA	23	DL
P. blakesleeanus	118496	-	-	+	+	+	-	+	PDA	23	DL
P. blakesleeanus	118497	-	-	+	+	+	-	-	PDA	23	DL
P. nitens	281611	-	-	-	-	-	+	-	PDA	23	DL
Phylosticta elettariae	277261	-	-	-	-	-	+	-	OA	23	BL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

		Storage method							Media & conditions		
	IMI Number	W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Phytophthora arecae	62655	+	-	-	-	-	-	+	OA	23	DL
P. arecae	62655	+	-	-	-	+	-	-	OA	23	DL
P. boehmeriae	32199	-	-	-	-	+	-	-	OA	23	DL
P. botryosa	136915	+	-	-	-	-	-	+	OA	23	DL
P. botryosa	136916	+	-	-	-	-	-	+	OA	23	DL
P. botryosa	189728	-	-	-	-	-	-	+	OA	23	DL
P. cactorum	21168	+	-	+	+	+	+	+	OA	23	DL
P. cactorum	49562	+	-	-	-	-	-	+	OA	23	DL
P. cactorum	62471	+	-	-	-	-	-	+	OA	23	DL
P. cactorum	129909	-	-	-	-	+	-	+	OA	23	DL
P. cactorum	242091	+	-	-	-	-	-	-	OA	23	DL
P. cambivora	40505	+	-	-	-	+	-	-	OA	23	DL
P. cambivora	77374	+	-	-	-	-	-	+	OA	23	DL
P. capsici	40502	-	-	-	-	-	-	+	OA	23	DL
P. capsici	45528	+	-	-	-	-	-	+	OA	23	DL
P. capsici	130937	-	-	-	-	-	-	+	OA	23	DL
P. cinnamomi	22938	+	-	-	-	-	-	+	OA	23	DL
P. cinnamomi	40506	-	-	-	-	+	-	-	OA	23	DL
P. cinnamomi	158786	+	-	-	-	-	-	+	OA	23	DL
P. cinnamomi	211105	+	-	-	-	-	-	+	OA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Phytophthora cinnamomi	230381	+	-	-	-	-	-	+	OA	23	DL
P. citricola	21173	+	-	-	-	-	-	+	OA	23	DL
P. citricola	45571	-	-	-	-	+	-	-	OA	23	DL
P. citricola	202319	+	-	-	-	-	-	+	OA	23	DL
P. citrophthora	132217	-	-	-	-	-	-	+	OA	23	DL
P. colocasiae	143253	+	-	-	-	-	-	+	OA	23	DL
P. cryptogea	21278	+	-	-	-	-	-	+	OA	23	DL
P. cryptogea	45168	+	-	-	-	+	-	+	OA	23	DL
P. cryptogea	152646	+	-	-	-	-	-	+	OA	23	DL
P. drechsleri	40499	+	-	-	-	-	-	+	OA	23	DL
P. drechsleri	40500	-	-	-	-	+	-	-	OA	23	DL
P. drechsleri	77969	+	-	-	-	-	-	+	OA	23	DL
P. drechsleri	136534	+	-	-	-	-	-	+	OA	23	DL
P. erythroseptica	17028	+	-	-	-	-	-	+	OA	23	DL
P. erythroseptica	34684	+	-	-	-	+	-	+	OA	23	DL
P. erythroseptica	139360	+	-	-	-	-	-	-	OA	23	DL
P. erythroseptica	181716	+	-	-	-	-	-	+	OA	23	DL
P. fragariae	131557	-	-	-	-	-	-	+	OA	23	DL
P. fragariae	181417	-	-	-	-	-	-	+	OA	23	DL
P. heveae	131093	+	-	-	-	-	-	+	OA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Phytophthora heveae	131372	+	-	-	-	-	-	-	OA	23	DL
P. heveae	147352	+	-	-	-	-	-	+	OA	23	DL
P. heveae	208224	-	-	-	-	-	-	+	OA	23	DL
P. heveae	210111	-	-	-	-	-	-	+	OA	23	DL
P. hibernalis	134760	+	-	-	-	-	-	-	OA	23	DL
P. infestans	181530	+	-	-	-	-	-	+	OA	23	DL
P. infestans	259084	-	-	-	-	-	-	+	OA	23	DL
P. iranica	158964	+	-	-	-	-	-	+	OA	23	DL
P. lateralis	40503	-	-	-	-	-	-	+	OA	23	DL
P. meadii	36529	+	-	-	-	+	-	-	OA	23	DL
P. meadii	129185	+	-	-	-	-	-	+	OA	23	DL
P. meadii	130427	+	-	-	-	-	-	-	OA	23	DL
P. megasperma	32035	+	-	-	-	-	-	+	OA	23	DL
P. megasperma	56348	-	-	-	-	+	-	+	OA	23	DL
P. megasperma	144023	+	-	-	-	-	-	-	OA	23	DL
P. megasperma	131375	+	-	-	-	-	-	+	OA	23	DL
P. megasperma	131555	+	-	-	-	-	-	+	OA	23	DL
P. mexicana	92550	-	-	-	-	-	-	+	OA	23	DL
P. nicotianae	21276	+	-	-	-	-	-	-	OA	23	DL
P. nicotianae	21279	+	-	-	-	+	-	+	OA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Phytophthora nicotianae	35087	+	-	-	-	+	-	-	OA	23	DL
P. nicotianae	77972	+	-	-	+	+	+	+	OA	23	DL
P. nicotianae	130899	+	-	-	-	-	-	-	OA	23	DL
P. nicotianae	158733	+	-	-	-	-	-	+	OA	23	DL
P. nicotianae	205751	+	-	-	-	-	-	-	OA	23	DL
P. nicotianae	207770	+	-	-	-	-	-	-	OA	23	DL
P. palmivora	46333	+	-	-	-	-	-	+	OA	23	DL
P. palmivora	63555	-	-	-	-	+	-	-	OA	23	DL
P. palmivora	80298	+	-	-	-	-	-	+	OA	23	DL
P. palmivora	182592	-	-	-	+	-	+	-	OA	23	DL
P. palmivora	189724	-	-	-	-	-	-	+	OA	23	DL
P. palmivora	189727	-	-	-	-	-	-	+	OA	23	DL
P. palmivora	198928	-	-	-	-	-	-	+	OA	23	DL
P. palmivora	202077	+	-	-	-	-	-	-	OA	23	DL
P. palmivora	202528	-	-	-	+	+*	+	+	OA	23	DL
P. palmivora	202542	-	-	-	-	-	-	+	OA	23	DL
P. palmivora	202544	-	-	-	-	-	-	+	OA	23	DL
P. palmivora	203531	-	-	-	-	-	-	+	OA	23	DL
P. palmivora	203532	-	-	-	-	-	-	+	OA	23	DL
P. palmivora	203536	-	-	-	-	-	-	+	OA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested; *, 2 stage cooling, vacuum drying technique;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Phytophthora palmivora	203538	-	-	-	-	-	-	+	OA	23	DL
P. palmivora	206790	+	-	-	-	-	-	+	OA	23	DL
P. palmivora	270386	-	-	-	-	-	-	+	OA	23	DL
P. porri	208979	+	-	-	-	-	-	-	OA	23	DL
P. syringae	38915	+	-	-	-	-	-	-	OA	23	DL
P. syringae	62472	+	-	-	-	-	-	-	OA	23	DL
P. syringae	131190	+	-	-	-	-	-	-	OA	23	DL
P. syringae	131191	+	-	-	-	-	-	-	OA	23	DL
P. vesicula	139645	+	-	-	-	-	-	-	OA	23	DL
Phytophthora sp.	241683	+	-	-	-	-	-	-	OA	23	DL
Piedraia quintanilhae	101644	-	-	-	-	+	-	-	OA	23	DL
Piptocephalis unispora	119340ii	-	-	-	-	-	-	+	YPSS	23	DL
P. virginiana	709101i	-	-	+	+	+	+	+	MA	23	DL
P. xenophila	156650	-	-	+	+	+	-	+	PDA	23	DL
Pithomyces valparadisiacus	281203	-	-	-	-	-	+	-	PDA	23	DL
Platysomum compressum	143286	-	-	-	-	+	-	-	PCA+FP	23	BL
Plectophomella visci	199474	-	-	-	-	-	-	+	CMA	23	DL
Pleospora herbarum	261031	-	-	-	-	-	+	-	TWA+W	23	BL
P. infectoria	173200	-	-	+	+	+	-	+	TWA+W	23	BL
P. scirpicola	117653	-	-	-	-	+	-	-	PCA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Podospora austro-americana	174498	-	-	-	-	-	-	+	RDA	23	DL
P. curvispora	175246	-	-	-	-	+	+	-	RDA	23	BL
P. horridula	263268	-	-	-	-	-	+	-	RDA	23	DL
P. pauciseta	280368	-	-	-	-	-	+	-	RDA	23	DL
P. pauciseta	280389	-	-	-	-	-	+	-	RDA	23	DL
Polypaecilium insolitum	75202	-	-	-	-	+	-	-	MA	30	NL
Polyschema olivacea	157787	-	-	-	-	+	-	-	PCA	23	DL
P. terricola	114592	-	-	-	-	+	-	-	PDA	23	DL
Polyscytalum pustulans	197208	-	-	-	-	-	+	-	CMA	23	DL
Polyporus betulina	144618	-	-	-	-	+	-	+	MA	23	DL
Poria vaillantii	147444	-	-	-	-	+	-	+	MA	23	DL
Preussia terricola	109539	-	-	-	-	-	-	+	CMA	15	NL
Protoachlya paradoxa	137391	-	-	-	-	-	-	+	ONION	23	DL
Pseudocercospora karaka	166178	-	-	-	-	+	-	+	V8	15	NL
Pseudophaeolus baudonii	ODA641	-	-	-	-	-	-	+	MA	23	DL
Puccinia graminis	174499	-	-	-	-	+	-	+	MA	23	DL
P. graminis	174502	-	-	-	-	-	-	+	MA	23	DL
P. graminis	174503	-	-	-	-	-	-	+	MA	23	DL
P. graminis	174504	-	-	-	-	-	-	+	MA	23	DL
P. graminis	174505	-	-	-	-	-	-	+	MA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Puccinia graminis	174507	-	-	-	-	-	-	+	MA	23	DL
P. graminis	174508	-	-	-	-	-	-	+	MA	23	DL
P. graminis	174509	-	-	-	-	+	-	+	MA	23	DL
P. paupercula	121021	-	-	-	-	+	-	+	MA	23	DL
Pycnoporus sanguineus	75002	-	-	-	-	+	-	-	MA	23	DL
Pyrenochaeta lycopersici	136604	-	-	-	-	+	-	-	OA	23	BL
Pyrenopeziza brassicae	204290	-	-	+	-	+	-	-	MA	15	NL
Pyrenophora avenae	134278	-	-	-	-	+	-	-	OA	23	DL
P. avenae	136454	-	-	-	-	+	-	-	TWA+W	23	BL
