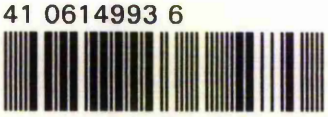


ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Evaluating the Use of Fungal Protoplasts to Investigate Fungicide Action.

Nicola Jane Spice

A thesis submitted in partial fulfilment of the requirements of
The Nottingham Trent University for the degree of Doctor of Philosophy.

October 2000

Supported by a BBSRC-CASE studentship in collaboration with AgrEvo UK Ltd, Chesterford Park Research Station, Saffron Walden, Essex.

Declaration

The material contained within this thesis has not been used in any other submission for academic award and is entirely the author's individual contribution. Due acknowledgement has been made of any assistance received. The author has attended the appropriate lectures and seminars in partial fulfilment of the requirements of the degree.

Signed ..... Nicola Price .....

(Candidate)

Signed ..... A H COBB .....

(Director of studies)

For Mum and Dad with love and thanks.

Acknowledgements

I would like to thank Professor Andy Cobb and Dr. Bill Carlile, Dr. Janice Pittis, and Dr. John Pillmoor for their continued help, encouragement, practical advice and supervision throughout this study. Also Dr. Dave Stock, Dr. Geoff Briggs and Dr. Steve Dunn for their valuable input, and Dr. Peter Holloway for supplying surfactant samples. Thanks also to Fiona Fletcher-Brown (AgrEvo) for HPLC analysis. The funding of this project by the BBSRC and AgrEvo UK Ltd. (now Aventis CropScience) is gratefully acknowledged.

I would like to thank all the staff at AgrEvo who made my time there enjoyable and worthwhile, especially Veronica (shiatsu queen!) for all her practical help and valued friendship. At Nottingham Trent University big smiles to MAGGIE MARTIN!!! for her photographic skills and pastoral care; Mark Cosgrove, Jane Braithwaite and Mike Brice for technical support; and all in the Plant Lab, especially Vic for their friendship. I must thank my family, especially: Mum & Jim; Dad, Alison, Holly & Robyn; Tracey, Steve & Ashleigh and Nan & Mo for their love, financial support and chocolate survival packages. Love and thanks to all my friends, but especially: Steph, Sue, Helen, Zoe and Kirsten for 'keeping it real'. Finally I am indebted to Pascal, for his endless love, encouragement and support - all the way to alpha Capricorni....and back. ☺

Publications

Spice, N.J., Cobb, A.H. and Carlile, W.R. (1996) The isolation of fungal protoplasts for studies of fungicide action. In: *Brighton Crop Protection Conference Proceedings,* volume 1, pp 433-434.

Spice, N.J., Carlile, W.R. and Cobb, A.H. (1998) Development of a rapid assay to determine viability of *Botrytis cinerea* protoplasts. In: *Proceedings of the 7th International Congress of Plant Pathology*, volume 2, section 1.3.34.

Contents

Abstract

Protoplasts have been successfully isolated from 3 economically important plant pathogenic fungi, *Botrytis cinerea*, *Phytophthora infestans* and *Stagonospora nodorum*, and characterised with respect to their metabolic capabilities using fluorescent microscopy and polarography techniques.

The effects of a series of linear alcohol ethoxylate surfactants upon respiration in protoplasts of *B. cinerea* have been examined. The length of the carbon chain within the molecules, rather than the ability of the surfactants to form micelles in solution was shown to be the predominant driving force leading to membrane disruption. The smaller molecules (C8 & C12) were able to solubilize the protoplast membrane, whilst the larger molecule (C16) did not cause protoplasts to rupture at the concentrations tested. The preferential carbon chain length for protoplast interaction was found to be C8, which caused membrane solubilization at 4mM, but not at 2mM.

The fluorescent dye fluorescein diacetate was successfully used spectrophotometerically to determine the viability of *B. cinerea* protoplasts. Concentrations of between 5×10^5 and 1×10^6 protoplasts ml^{-1} incubated with 10µM FDA over a 10min time period were optimal. Low levels of background fluorescence could be accounted for using simple mathematical formulae. Protoplasts subjected to treatments expected to significantly reduce their viability showed a corresponding reduction in FDA-derived fluorescence.

The assay was used to determine protoplast tolerance of different organic solvents. Protoplasts were found to tolerate acetone, ethanol, methanol and DMSO at concentrations up to and including 1% (v/v aq.). DMSO and methanol did not significantly reduce protoplast viability, even at 10%. The effects of 2 commercially available fungicides on protoplast fluorescence were investigated. Incubation with azoxystrobin was found to significantly reduce FDA derived fluorescence at 10 and

100μM (at 30 and 60min) and at 1μM (60min only). Incubation with pyrimethanil did not cause a significant reduction in protoplast viability, which could be linked to the mode of action of this compound.

Radiolabelled amino acids were incorporated into proteins within viable protoplasts, but not non-viable protoplasts of *B. cinerea*. A suitable assay was developed for the detection of this incorporation. A preferential growth medium which stimulated protoplast regeneration and hence protein synthesis was determined. Protoplast association with the radiolabelled experimental fungicide "AG1" was found to be higher in non-viable protoplasts. A high percentage of the compound was found to be associated with the protoplast samples, probably linked to the lipophilicity of the compound.

An assay was developed and optimised to investigate the association of a range of radiolabelled compounds with *B. cinerea* protoplasts. Compounds were chosen with reference to log P values. A relationship between compound log P value and association with a protoplast population was discovered; association of the compound with the protoplasts increased with increasing log P values. A "standard curve" was generated by the assay, which could potentially provide a useful marker to study structure optimisation and delivery of radiolabelled experimental compounds.

The effectiveness of known and experimental fungicides against *B. cinerea* was investigated using a variety of assays, in order to establish the usefulness of protoplasts in this context. Large discrepancies were discovered between compound effectiveness *in planta*, where no compound offered good protection against *B. cinerea* infection, and *in vitro* where most compounds inhibited the pathogen to various extents depending on the assays employed (measurement of growth on agar, oxygen uptake by germlings and protoplasts, and cytochrome c reduction in the electron transport chain of isolated mitochondria). Potentially useful applications of protoplast technology within the agrochemical industry have been discussed.

Abbreviations

AG1	An experimental fungicide
ANOVA	Analysis of variance
approx.	Approximately
cm	Centimetre(s)
CMC	Critical micelle concentration
°C	Degrees Celsius
d	Day(s)
dpm	Disintegrations per minute
EC50	Concentration that causes a measurable inhibitory effect on one half of a given population
Eds	Editors
e.g.	*exampli gratia* (Latin; for example)
et al.	*Et alia* (Latin; and others)
ETI	Electron transport inhibition
F	F statistic used in analysis of variance
FDA	Fluorescein diacetate
g	Gram(s)
GGM	Glucose growth medium
h	Hour(s)
INC	Incorporated
i.e.	*Id est* (Latin; for instance)
KBq	Kilobequerels
L	Litre(s)
log P	Octinol water partition coefficient
Ltd.	Limited
m	Metre(s)
M	Molar
MBq	Megabequerel(s)
mCi	Millicurie(s)

MEB	Malt extract broth
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar
mV	Millivolt(s)
nm	Nanometre(s)
pers.comm.	Personal communication
pH	Negative logarithm of hydrogen ion concentration
Phos-KCl	0.8M KCl buffered to pH 5.8 with a 10mM potassium phosphate buffer
pp	Pages
ppm	Parts per million
RFU	Relative fluorescent units
rpm	Revolutions per minute
r_s	r_s statistic used in Spearman's rank correlation coefficient
s	Seconds
SDW	Sterile distilled water
SE	Standard error
TCA	Trichloroacetic acid
Tris	2-amino-2 (hydroxymethyl)-1,3-propanediol
UK	United Kingdom
USA	United States of America
UV	Ultra violet
w/v	Weight to volume ratio
µCi	Microcurie(s)
µm	Micrometer(s)
µM	Micromolar
µl	Microlitre(s)
µg	Microgram(s)
>	Greater than
<	Less than