P. dictyoides	135811	-	-	-	-	+	-	+	TWA+W	23	BL
P. erythrospila	129760	-	-	+	+	+	-	-	OA	23	BL
P. graminea	135815	-	-	-	-	-	-	+	TWA+W	23	BL
Pyricularia zingiberi	195407	-	-	-	-	-	-	+	PCA	23	BL
Pyronema domesticum	57472	-	-	+	+	+	-	+	PCA+FP	23	DL
Pythium acanthicum	139143	-	-	-	-	-	-	+	OA	23	DL
P. aphanidermatum	58847	+	-	-	-	-	-	-	OA	23	DL
P. aphanidermatum	104926	+	-	-	-	-	-	+	OA	23	DL
P. aristosporum	209670	-	-	-	-	-	-	+	OA	23	DL
P. coloratum	181938	+	-	-	-	-	-	+	OA	23	DL
P. debaryanum	48558	+	-	+	+	+	-	-	OA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Pythium flevoense	176046	-	-	+	+	-	-	+	OA	23	DL
P. fluminum	212948	-	-	-	-	-	-	+	OA	23	DL
P. fluminum	212949	-	-	-	-	-	-	+	OA	23	DL
P. graminicola	34768	-	-	-	-	+	-	+	OA	23	DL
P. graminicola	91329	-	-	-	-	-	-	+	OA	23	DL
P. helicoides	61433	-	-	-	-	+	-	-	OA	23	DL
P. hydnosporum	147441	-	-	-	-	-	-	+	OA	23	DL
P. irregulare	203387	-	-	-	-	-	-	+	OA	23	DL
P. iwagamai	209669	-	-	-	-	-	-	+	OA	23	DL
P. mamillatum	45622	+	-	-	-	-	-	-	OA	23	DL
P. middletonii	42098	+	-	+	+	+	+	+	OA	23	DL
P. oligandrum	78731	-	-	-	-	-	-	+	OA	23	DL
P. oligandrum	133857	+	-	-	-	-	-	-	OA	23	DL
P. paroecandrum	92552	+	-	-	-	-	-	-	OA	23	DL
P. periplocum	202312	-	-	-	-	-	-	+	OA	23	DL
P. polymorphon	48559	-	-	-	-	+	-	+	OA	23	DL
P. splendens	61523	-	-	-	-	+	-	-	OA	23	DL
P. sulcatum	197678	-	-	-	-	-	-	+	OA	23	DL
P. sulcatum	197679	-	-	-	-	-	-	+	OA	23	DL
P. sylvaticum	248394	+	-	-	-	+*	-	+	OA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested; *, 2 stage cooling, vacuum drying technique;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Pythium sylvaticum	248395	+	-	-	-	+*	-	+	OA	23	DL
P. ultimum	82514	+	-	-	-	+	-	-	OA	23	DL
P. undulatum	35558	-	-	-	-	+	-	+	OA	23	DL
Quaternaria dissepta	146056	-	-	-	-	+	-	-	MA	23	BL
Ramichloridium subulatum	273184	-	-	-	-	-	+	-	MA	23	DL
R. subulatum	276193a	-	-	-	-	-	+	-	MA	23	BL
Ramularia deusta	101370	-	-	-	-	+	-	-	PCA	23	DL
Ramulispora sorghi	81785	-	-	-	-	+	-	-	MA	23	DL
R. zonata	129672	-	-	-	-	+	-	-	PCA	23	DL
Rhinocladiella atrovirens	277647	-	-	-	-	-	+	-	OA	23	DL
Rhizoclosmatium globosum	197677	-	-	-	-	-	-	+	OA	23	DL
Rhizoctonia carotae	162910	-	-	+	+	-	-	-	PDA	15	NL
R. lamellifera	83473	-	-	-	-	+	-	-	RDA	23	DL
R. solani	20697	-	-	+	+	+	-	+	PDA	23	DL
Rhizomucor meihei	125823	-	-	-	-	+	-	-	PDA	40	NL
R. meihei	126334	-	-	-	-	+	-	-	PDA	40	NL
R. pusillus	57407	-	-	-	-	+	+	-	PCA	37	NL
R. tauricus	137380	-	-	-	-	+	-	+	PDA	23	DL
Rhizophydium biporosum	170357	-	-	-	-	-	-	+	ONION	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested; *, 2 stage cooling, vacuum drying technique;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Rhizophydium capillaceum	143634	-	-	-	-	-	-	+	ONION	23	DL
R. capillaceum	155838	-	-	-	-	-	-	+	ONION	23	DL
R. chlorogonii	170538	-	-	-	-	-	-	+	ONION	23	DL
R. granulosporum	170356	-	-	-	-	-	-	+	ONION	23	DL
R. haynaldii	170359	-	-	-	-	-	-	+	ONION	23	DL
R. karlingii	143632	-	-	-	-	-	-	+	ONION	23	DL
R. patellarium	155839	-	-	-	-	-	-	+	ONION	23	DL
R. sphaerocarpum	143631	-	-	-	-	-	-	+	ONION	23	DL
R. sphaerotheca	143633	-	-	+	+	+	-	+	HEMP	23	DL
Rhizopus arrhizus	16641	-	-	-	-	-	+	-	PDA	23	DL
R. arrhizus	57412	-	-	-	-	+	+	-	PDA	23	DL
R. arrhizus	280098	-	-	-	-	-	+	-	PDA	23	DL
R. cohnii	39698	-	-	-	-	+	+	-	PDA	23	DL
R. homothallicus	89714	-	-	+	+	+	-	+	PDA	23	DL
R. homothallicus	280658	-	-	-	-	-	+	-	PDA	23	DL
R. microsporus	202612	-	-	-	-	+	+	-	MA	23	DL
R. oryzae	50109b	-	-	-	-	+	-	-	PDA	23	DL
R. rhizopodiformis	158738	-	-	+	+	+	+	-	PDA	23	DL
R. sexualis	103481	-	-	-	-	+	+	-	PDA	23	DL
R. tonkinensis	21601	-	-	-	-	-	+	-	PDA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Rhodotorula rubra	38784	-	-	+	+	+	-	-	MA	23	DL
Rigidoporus lignosus	83027	-	-	-	-	+	-	-	MA	23	DL
Robillarda sessilis	276578	-	-	-	-	-	+	-	PDA	23	BL
Rosellinia aquila	107929	-	-	-	-	+	-	+	MA	23	DL
R. aquila	146522	-	-	-	-	+	-	-	MA	23	DL
R. arcuata	107930	-	-	-	-	+	-	-	MA	23	DL
R. bunodes	107931	-	-	-	-	+	-	-	MA	23	DL
R. buxi	198634	-	-	-	-	+	-	+	MA	23	DL
R mammiformis	146524	-	-	-	-	+	-	-	PCA	23	DL
R. necatrix	107934	-	-	-	-	+	-	-	MA	23	DL
R. necatrix	108006	-	-	-	-	+	-	-	MA	23	DL
R. necatrix	108336	-	-	-	-	+	-	-	OA	23	DL
R. pepo	107935	-	-	-	-	+	-	+	MA	23	DL
Ryparobius polysporus	75299	-	-	+	+	+	-	-	RDA	23	DL
Saccharomyces cerevisiae	140023	-	-	+	+	+	-	-	MA	23	DL
Sagenomella griseoviridis	113160	-	-	-	-	+	-	-	V8	23	DL
Saprolegnia ferax	146489	-	-	+	+	+	-	+	ONION	23	DL
S. glomerata	146490	-	-	-	-	-	-	+	ONION	23	DL
S. litoralis	137393	-	-	-	-	-	-	+	ONION	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Saprolegnia parasitica	140977	-	-	-	-	+	-	-	ONION	23	DL
S. parasitica	169621	-	-	-	+	+*	+	-	ONION	23	DL
S. parasitica	191668	-	-	-	-	-	-	+	ONION	23	DL
Schizophylum commune	89295ii	-	-	+	+	+	-	+	MA	23	DL
S. radiatum	90347	-	-	+	+	+	-	-	MA	23	DL
Scirrhia pini	187703	-	-	-	-	+	-	-	MA	23	DL
Sclerotinia sclerotiorum	147201	-	-	+	+	-	-	+	OA	15	DL
Sclerotium delphinii	159926	-	-	+	+	+	-	+	PCA	23	DL
S. hydrophilum	253211	-	-	-	-	-	+	-	PDA	23	BL
S. hydrophilum	274690	-	-	-	-	-	+	-	PDA	23	BL
S. wakkeri	103548	-	-	-	-	+	+	+	OA+R	23	DL
Scopulariopsis canadensis	86938	-	-	-	-	+	-	-	MA	23	DL
S. carbonaria	86941	-	-	-	-	+	-	-	OA	23	DL
S. halophilica	184617	-	-	-	-	-	-	+	M/40	23	DL
Searchomyces caprophiloides	91832	-	-	-	-	+	-	-	MA	23	DL
S. caprophiloides	138638	-	-	-	-	+	-	-	MA	23	DL
Selinia pulchra	67947	-	-	-	-	+	-	-	MA	23	DL
Septofusidium elegantulum	147205	-	-	-	-	+	-	-	OA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested; *, 2 stage cooling, vacuum drying;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Septoria apiicola	92628	-	-	-	-	-	+	-	PDA	23	BL
S. apiicola	110277	-	-	-	-	+	+	-	PDA	23	DL
S. chrysanthemella	145558	-	-	-	-	-	-	+	OA+LUP	23	DL
S. cucurbitarum	275865	-	-	-	-	-	+	-	OA	23	BL
S. leucanthemi	91322	-	-	-	-	+	-	-	OA	23	DL
S. menthae	100278	-	-	-	-	+	-	-	CMA+W	23	DL
Serpula lacrimans	130942	-	-	-	-	-	-	+	MA	23	DL
S. lacrimans	152233	-	-	+	+	-	-	+	MA	23	DL
Setosphaeria prolata	181085	-	-	-	-	-	-	+	OA	23	BL
S. turcica	77392	-	-	-	-	+	+	+	OA	23	BL
S. turcica	113848	-	-	-	-	+	-	-	MA	23	DL
Sigmoidea marina	239282	-	-	-	-	+	-	-	PCA	23	DL
Sordaria bosensis	90324	-	-	-	-	+	-	-	RDA	23	DL
S. bosensis	110455	-	-	-	-	+	-	-	RDA	23	DL
S. fimicola (+)	105390	-	-	+	+	+	-	+	RDA	23	DL
S. fimicola (-)	105391	-	-	+	+	+	-	-	RDA	23	DL
Sphaceloma embeliae	92304	-	-	-	-	+	-	-	MA	15	NL
S. ixorae	92303	-	-	-	-	+	-	+	MA	15	NL
Sphaerobolus stellatus	155101	-	-	+	+	+	-	+	OA	25	DL
S. stellatus	155102	-	-	-	-	+	-	+	OA	23	DL