=	Equal to
≡	Equivalent to
%	Percent/percentage
x g	Acceleration due to gravity
±	Plus or minus
#	Number of

Chapter One: General introduction

1.1 Sustaining the food supply

An estimated 800 million people world-wide do not have enough food (James, 1998). This figure will continue to rise if the world population increases as predicted from the current 5.8 billion to 8 billion by 2020 (United Nations statistics, 1994). The need for an increased sustainable global food supply is therefore clearly evident. Productivity can be increased by planting more crops, however suitable land may not be readily available and the clearing of biodiverse areas to create additional agricultural space is undesirable on a global scale. Other means of raising productivity are becoming increasingly important - reducing those factors which result in yield losses can result in significant additional yields.

Annually, approximately one third of food crops world-wide are destroyed by pests and diseases (fungal pathogens contribute substantially to this figure), in spite of the different controls employed to prevent such losses. Indeed, some modern agricultural practices designed to increase productivity, such as growing large areas of monocultures, transporting crops over long distances, and storing crops for extended periods, may have increased the likelihood of pathogen infection (Lucas, 1998), adding to the burden of disease prevention and control.

Whilst an increase in global food supply could be considered to be the ultimate rationale for the development of new chemical control agents, it should be recognised that economic factors and consumer demand for high quality produce, in the developed world at least, are equally important. Whatever the driving force behind plant disease control, the dynamic interaction between the plant, the pathogen and the environment, ensures that there is a constant need for the development of new, effective control measures to sustain and increase productivity.

1.2 A brief introduction to the pathogens used in this investigation

Three plant pathogenic fungi were initially chosen for this study due to their economic importance, taxonomic diversity and routine inclusion in agrochemical empirical screening procedures.

1.2.1 *Botrytis cinerea* (Pers.) ex Fr.

The deuteromycotina fungus *Botrytis cinerea* is a ubiquitous plant pathogen causing disease on a wide range of economically important crop and ornamental plant species (Coley-Smith, Verkhoeff & Jarvis, 1980). *Botrytis* spp. are regarded as the most damaging of all pathogens to peas and field beans (Carlile, 1995). A serious problem in glasshouse-grown crops, infecting lettuce, tomato and cucumber, *B. cinerea* causes major crop losses with produce failing to meet consumer quality controls (Reglinski, Lyon & Newton, 1995).

Botrytis cinerea is a necrotrophic pathogen which often infects healthy plant tissues. Subsequent mycelial growth and conidial spore production within the induced necrotic lesions, gives rise to polycyclic epidemics of sporulation and infection in the field (Köhl, Molhoek, van der Plas & Fokkema, 1995). The conidia of this fungus are widespread and may be present at a background level even when a crop is not infected. The initiation of an epidemic within a field from background inoculum provides airborne spores from infected tissue, whilst plant debris, wild vegetation and the over-wintering sclerotia structures act as further sources of infection (Köhl *et al.*, 1995).

1.2.2 *Phytophthora infestans* (Mont.) de Bary

The oomycete fungus *Phytophthora infestans* causes late blight disease in the Solanaceae and is considered one of the most important of all potato pathogens (Parry, 1990). Unlike *B. cinerea, P. infestans* is host-range restricted, infecting mainly potato and tomato crops. Since causing the Potato Famine in Ireland in the mid 1840's, *P. infestans* has continued to result in large yield losses in European crops, sometimes causing total destruction of all plants within a field under favourable weather conditions (Agrios, 1988).

The fungus over-winters as mycelium in infected potato tubers, either in stored crops or in those left in the ground after harvest. The diseased tuber gives rise to infected shoots in the spring and sporangia produced from these shoots are able to infect the new crop (Carlile, 1995). Sporangia may germinate directly and infect healthy leaf tissue, where, after as little as 4 days, more sporangia will be produced. In cooler wetter conditions the sporangia may differentiate into motile biflagellate zoospores which are able to swim in soil moisture to infect tubers near the soil surface (Parry, 1990).

1.2.3 *Stagonospora nodorum* (Berk.)

The ascomycete fungus *Stagonospora nodorum* has a world-wide distribution, infecting mainly, but not exclusively, wheat and barley (King, Cook & Melville, 1983). In wet years in the UK, *Septoria* diseases of wheat are the most destructive of this crop (Carlile, 1995), with average annual losses of approximately 10% (Parry, 1990). The fungus overwinters as dormant mycelium, pycnidia and pseudothecia (sporing structures) on seed, crop stubble and debris as well as surviving on autumn-sown crops and volunteer plants (Parry, 1990). Spores from pycnidia are released and dispersed to

to new plants by rain splash (Faulkner & Colhoun, 1976), whereupon they germinate and infect leaves and glumes. Epidemics can arise within 10-14 days under optimal conditions of 20-27°C and 100% relative humidity (Parry, 1990). Ascospores produced from pseudothecia, and infected seeds are also sources of transmission of the disease from one crop to the next (King *et al.*, 1983), although the level of seed infection may not always relate to the severity of the disease (Cunfer & Johnson, 1981).

1.3 Disease control and the problem of fungicide resistance

Plant disease control is achieved either directly, by targeting and destroying the pathogen, or indirectly, by minimising the risk of infection using physical, chemical and biological means. Disease management is most effective when all information regarding the pathogen, environment and available means of control is considered and utilised to implement an integrated protection strategy. Such strategies involve the use of cultural control methods to reduce the likelihood of infection, for example *S. nodorum* infection can be reduced by ploughing infected stubble deep into the field (Brown & Rosielle, 1980) whilst removing waste potatoes from the field after harvest can reduce infection by *P. infestans* (Parry, 1990). Other simple measures such as avoiding planting early crops next to late ones and ensuring that crop emergence does not coincide with conditions favourable to pathogen attack, may also be employed.

Disease forecasting using models often based on the acquisition of rainfall and temperature data, are also used in pathogen control, particularly for *P. infestans* which is very weather dependent (Carlile, 1995). Forecasting models provide information regarding the likelihood of a disease outbreak, advising on the best time for fungicide spraying. They are therefore not only valuable in preventing disease, but also in the economic application of chemicals. Cultivar choice is an important element in disease

control - avoiding planting those varieties most susceptible to attack in high risk areas, and where appropriate choosing resistant ones. Examples include King Edward potatoes which are very susceptible to blight (Parry, 1990) and shorter wheat varieties which are more susceptible to attack by *S. nodorum* (Scott, Benedikz, Jones & Ford, 1985). Despite the employment of cultural practices, control of all 3 pathogens relies heavily on the application of fungicides (for reviews see Carlile, 1995; King *et al.*, 1983 & Platt, 1994), although some advances in biological control of *B. cinerea* have been made in recent years.

Fungicides are chemical compounds that kill or inhibit the growth of fungi and are classified as protectant and/or systemic depending on their chemical properties. Protectant fungicides are not taken up by the plant and work at the site of application only to prevent fungal colonisation of the leaf surface. They are therefore susceptible to breakdown due to weathering, and require frequent re-application. Protectant fungicides often act by inhibition of spore germination, as the fungicide is selectively taken up into spores where accumulation proves toxic.

Most protectant fungicides are non-specific toxins that inhibit enzymes in any number of metabolic pathways, for example the dithiocarbamate fungicides bind to thiol groups on proteins and enzyme inactivation eventually proves fatal to the fungal cell. Plants are not affected because the chemicals are not able to penetrate the plant cuticle. Protectant fungicides broadly fall into one of three types; metal-based, sulphur-based and aromatic hydrocarbons, and represent some of man's earliest attempts to control diseases on plants. Millardet's use of copper formulations to control downey mildew on grapes in the late nineteenth century saw the beginning of pathogen control using fungicides. Copper-based fungicides are particularly effective at controlling oomycete diseases and are still in use today - often in conjunction with systemic fungicides for this purpose (Carlile, 1995).