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Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Sphaerostilbe repens	84360	-	-	-	-	+	-	-	CMA	23	DL
S. repens	135503	-	-	-	-	+	-	+	PCA	23	DL
Spondylocladiopsis cupulicola	200611	-	-	-	-	-	-	+	PCA	23	DL
Sporendonema casei	68748a	-	-	-	-	+	-	-	M/20	23	DL
Sporidesmium flexum	246524	-	-	-	-	+	-	-	PCA	23	DL
S. tropicale	275328	-	-	-	-	+	+	-	PCA	23	BL
Sporobolomyces roseus	43529	-	-	+	+	+	-	-	MA	23	DL
Sporormiella intermedia	148830	-	-	+	+	+	-	-	PDA	23	DL
Sporothrix catenata	154711	-	-	-	-	+	-	-	PCA	23	DL
S. cyanescens	275373	-	-	-	-	-	+	-	MA	30	DL
S. cyanescens	276602	-	-	-	-	-	+	-	MA	30	DL
S. schenkii	275251	-	-	-	-	-	+	-	MA	30	DL
Sporotrichum pulverulenta	174727	-	-	-	-	+	+	-	MA	23	DL
Stachybotryna columnare	158980	-	-	+	+	-	-	-	TWA+W	23	BL
Stachtbotrys atra	82021	-	-	+	+	+	-	-	PCA+FP	23	DL
Stauronema cruciferum	275018	-	-	-	-	-	+	-	MA	23	DL
Stemphylium solani	280800	-	-	-	-	-	+	-	PCA	23	DL
S. triglochinicola	135460	-	-	-	-	+	-	-	PCA	23	DL
Stereum purpureum	62469	-	-	-	-	+	-	-	MA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Stereum purpureum	145560	-	-	-	-	+	-	-	MA	23	DL
Stilbella buquetii	268568	-	-	-	-	+	+	-	PDA	23	DL
S. thermophila	173315	-	-	-	-	+	-	-	YPSS	45	NL
Stilbum macrosporum	163252	-	-	+	+	+	-	-	MA	23	DL
Streptomyces griseus	50967	-	-	+	+	+	-	-	MA	23	DL
S. lisandri	137178	-	-	+	+	+	-	-	MA	23	DL
Sympodiella multiseptata	158984	-	-	-	-	+	-	+	MA	23	DL
Sympodiophora mycophila	158792	-	-	-	-	+	-	-	PCA	23	DL
S. stereicola	158407	-	-	-	-	-	-	+	MA	23	DL
Syncephalis sphaerica	212171	-	-	-	-	-	-	+	MA	23	DL
Syzigites megolocarpus	122577	-	-	-	-	+	-	-	PDA	23	DL
S. megalocarpus	231978	-	-	+	-	+	-	+	PDA	23	DL
Testudina terrestris	197216	-	-	-	-	-	-	+	MA	23	DL
Tetranacrium gramineum	83001	-	-	-	-	+	-	-	TWA+W	23	DL
Thamnidium elegans	43624	-	-	-	-	+	-	-	PDA	23	DL
Thanatephorus cucumeris	44226	-	-	-	-	+	-	-	RDA	23	DL
T. cucumeris	70816	-	-	-	-	+	-	-	RDA	23	DL
T. cucumeris	70827	-	-	-	-	+	-	-	RDA	23	DL
T. cucumeris	78354	-	-	-	-	+	-	-	RDA	23	DL
T. cucumeris	78358	-	-	-	-	+	-	-	RDA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Thanatephorus cucumeris	82071	-	-	-	-	+	-	-	RDA	23	DL
T. cucumeris	172732	-	-	-	-	+	+	+	RDA	23	DL
T. cucumeris	230993	-	-	-	-	+	+	-	PDA	23	DL
Thedgonia ligustrina	161114	-	-	-	-	-	-	+	PCA	4	NL
Thelebolus crustaceus	144389	-	-	-	-	+	-	-	RDA	23	DL
Thermoascus crustaceus	158740	-	-	-	-	+	-	-	YPSS	37	NL
T. crustaceus	158741	-	-	+	+	+	-	-	YPSS	37	NL
T. thermophilus	123299ii	-	-	-	-	-	-	+	OA	37	NL
Thielavia albomyces	125815	-	-	-	-	-	-	+	YPSS	30	NL
T. albomyces	126326	-	-	+	+	+	-	-	YPSS	40	NL
T. albomyces	131015	-	-	-	-	-	-	+	PDA	45	NL
T. albomyces	204245	-	-	-	-	-	-	+	OA	37	NL
T. fimeti	116692	-	-	+	+	+	-	-	PDA	23	DL
T. setosa	21599	-	-	-	-	+	-	-	RDA	23	DL
T. terricola	104951	-	-	+	+	+	-	+	TWA+W	23	BL
T. terricola	153731	-	-	+	+	+	-	+	TWA+W	23	BL
T. trichorobusta	130230	-	-	-	-	+	-	-	RDA	23	DL
Thielaviopsis basicola	125845	-	-	-	-	+	+	-	PCA	23	DL
T. basicola	278656	-	-	-	-	-	+	-	PCA	23	DL
Tolypocladium niveum	187376	-	-	-	-	+	+	-	MA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Tilletiopsis minor	56590	-	-	-	-	+	-	-	MA	23	DL
Trachysphaera fructigena	33913	-	-	-	-	+	-	-	OA	23	DL
Tretopileus sphaerophorus	141217	-	-	-	-	+	-	-	PCA	23	BL
Trichocladium lobatum	188290	-	-	-	-	+	-	-	MA	23	BL
Trichoderma harzianum	274332	-	-	-	-	-	+	-	PCA+FP	23	DL
T. harzianum	281112	-	-	-	-	-	+	-	PCA	23	DL
T. viride	45548	-	-	-	-	+	-	-	PCA	23	DL
T. viride	57421	-	-	+	+	+	-	-	PCA+FP	23	DL
T. viride	110138	-	-	-	-	+	+	-	PCA	23	DL
Trichophyton tehraniensis	223562	-	-	-	-	-	-	+	MA	23	DL
T. terrestre	277732	-	-	-	-	-	+	-	MA	23	DL
Trichothecium roseum	129425	-	-	+	+	+	-	-	PDA	23	DL
Tricladium malaysianum	177449	-	-	-	-	-	-	+	MA	23	DL
T. terrestre	177677	-	-	-	-	-	-	+	MA	23	DL
Tripospermum myrti	280413b	-	-	-	-	-	+	-	OA	25	NL
Tritirachium roseum	169856	-	-	+	+	+	-	-	PDA	23	DL
Tubakia japonica	157600	-	-	-	-	+	-	+	MA	30	NL
T. subglobosa	157596	-	-	-	-	+	-	+	MA	30	NL
Ustilaginoidea virens	165999	-	-	-	-	+	-	+	PCA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Ustilago hordei	161944	-	-	-	-	+	-	-	MA	23	DL
U. scitaminea	35616	-	-	-	-	+	-	-	PCA	23	DL
Ustulina deusta	108703	-	-	-	-	+	-	-	MA	23	DL
U. deusta	121510	-	-	-	-	+	+	+	MA	23	DL
U. deusta	146057	-	-	-	-	+	-	-	TWA+W	23	DL
U. deusta	193239	-	-	-	-	+	-	-	MA	23	DL
Venturia inaequalis	61538	-	-	-	-	+	-	+	MA	23	DL
Verticillium dahliae	45492	-	-	-	-	-	+	-	PCA	23	DL
V. dahliae	81822	-	-	+	+	+	-	-	OA	23	DL
V. dahliae	88631	-	-	-	-	+	-	-	MA	23	DL
V. nigrescens	2275294	-	-	-	-	-	+	-	OA	23	DL
V. nigrescens	278733	-	-	-	-	-	+	-	OA	23	DL
V. nubilum	130212	-	-	-	-	+	-	-	MA	23	DL
V. nubilum	278734	-	-	-	-	-	+	-	PCA	23	DL
V. psalliotae	276191	-	-	-	-	-	+	-	OA	23	DL
V. rexianum	276149	-	-	-	-	-	+	-	PCA	23	DL
V. theobromae	280163	-	-	-	-	-	+	-	PCA	23	DL
V. tricorpus	273799	-	-	-	-	-	+	-	OA	23	DL
V. tricorpus	276674	-	-	-	-	-	+	-	OA	23	DL
V. tricorpus	276676	-	-	-	-	-	+	-	OA	23	DL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