Systemic fungicides enter the plant, either partially (e.g., dicarboximides) or completely (e.g. many triazoles), with some compounds taken up and dispersed throughout the plant by the xylem vessels. Systemic fungicides can therefore be used to cure as well as prevent disease. Most systemic fungicides act by inhibition of specific biochemical pathways; for example the carboxamide group of compounds bind to succinate dehydrogenase in basidiomycete fungi, which differs to this enzyme in plants, mammals and other fungi by just 5 amino acids (Ragsdale & Sisler, 1970).

Some systemic fungicides therefore have quite narrow applications, whilst others e.g., the inhibitors of sterol biosynthesis have a broader spectrum of activity as they inhibit a stage in ergosterol synthesis. Ergosterol is the principle sterol in most fungal membranes (with the exception of the Oomycota) whilst stigmasterol and cholesterol are the principle plant and mammalian sterols. As systemic fungicides usually act by inhibition of specific biochemical pathways within the pathogen, resistance through mutation of the fungal gene (or genes) controlling these pathways arises frequently under high selection pressure.

The emergence of fungi resistant to previously effective fungicides, depends on the nature of the fungicide (*e.g.*, single-site, multisite), how that fungicide has been used and the biology of the pathogen (Lucas, 1998). Fungicides that act on a single site are more likely to fail than those that act on multiple sites, as a single corresponding mutation in the pathogen can render the active site inactive. The greater the exposure of a pathogen population to a fungicide, the more likely that resistance will develop as mutants and/or naturally resistant organisms spread.

Since the introduction of systemic fungicides in the late 1960's, high levels of disease control have been achieved and then lost as resistance developed. Isolates of *B. cinerea* resistant to the benzimidazole, dicarboximide and *N*-phenylcarbamate fungicide classes are now common (Elad, Yunis & Katan, 1992). Isolates of *P infestans* resistant to the phenylamide fungicide metalaxyl (Dowley & O'Sullivan,

1991) and isolates of *S. nodorum* resistant to the benzimidazoles (Horsten & Fehrmann, 1980), amongst others have also become commonplace.

Across the spectrum of plant pathogenic fungi and the chemicals developed to control them, it would seem that the announcement of a new fungicide is often closely followed by the reporting of emerging resistance to that compound. Research has focused on the alteration and improvement of existing compounds, for example the sterol 14α-demethylation inhibitors (Kapteyn, Milling, Simpson & de Waard, 1994) in addition to the development of new compounds e.g., pyrimethanil (Milling & Richardson 1995), to provide effective disease control.

In practice, the judicious use of fungicides can help to prevent resistance from developing. This may involve using a mixture of fungicides (for example metalaxyl and mancozeb for the control of *P. infestans*) or alternating fungicides so that pathogen populations are not exposed to a single chemical for extended periods. When fungicides are used in this way as part of a larger, integrated approach to disease control, which might include simple cultural practices or more complex disease forecasting, disease management can be effective and fungicide resistance minimised. The process of fungicide discovery and development is discussed in Chapter 6.

1.4 Fungal protoplasts

A protoplast is described as a "naked cell", completely devoid of cell wall residues (Davis, 1985). The fungal cell wall forms a rigid mechanical barrier on the surface of the protoplast and acts as an osmotic protector, preventing bursting in the normally hypotonic environment (Farkaš, 1985). Removal of the cell wall in an osmotically supportive medium yields the production of spherical protoplasts. The isolated protoplast represents the organised entities of the living components of cells, which are capable of active metabolism (Davis, 1985).

Following their initial isolation in 1958 (Emerson & Emerson), fungal protoplasts have been produced from representatives of all the major fungal taxonomic classes and extensively reviewed (e.g., see Villanueva & Garcia-Acha, 1971; Peberdy, 1972; 1976; 1978; 1979a; 1979b; Wostemeyer & Wostemeyer, 1998). The stimulus for protoplast production in yeasts, was to allow the preparation of undamaged cell contents as the mechanisms employed to break the cell wall also damaged organelles (Peberdy, 1979a). Subsequently protoplasts have been deemed worthy of study in their own right providing novel means of investigating morphological, biochemical and physiological properties of fungi.

Most early protoplast studies were concerned with cell wall regeneration and reversion to normal hyphal form, in attempt to understand the process of wall polymer biogenesis in the intact cell (reviewed by Peberdy, 1979b, Necas & Svoboda, 1985, Wostemeyer & Wostemeyer, 1998). Wall regeneration studies have been undertaken more recently (Sietsma, Beth Din, Ziv, Sjollema & Yarden, 1996), with interest in chitin synthase as a potential fungicidal target.

Protoplast fusion is an important area of research with widespread purposes (reviewed by Gokhale, 1992). Protoplast fusion is an efficient way of inducing sexual recombination in fungi which lack a sexual cycle, resulting for example, in an increased productivity of penicillin in recombinants derived from crosses in *P. chrysogenum* (Gokhale, 1992). Protoplasts have facilitated the improvement of industrial strains of fungi, (for example by increasing the secretion of extracellular enzymes or metabolites, Morgan 1983), have aided genetic characterisation resulting in improved diagnosis of strains (Ersek, English & Schoelz, 1995) and have allowed the elucidation of certain biochemical pathways (Becher, Wedler, Schulze, Bode, Kasuske & Samsonova, 1991).

More recently, regenerating protoplast systems have been developed in order to achieve genetic transformation, where regeneration is a pre-requisite for recovering

transformants. Successful transformation systems have been developed for many fungi, including all three pathogens used in this study - *B. cinerea* (Santos, Vallejo, Rebordinos, Gutiérrez, Collado & Cantoral, 1996), *P. infestans* (Judelson, Tyler & Michelmore, 1991; Judelson & Michelmore, 1991; Judelson, 1993) and *S. nodorum* (Cooley, Shaw, Franklin & Caten, 1988; Cooley, Franklin & Caten, 1990; Cooley, van Gorcom, van der Hondel & Caten, 1991), amongst others. Genetic transformation of fungal plant pathogens has facilitated the analysis of molecular interactions between the fungus and the plant, with the aim of characterising genes controlling specificity and pathogenicity (Judelson *et al.*, 1991), and generating plants resistant to disease (Bailey, Mena & Herrera-Estrella, 1991).

Using protoplasts as the starting material for isolating subcellular fractions, for example nuclei, chromosomes and mitochondria, has also proved successful (Wostemeyer & Wostemeyer, 1998). Osmotically lysing protoplasts to release the cell contents can result in the production of organelles that are more intact than those produced by other isolation procedures that rely on mechanical pressure. Intact nuclei were recovered from *A. nidulans* in this way (Vagvolgyi & Ferenczy, 1991). Fungal protoplasts have also been used to study the heat shock response in *A. nidulans* (Newbury & Peberdy, 1996), enzyme secretion in *A. nidulans* (Andres & Peberdy, 1974) and transport processes, macromolecule synthesis, respiration and metabolite production in a range of species (reviewed by Isaac 1985).

For clarity, work undertaken in each section of this thesis has been introduced separately at the beginning of each chapter, and includes: practical aspects of protoplast isolation (Chapter 2); fungicide formulations (Chapter 3), fluorescent probes and their applications (Chapter 4), radioactivity and protoplasts (Chapter 5) and fungicide development/screening (Chapter 6).

1.5 Thesis aims

The aims of this research were:

- firstly to establish reliable, reproducible procedures for the isolation of protoplasts from 3 economically important plant pathogenic fungi, *B. cinerea*, *P. infestans* and *S. nodorum*;

- secondly to characterise the isolated protoplasts with respect to metabolic capabilities, employing the polarographic measurement of respiration and using fluorescent viability probes;

- thirdly to investigate the potential uses of protoplasts isolated from one of the pathogens in the context of fungicide research and development.