Appendix III (continued) The organisms tested, storage methods, growth media, temperature of incubation and light requirement

	IMI Number	Storage method							Media & conditions		
		W	S	SG	OIL	CFD	SFD	LN	MEDIUM	T	LIGHT
Volvariella esculenta	63833ii	-	-	-	-	+	-	-	MA	23	DL
V. esculenta	70680	-	-	-	-	+	-	-	MA	23	DL
Wallemia sebi	86292	-	-	-	-	-	-	+	M/20	23	DL
W. sebi	156385a	-	-	-	-	+	+	-	M/40	23	DL
Westerdykella multispora	276056	-	-	-	-	-	+	-	PDA	23	DL
Xylaria carpophila	146428	-	-	-	-	+	+	+	MA	23	DL
X. longipes	146055	-	-	-	-	+	-	+	MA	23	DL
X. polymorpha	146529	-	-	-	-	+	-	+	MA	23	DL
X. polymorpha	193238	-	-	-	-	+	-	-	MA	23	DL
X. sicula	73034	-	-	-	-	+	-	-	MA	23	DL
Zalerion maritima	81620	-	-	+	+	+	-	+	PCA	23	DL
Z. maritima	89317	-	-	-	-	+	-	-	MA	23	BL
Zopfiella vehmii	185021	-	-	-	-	+	-	-	EGG	23	DL
Z. leucotricha	153733	-	-	+	+	+	-	+	TWA+W	23	BL

W, water storage; S, Soil storage; SG, silica gel storage; CFD, centrifugal freeze drying; SFD, shelf freeze drying; LN, liquid nitrogen storage; T, temperature in degrees centigrade; Media abbreviations are given in section 2.02. BL, near ultraviolet light, DL, daylight; NL, incubated in a non-illuminated incubator; +, tested; -, not tested;

APPENDIX IV

A list of species that have been discarded from liquid nitrogen and have no representatives stored by this means at CMI

Name	IMI Number	Number attempts	Number viable in genus		
			species	isolates viable/tested	%
Achlya bisexualis	141473	3	3	6/42	14
A. bisexualis	141474	2	3	6/42	14
A. bisexualis	146646	2	3	6/42	14
A. bisexualis	146647	3	3	6/42	14
A. debaryana	161801	3	3	6/42	14
A. radiosa	137645	1	3	6/42	14
A. radiosa	137966	3	3	6/42	14
Allomyces arbuscula	152201	1	3	7/8	88
Anixiella endodonta	148368	1	5	6/8	75
A. endodonta	148369	2	5	6/8	75
Anthostomella spartii	185019	5	1	1/2	50
Arthrobotrys arthrobotryoides	96726	2	6	14/15	93
Arxiella terrestris	176392	2	0	0/1	0
Ascobolus winteri	145962	1	1	1/2	50
Ascosphaera major	160841	1	0	6/7	86
Balansia sclerotica	138634	2	0	0/1	0
Battarraea phalloides	151693	1	0	0/1	0
Beltraniella portoricensis	90992ii	1	1	1/2	50
Caloscypha fulgens	144877	1	0	0/1	0
Catenaria anguillulare	175996	1	0	0/1	0
Chlamydomyces palmarum	130823	1	0	0/1	0

Appendix IV (continued) A list of species that have been discarded from liquid nitrogen and have no representatives stored by this means at CMI

Name	IMI Number	Number attempts	Number viable in genus		
			species	isolates viable/tested	%
Chytridium olla	86666	2	0	0/3	0
C. ottariense	194741	1	0	0/3	0
Clathrospora diplospora	68086	1	0	0/1	0
Claviceps paspali	82999	1	1	1/2	50
Colletotrichum ampelinum	82267	1	17	25/26	96
Conidiobolus khandalensis	102045	3	11	12/14	86
C. stromatoideus	92298ii	1	11	12/14	86
Coprinus atramentarius	132648	3	10	11/14	79
C. macrocephalus	182008	3	10	11/14	79
Cunninghamella vesiculosa	93346	1	4	12/13	92
Dendryphiella infuscans	144005	1	1	1/2	50
Dictyuchus sterile	182416	1	1	0/2	0
Dispira cornuta	77590ii	1	1	1/2	50
Elaphomyces variegatus	213783	1	0	0/1	0
Entomophthora oligolophi	225446	1	4	4/5	80
Entosordaria perfidiosa	185020	1	0	0/1	0
Ganoderma philippii	108700	2	2	3/4	75
Glomerella tucumanensis	78362	1	1	3/5	60
Gymnoascus petalosporus	183752	1	0	0/1	0
Hymenula cerealis	80179	1	0	0/1	0
Hypoxylon confluens	93350	2	9	18/23	78

Appendix IV (continued) A list of species that have been discarded from liquid nitrogen and have no representatives stored by this means at CMI

Name	IMI Number	Number attempts	Number viable in genus		
			species	isolates viable/tested	%
Hypoxylon confluens	146053	3	9	18/23	78
H. confluens	193350	2	9	18/23	78
H. howeianum	192590	1	9	18/23	78
H. rutilum	198628	1	9	18/23	78
Idriella australiensis	149915	1	2	2/3	67
Isoachlya eccentrica	146648	1	0	0/1	0
Isthmotricladia gombakiensis	184588	2	1	1/2	50
Monacrosporium gephyrophagum	143688	1	5	8/9	89
Nematoctonus haptocladus	129984	1	1	1/2	50
Neurospora sitophila	60354	1	2	15/16	94
Penicillium dimorphosporum	149680	1	152	307/308	99
Peziza trachycarpa	59800	1	1	3/4	75
Phlyctochytrium plurigibbosum	143638	1	3	4/12	33
P. plurigibbosum	143639	1	3	4/12	33
P. reinboldtae	143635	1	3	4/12	33
Phoma glomerata	202773	1	24	43/44	98
Phomopsis sclerotioides	151823	1	14	27/28	96
Phytophthora citrophthora	132217	2	19	111/210	53
P. fragariae	131557	1	19	111/210	53
P. fragariae	181417	4	19	111/210	53
Phytophthora iranica	158964	1	19	111/210	53

Appendix IV (continued) A list of species that have been discarded from liquid nitrogen and have no representatives stored by this means at CMI

Name	IMI Number	Number attempts	Number viable in genus		
			species	isolates viable/tested	%
Phytophthora lateralis	40503	1	19	111/210	53
P. meadii	129185	1	19	111/210	53
P. mexicana	92550	1	19	111/210	53
P. palmivora	203532	2	19	111/210	53
Piptocephalis unispora	119340ii	1	14	24/25	96
Plectophomella visci	199474	1	1	2/3	67
Podospora austro-americana	174498	1	3	3/4	75
Poria vaillantii	147444	2	1	1/2	50
Preussia terricola	109539	1	4	4/5	80
Protoachlya paradoxa	137391	3	0	0/3	0
Puccinia graminis	174499	4	0	0/8	0
P. graminis	174502	1	0	0/8	0
P. graminis	174503	1	0	0/8	0
P. graminis	174504	1	0	0/8	0
P. graminis	174505	1	0	0/8	0
P. graminis	174507	1	0	0/8	0
P. graminis	174508	5	0	0/8	0
P. paupercula	121021	1	0	0/8	0
Pyrenophora graminea	135815	1	4	7/8	88
Pyricularia zingiberi	195407	2	2	2/3	67
Pythium acanthicum	139143	1	18	29/58	50

Appendix IV (continued) A list of species that have been discarded from liquid nitrogen and have no representatives stored by this means at CMI

Name	IMI Number	Number attempts	Number viable in genus		
			species	isolates	
				viable/tested	%
Pythium aristosporum	209670	4	18	29/58	50
P. fluminum	212948	2	18	29/58	50
P. fluminum	212949	2	18	29/58	50
P. graminicola	34768	1	18	29/58	50
P. graminicola	91329	1	18	29/58	50
P. hydnosporum	147441	2	18	29/58	50
P. iwagamai	209669	5	18	29/58	50
P. oligandrum	78731	1	18	29/58	50
P. oligandrum	133857	1	18	29/58	50
P. periplocum	202312	1	18	29/58	50
P. sulcatum	197678	1	18	29/58	50
P. sulcatum	197679	1	18	29/58	50
Rhizoclosmatium globosum	197677	3	0	0/1	0
Rhizophidium capillaceum	143634	1	1	1/20	5
R. capillaceum	155838	2	1	1/20	5
R. chlorogonii	170538	1	1	1/20	5
R. granulosporum	170356	3	1	1/20	5
R. haynaldii	170359	3	1	1/20	5
R. karlingii	143632	1	1	1/20	5
R. patellarium	155839	3	1	1/20	5
R. sphaerocarpum	143631	2	1	1/20	5

Appendix IV (continued) A list of species that have been discarded from liquid nitrogen and have no representatives stored by this means at CMI

Name	IMI Number	Number attempts	Number viable in genus		
			species	isolates	
				viable/tested	%
Rhizophidium sphaerotheca	143633	6	1	1/20	5
Rosellinia buxi	198634	2	3	5/6	83
Saprolegnia glomerata	146490	1	3	4/16	25
S. litoralis	137393	3	3	4/16	25
Scopulariopsis halophilica	184617	2	8	9/10	90
Septoria chrysanthemella	145558	1	4	5/6	83
Serpula lacrimans	152233	4	0	1/2	50
Setosphaeria turcica	77392	1	0	0/1	0
Spondylocladiopsis cupulicola	200611	1	0	0/1	0
Sympodiophora stereicola	158407	1	1	1/2	50
Syncephalis sphaerica	212171	2	4	6/7	86
Thedgonia ligustrina	161114	1	32	42/48	88
Thermoascus thermophilus	123299ii	1	2	13/14	93
Thielavia albomyces	125815	1	10	17/21	81
T. albomyces	126326	1	10	17/21	81
T. albomyces	131015	1	10	17/21	81
T. albomyces	204245	1	10	17/21	81
Trichophyton tehraniensis	223562	1	2	12/13	92
Tricladium malaysianum	177449	2	2	2/4	50
T. terrestre	177677	1	2	2/4	50
Wallemia sebi	86292	2	0	0/1	0
Xylaria polymorpha	146529	1	3	6/7	86

APPENDIX V

Viabilities of fungi freeze dried by the developed routine technique on the Minifast 3400 compared with centrifugal freeze drying

Name	IMI Number	Freeze drying method Growth and sporulation		
		Centrifugal and period stored before test (years)		Minifast
Absidia spinosa	68077	N	5	N
Acremonium alternatum	166204	F	11	-
A. strictum	178506	R	10	N
A. strictum	276794	-		-
A. padwickii	136436a	AN	10	N
Akenomyces costatus	279296	N	1	N
Arthrocristula hyphenata	272976	-		-
Ascochyta fabae	156657	R	9	F
A. pisi	141220	N	14	F
Aspergillus flavus	91019b	N	7	N
A. flavus	91019bii	N	10	N
A. flavus	91456	N	1	N
A. fumigatus	174456	N	10	N
A. niger	75353ii	N	16	N
A. niger	149007	N	6	N
A. quercinus	95251	R	15	R
A. sclerotiorum	67759b	N	15	N
A. sclerotiorum	112328	F	12	N

Growth and sporulation, Normal. N; Normal but reduced, R; Further reduced but normal, F; Abnormal, AN; Failed, -;

Appendix V (continued) Viabilities of fungi freeze dried by the developed routine technique on the Minifast 3400 compared with centrifugal freeze drying

Name	IMI Number	Freeze drying method Growth and sporulation		
		Centrifugal and period stored before test (years)		Minifast
Aspergillus sclerotiorum	191603	R	8	N
A. stellatus	136778	N	7	N
Aureobasidium pullulans	70103	R	6	R
Auxarthron zuffianum	76603	R	7	R
Bipolaris indica	164633	N	(11D)10	N
B. nicotiae	202589	N	8	N
Botrytis cinerea	100465	N	4	N
Chaetomium bostrychodes	139638	N	14	N
C. gracile	84227	F	9	R
C. globosum	16203	N	6	N
Choanephora cucurbitarum	164967	N	11	N
Cladosporium cladosporioides	45534	N	5	N
C. cucumerinum	249540	R	3	N
C. herbarum	49627	R	10	N
Cochliobolus tuberculata	99410	N	11	N
Coemansia pectinata	142377	N	6	N
Coniothyrium minitans	134523ii	N	6	N
Cunninghamella echinulata	199844	N	7	N
C. elegans	188670	N	9	N

Growth and sporulation, Normal. N; Normal but reduced, R; Further reduced but normal, F; Abnormal, AN; Failed, -;

Appendix V (continued) Viabilities of fungi freeze dried by the developed routine technique on the Minifast 3400 compared with centrifugal freeze drying

Name	IMI Number	Freeze drying method Growth and sporulation		
		Centrifugal and period stored before test (years)		Minifast
Curvularia borreriae	155733	N	12	N
C. crepinii	605	N	7	R
C. deightonii	148188	N	13	F
Diaporthe phaseolorum	158864	N	13	N
Drechslera heveae	163331	F	(4D)10	R
Eremascus albus	100446a	N	7	N
Fulvia fulva	54976	N	10	N
Fusarium avenaceum	272884	N		-
F. culmorum	14764	R	4	-
F. culmorum	272853	N	1	-
F. fujikuroi	202879	N	1	N
F. graminearum	160243	N	7	N
F. oxysporum	159029	N	(4P)10	N
F. poae	272890	N	(5.5%)	-
Ganoderma applanatum	157818	-		-
Humicola grisea	149015	N	7	N
H. insolens	126330	R	11	N
Martensiomyces pterosporus	60573	AN	4	-
Metarhizium anisopliae	152223	N	12	N

Growth and sporulation, Normal. N; Normal but reduced, R; Further reduced but normal, F; Abnormal, AN; Failed, -;

Appendix V (continued) Viabilities of fungi freeze dried by the developed routine technique on the Minifast 3400 compared with centrifugal freeze drying

Name	IMI Number	Freeze drying method Growth and sporulation		
		Centrifugal and period stored before test (years)		Minifast
Metarhizium anisopliae	177416	N	8	N
Monacrosporium mutabile	138221	N	7	N
Monilinia fructigena	143628	N	6	R
Mortierella ramanniana	144619	R	12	N
Mucor hiemalis	21216	N	16	N
M. hiemalis	21217	N	9	N
M. mucedo	184726	N	9	R
M. racemosus	17364	N	9	N
Myxotrichum deflexum	109888	N	13	N
Nectria mammoidea	120337	F	15	N
Oidiodendron flavum	184623	N	9	N
O. periconioides	131497	R	15	N
Penicillium aculeatum	133243	N	15	N
P. aculeatum	186297	N	8	N
P. aromaticum	129964	N	12	N
P. chrysogenum	41606ii	N	15	N
P. chrysogenum	92241	N	13	N
P. daleae	89338	N	8	N
P. digitatum	92217	N	8	N

Growth and sporulation, Normal. N; Normal but reduced, R; Further reduced but normal, F; Abnormal, AN; Failed, -;

Appendix V (continued) Viabilities of fungi freeze dried by the developed routine technique on the Minifast 3400 compared with centrifugal freeze drying

Name	IMI Number	Freeze drying method Growth and sporulation		
		Centrifugal and period stored before test (years)		Minifast
Penicillium expansum	39761	N	8	N
P. funiculosum	114933	N	13	N
P. isariforme	205074	N	7	N
P. italicum	181050	N	9	N
P. notatum	39759	N	3	R
P. oxalicum	39750	N	12	N
P. purpurogenum	90178	N	6	N
P. rugulosum	40041	N	4	N
P. stolkiae	136210	N	13	N
P. stoloniferum	143520	N	6	N
P. viridicatum	200310	N	7	R
Phoma destructiva	188639	N	9	N
Phycomyces blakesleeanus	63219	N	7	N
Piptocephalis virginiana*	70910ii	N	7	N
Podospora curvispora	175246	R	10	N
Rhizomucor pusillus	57407	N	7	R
Rhizopus arrhizus	57412	N	7	N
R. cohnii	39698	N	10	N

Growth and sporulation, Normal. N; Normal but reduced, R; Further reduced but normal, F; Abnormal, AN; Failed, -;
*Preserved with its host Circinella sp.

Appendix V (continued) Viabilities of fungi freeze dried by the developed routine technique on the Minifast 3400 compared with centrifugal freeze drying

Name	IMI Number	Freeze drying method Growth and sporulation		
		Centrifugal and period stored before test (years)		Minifast
Rhizopus microsporus	202612	N	7	N
R. rhizopodiformis	158738	R	12	R
R. sexualis	103481	N	5	N
Septoria apiicola	110277	N	6	F
Setosphaeria turcica	77392	R	16	N
Sporotrichum pulverulentum	174727	N	10	N
Stilbella buquetii	268568	N		-
Thanatephorus cucumeris	230993	-		-
Thielaviopsis basicola	125845	N	5	N
Tolypocladium niveum	187376	N	9	N
Trichoderma viride	110138	R	13	N
Wallemia sebi	156385a	N	11	N

Growth and sporulation, Normal. N; Normal but reduced, R; Further reduced but normal, F; Abnormal, AN; Failed, -;

APPENDIX VI

Viabilities of fungi freeze dried by the developed routine technique on the Minifast 3400

Name	IMI Number	Growth and sporulation
Acremonium recifei	277456	N
Acrospeira mirabilis	278058	N
Alternaria dianthi	280151	N
A. poonensis	278420	N
A. radicina	279636	N
A. zinnae	278126d	R
Arthroderma lenticularum	113772	N
Ascochytula obiones	282137	N
Ascosphaera osmophila	277755	N
Aspergillus alliaceus	275535	R
A. flavus	277248	N
A penicillioides	274334	N
A. sejunctus	274335	N
Aureobasidium microstictum	275632	R
A. pullulans	278350	N
Beauveria alba	278647	N
B. bassiana	262947	N
B. bassiana	282533	N
Bipolaris australis	261917	N
B. crustacea	276037	N
B. multiformis	281320	N

Growth and sporulation, normal, N; Reduced but normal, R; Further reduced but normal, F; Abnormal, AN.

Appendix VI (continued) Viabilities of fungi freeze dried by the developed routine technique on the Minifast 3400

Name	IMI Number	Growth and sporulation
Bipolaris ovariicola	261919	R
Blakeslea trispora	283659	N
Calyptella campanula	262248	AN
Ceratocystis autographa	173177	N
C. falcata	274631	N
C. pilifera	274632	N
Chaetomium anguipilium	281165	N
C. cochliodes	279189	N
C. cupreum	279208	N
C. dreyfussii	281164	N
C. elatum	280816	N
C. jodhpurense	276987	N
C. mollicellum	276168	N
C. murorum	279726	N
C. quizotiae	279573	N
C. reflexum	279252	N
C. robustum	281166	N
Chlamydomyces palmarum	278355	N
Choanephora cucurbitarum	276406	N
Chrysosporium synchronum	282433	N
Circinella mucoroides	276533	N
Cladosporium allii-cepae	275851	N

Growth and sporulation, normal, N; Reduced but normal, R; Further reduced but normal, F; Abnormal, AN.

Appendix VI (continued) Viabilities of fungi freeze dried by the developed routine technique on the Minifast 3400

Name	IMI Number	Growth and sporulation
Cladosporium allii-cepae	275852	R
Claviceps purpurea	126133	F
Cochliobolus dactyloctenii	276040	N
C. dactyloctenii	276041	N
C. peregiarensis	264355	N
C. peregianensis	276042	N
C. peregianensis	276043	N
C. perotidis	264356	N
Coemansia erecta	279145	N
Colletotrichum sublineolum	275716	R
C. sublineolum	275720	N
C. sublineolum	279189	N
C. trichellum	279999	R
Cryphonectria cubensis	279614	N
C. eugeniae	279035	N
C. eugeniae	279036	R
C. eugeniae	279618	N
Cryptocline cinerescens	275743	N
Didymella bryoniae	280801	N
Dimargaris verticillata	278511	N
Drechslera brizae	276557	R
Embellisia abundans	279172	R

Growth and sporulation, normal, N; Reduced but normal, R; Further reduced but normal, F; Abnormal, AN.

Appendix VI (continued) Viabilities of fungi freeze dried by the developed routine technique on the Minifast 3400

Name	IMI Number	Growth and sporulation
Embellisia abundans	279181	F
E. hyacinthi	279179	N
E. indefessa	279175	N
E. planifunda	115034	N
E. planifunda	279178	N
E. telluster	279180	N
E. turnida	279176	N
Exophiala jeanselmei	279566	R
Fusariella concinna	277515	N
Fusarium avenaceum	273040	N
F. avenaceum	273492	N
F. culmorum	272853	N
F. culmorum	273711	N
F. crookwellense	281151	N
F. merismoides	279297	N
F. merismoides	280230	N
F. oxysporum	264170	N
F. poae	273481	N
F. solani	63862	R
F. sporotrichioides	281904	N
F. stilboides	276798	N
F. udum	275452	N

Growth and sporulation, normal, N; Reduced but normal, R; Further reduced but normal, F; Abnormal, AN.

Appendix VI (continued) Viabilities of fungi freeze dried by the developed routine technique on the Minifast 3400

Name	IMI Number	Growth and sporulation
Gaeumannomyces graminis	280418	R
Gelasinospora kobi	281199	N
Gilbertella persicaria	280841	N
Gliocladium roseum	278745	N
G. vermoesenii	279785	R
Glomerella cingulata	280101	N
Goidanichiella scopula	278477	AN
Gonytrichum macrocladium	278340	N
Guignardia cocoicola	280132	F
Heterocephalum aurantiacum	276848a	N
Macrophoma mangiferae	278638	N
Massariothea attenuata	280903	R
Metarhizium anisopliae	274954ii	R
Microdochium bolleyi	277137	N
M. bolleyi	278995	N
Mucor flavus	280011	N
M. hiemalis	276667	N
M. piriformis	276599	N
Mycosphaerella mori	281787	N
Mycotypha microspora	282443	N
Myriodontium Keratinophilum	273431	N
Nectria fuckeliana	277828	R

Growth and sporulation, normal, N; Reduced but normal, R; Further reduced but normal, F; Abnormal, AN.

Appendix VI (continued) Viabilities of fungi freeze dried by the developed routine technique on the Minifast 3400

Name	IMI Number	Growth and sporulation
Neocosmospora vasinfecta	277708	N
Onychophora coprophila	275663	R
Paecilomyces farinosus	276202	N
P. marquandii	39815	N
P. variottii	276211	N
Papulaspora byssina	275864	F
Penicillium capsulatum	272959	N
P. capsulatum	274311	N
P. chrysogenum	277731	N
P. citrinum (white mutant)	274774	N
P. cyclopium	276203	N
P. implicatum	99927	N
P. janthinellum	75589	R
P. melinii	119893	N
P. olsonii	274654	N
Pezicula cinnamomea	280102	N
Phaeodactylium alpiniae	276708	F
Phoma eupyrena	282253	N
P. herbarum	276968	N
P. nebulosa	282252	N
Phomopsis castanea	278057	R
P. cocoina	277002	N

Growth and sporulation, normal, N; Reduced but normal, R; Further reduced but normal, F; Abnormal, AN.

Appendix VI (continued) Viabilities of fungi freeze dried by the developed routine technique on the Minifast 3400

Name	IMI Number	Growth and sporulation
Phomopsis cocoina	280133	N
P. coffeae	277260	AN
P. folliculicola	279970	N
P. malvacearum	279619	R
P. mangiferae	280033	N
Phycomyces nitens	281611	N
Phylosticta elettariae	277261	N
Pithomyces valparadisiacus	281203	F
Podospora horridula	263268	R
P. pauciseta	280368	N
P. pauciseta	280389	N
Ramichloridium subulatum	276193a	N
Rhinocladiella atrovirens	277647	N
Rhizopus arrhizus	16641	N
R. arrhizus	280098	N
R. homothallicus	280658	N
R. tonkinensis	21601	N
Robillarda sessilis	276578	N
Sclerotium hydrophilum	253211	AN
S. hydrophilum	274690	AN
Septoria apiicola	92628	R
S. cucurbitarum	275865	N

Growth and sporulation, normal, N; Reduced but normal, R; Further reduced but normal, F; Abnormal, AN.

Appendix VI (continued) Viabilities of fungi freeze dried by the developed routine technique on the Minifast 3400

Name	IMI Number	Growth and sporulation
Sporothrix cyanescens	275373	N
S. cyanescens	276602	R
S. schenkii	275251	R
Stauronema cruciferum	275018	R
Stemphylium solani	280800	R
Syzigites megalocarpus	231978	N
Thielaviopsis basicola	278656	N
Trichoderma harzianum	274332	N
T. harzianum	281112	N
Trichophyton terrestre	277732	N
Tripospermum myrti	280413b	N
Verticillium dahliae	45492	N
V. nubilum	278734	N
V. nigrescens	2275294	R
V. nigrescens	278733	N
V. psalliotae	276191	R
V. rexianum	276149	R
V. theobromae	280163	N
V. tricorpus	273799	R
V. tricorpus	276674	N
V. tricorpus	276676	N
Westerdykella multispora	276056	N

Growth and sporulation, normal, N; Reduced but normal, R; Further reduced but normal, F; Abnormal, AN.

APPENDIX VII

The comparison of longevity in storage within species preserved by centrifugal freeze drying. A list of isolates that died in storage giving their successful storage period and the maximum recorded storage period of other strains of the same species that remain viable

Name	IMI Number	Survival period (years)	Other isolates processed remaining viable in storage	
			Number	Maximum storage period (years)
Acremonium chrysogenum	91579	3	6	5
A. furcatum	57398	5	1	4
Actinomucor elegans	56159	0.5	6	10
Alternaria alternata	89345	5	19	15
A. brassicae	156655	3	1	9
A. solani	129087	4	5	6
Anthostomella spartii	185019	2	0	0
Apiocrea chrysosperma	109891	0.25	3	6
Apiosordaria verruculosa	51496	6	3	8
Arthrobotrys cylindrospora	140015	6	1	5
A. scaphoides	50675	4	1	1
Ascochyta fimbriata	87300	0.5	0	0
Aspergillus amstelodami	17455	7	12	7
A. amstelodami	140508	0.3	12	7
A. amylovorus	129961	1	1	10
A. arenarius	55632ii	2	1	8
A. asperescens	46813	0.5	1	8
A. athecius	32048	3	2	8
A. caespitosus	16034	3	2	8

Appendix VII (continued) The comparison of longevity in storage within species preserved by centrifugal freeze drying. A list of isolates that died in storage giving their successful storage period and the maximum recorded storage period of other strains of the same species that remain viable

Name	IMI Number	Survival period (years)	Other isolates processed remaining viable in storage	
			Number	Maximum storage period (years)
Aspergillus crystallinus	139270	4	1	6
A. glaucus	53242	4	3	5
A. itaconicus	16119	6	1	6
A. nidulans	61454	6	63	15
A. parasiticus	15957ii	6	10	15
A. penicillioides	144121	5	9	4
A. peyronellii	139272	3	2	6
A. pulverulentus	91886	6	5	8
A. restrictus	140815	8	7	8
A. sejunctus	91862	2	14	8
Bartalinia bischofiae	81612	1	3	9
Basidiobolus microsporus	93345	6	0	0
Botryodiplodia ricinicola	145809	2	1	9
Calcarisporium thermophilum	144750	1	1	5
Calonectria rigiduscula	174223	2	0	0
Caloscypha fulgens	144877	0.6	0	0
Ceratocystis cainii	176523	2	1	3
C. doluminuta	176538	2	1	4
Cercospora fusimaculans	167426	2	0	0

Appendix VII (continued) The comparison of longevity in storage within species preserved by centrifugal freeze drying. A list of isolates that died in storage giving their successful storage period and the maximum recorded storage period of other strains of the same species that remain viable

Name	IMI Number	Survival period (years)	Other isolates processed remaining viable in storage	
			Number	Maximum storage period (years)
Cercospora olivascens	124975	3	0	0
Chaetocladium jonesii	190954	4	3	4
Chaetomium tenuissimum	81769	2	1	8
Choanephora cucurbitarum	121212	6	15	8
Circinella simplex	101093	3	8	11
Coprinus alkalinus	133856	2	0	0
Conidiobolus coronatus	145949	0.25	1	3
Coniochaeta leucoplaca	144079	5	1	3
Corynespora cassiicola	56007	4	3	9
Culcitalna achraspora	132773	2	1	5
Cunninghamella blakesleeana	53586	8	10	6
C. vesiculosa	93346	4	0	0
C. vesiculosa	93346ii	1	0	0
Curvularia fallax	79737	3	4	7
Cylindrocarpon obtusisporum	96731	5	2	5
C. olidum	182099	4	3	8
Dictyoarthrinium rabaulense	51264	4	1	7
Dipodascopsis uninucleatus	86676	7	0	0

Appendix VII (continued) The comparison of longevity in storage within species preserved by centrifugal freeze drying. A list of isolates that died in storage giving their successful storage period and the maximum recorded storage period of other strains of the same species that remain viable

Name	IMI Number	Survival period (years)	Other isolates processed remaining viable in storage	
			Number	Maximum storage period (years)
Doratomyces purpureofuscus	137993	7	8	12
D. stemonitis	43604	4	5	4
Ellisiella caudata	176619	4	3	8
Emericellopsis minima	69015	5	8	13
Epicoccum purpurascens	79496	2	8	10
Eremascus albus	100446a	2	1	10
Fulvia fulva	54976	2	6	8
Fusarium dimerum	121317	4	3	13
F. oxysporum	141140	2	85	8
F. semitectum	135410	5	2	6
Gaeumannomyces graminis	187782	1	8	7
G. graminis	189119	3	8	7
Gelasinospora cerealis	76253a	2	1	8
G. cerealis	45147	5	12	13
Geosmithia namyslowskii	40033	12	0	0
G. swiftii	40045	9	2	9
Geotrichum amycelicum	96824	7	0	0
Hansfordia pulvinata	20743	3	7	5

Appendix VII (continued) The comparison of longevity in storage within species preserved by centrifugal freeze drying. A list of isolates that died in storage giving their successful storage period and the maximum recorded storage period of other strains of the same species that remain viable

Name	IMI Number	Survival period (years)	Other isolates processed remaining viable in storage	
			Number	Maximum storage period (years)
Harposporium helicoides	87013	4	1	3
Helicodendron tubulosum	92743	2	1	5
Heslicus submersus	82609	4	1	8
Hymenula cerealis	135525	4	2	9
Iodophanus cervineus	70912ii	1	0	0
Kickxella alabastrina	139630	2	2	4
Macrophomina phaseolina	147229	2	2	7
Malbranchea sulfurea	126327	8	9	12
Melanospora zamiae	68202	3	4	5
Metarhizium anisopliae	98375	1	65	5
M. anisopliae	129065	5	65	5
M. anisopliae	170138	1	65	5
Monacrosporium oxysporum	78728	1	0	0
Monascus purpureus	123954	3	5	6
Monilinia fructigena	103791	0.75	2	2
M. fructigena	162408	0.5	2	2
Mortierella nantahalensis	158113	3	1	3
M. polycephala	144610	5	3	7
Mucor mucedo	133298	2	9	10

Appendix VII (continued) The comparison of longevity in storage within species preserved by centrifugal freeze drying. A list of isolates that died in storage giving their successful storage period and the maximum recorded storage period of other strains of the same species that remain viable

Name	IMI Number	Survival period (years)	Other isolates processed remaining viable in storage	
			Number	Maximum storage period (years)
Mycocentrospora acerina	128980	8	2	10
M. acerina	142050	9	2	10
Mycosphaerella fijiensis	162753	5	1	5
Myrothecium cinctum	45148	0.5	22	10
Neurospora crassa (anamorph)	53239	5	32	13
Paecilomyces niphetodes	136368	1	2	7
Penicillium asperosporum	80450ii	1	1	11
P. digitatum	91956	9	6	8
P. fellutanum	68224	9	5	5
P wortmanni	40047	5	6	6
Pestalotiopsis gracilis	69749	3	1	10
P. sydowiana	82405a	9	0	0
Peziza ostracoderma	61802	3	5	4
Phaeoseptoria musae	187050	2	0	0
Phialophora lignicola	96746	4	2	7
Polypaecilium insolitum	75202	3	1	4
Pyrenophora avenae	134278	1	5	5
P. avenae	136454	3	5	5
Rhizomucor meihei	125823	7	1	1

Appendix VII (continued) The comparison of longevity in storage within species preserved by centrifugal freeze drying. A list of isolates that died in storage giving their successful storage period and the maximum recorded storage period of other strains of the same species that remain viable

Name	IMI Number	Survival period (years)	Other isolates processed remaining viable in storage	
			Number	Maximum storage period (years)
Rhizomucor meihei	126334	8	1	1
Rhizopus oryzae	50109b	3	18	9
R. sexualis	103481	2	5	4
Scirrhia pini	187703	0.25	1	3
Septoria leucanthemi	91322	4	1	5
Sporendonema casei	68748a	3	0	0
Sporothrix catenata	154711	9	1	1
Stilbella thermophila	173315	5	1	8
Sympodiophora mycophila	158792	0.25	1	4
Thamnidium elegans	43624	4	5	9
Thelebolus crustaceus	144389	1	5	10
Thermoascus crustaceus	158740	1	9	13
Tilletiopsis minor	56590	3	1	9
Trachysphaera fructigena	33913	3	0	0
Tretopileus sphaerophorus	141217	2	0	0
Trichocladium lobatum	188290	3	1	3
Trichoderma viride	45548	5	18	6
Ustilago hordei	161944	6	1	1
Venturia inaequalis	61538	4	1	2

Appendix VII (continued) The comparison of longevity in storage within species preserved by centrifugal freeze drying. A list of isolates that died in storage giving their successful storage period and the maximum recorded storage period of other strains of the same species that remain viable

Name	IMI Number	Survival period (years)	Other isolates processed remaining viable in storage	
			Number	Maximum storage period (years)
Verticillium dahliae	88631	3	9	4
V. nubilum	130212	1	4	6
Zalerion maritima	89317	3	0	0

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