Investigating the potential uses of *B. cinerea* protoplasts in fungicide research and development involved:

- developing a quantifiable fluorescence-based viability assay and using it to determine i) tolerance to organic solvents such that suitable solvents could be chosen for further experiments and ii) protoplast sensitivity to known fungicides;

- investigating protoplast interaction with a series of alcohol ethoxylate surfactants;

- developing methods of incorporating and detecting radiolabelled compounds within the protoplasts;

- undertaking a range of *in planta* and *in vitro* studies at the whole organism, whole cell and subcellular level to assess the value of using protoplasts in fungicide screening.

Chapter Two: Protoplast isolation and characterisation

2.1 Introduction

Protoplasts have been isolated from *B. cinerea* (Shirane & Hatta, 1986; Braun & Heisler, 1990; Santos *et al.*, 1996); *P. infestans* (Kinghorn, Moon, Unkles & Duncan, 1991; Judelson *et al.*, 1991; Judelson & Michelmore, 1991; Judelson, 1993) and *S. nodorum* (Cooley *et al.*, 1988; Cooley *et al.*, 1990; Cooley *et al.*, 1991), and have been used mainly for genetic transformation purposes. Procedures for the isolation of protoplasts from *P. infestans* and *S. nodorum* are well characterised, with repetition across different research groups. Protoplast isolation from *B. cinerea* is less consistent and therefore requires the development of reliable, reproducible methods for growth and storage on agar, growth in liquid culture and protoplast isolation.

Many factors have been shown to influence protoplast isolation, including the lytic enzyme, osmotic stabiliser, buffer, culture conditions and age of culture (as reviewed by Peberdy, 1978; Davis, 1985). Initially, enzymes were prepared from the digestive juices of the snail *Helix pomatia* and were found to effectively release protoplasts from various yeasts (as reviewed by Peberdy, 1979). Following this, enzymes were isolated from the pathogenic fungus *Trichoderma harzianum* which are now available as commercial preparations (Hamlyn, Bradshaw, Mellon, Santiago, Wilson & Peberdy, 1981). The commercial preparations consist of cellulases, glucanases, chitinases and proteases which can be used alone, or in combination. Enzymes prepared from *T. harzianum* (e.g., Novozym 234 or equivalent) often have all of these activities and have proved to be the enzymes of choice for many researchers.

There is no one osmotic stabiliser suitable for use with all fungi, although some such as KCl, $MgSO_4$ and mannitol are used more often than others (Davis, 1985). Generally inorganic salts are preferred for filamentous fungi, whilst sugar alcohols are favoured for yeasts. The age of the mycelial culture affects protoplast isolation, with higher yields obtained when the fungus is in the exponential rather than the stationary growth phase. Young mycelia (approx. 48h) are therefore normally used (Peberdy *et al.*, 1976). The aim of these experiments was to establish reliable, reproducible procedures for the isolation of protoplasts from *B. cinerea*, *P. infestans* and *S. nodorum* and to characterise the isolated protoplasts with respect to metabolic capabilities, using both the polarographic measurement of respiration and fluorescent viability probes.

2.2 Materials and methods

2.2.1 Sterile technique

Sterile technique was employed throughout. This involved working in a laminar air flow cabinet, flaming all implements in methanol, autoclaving all glassware, sieves, solutions and media at 121°C for 15min; and filter-sterilising solutions unsuitable for autoclaving using a 0.2μm Acrodisc® 32 syringe filter, Gelman Sciences, Michigan, USA.

2.2.2 Strains and media

2.2.2.1 *Botrytis cinerea*

2.2.2.1.1 Isolation and growth

Nine field isolates of *B. cinerea* (BC1-BC9, Nottingham 1995) were cultured onto potato dextrose agar (PDA [Oxoid, Unipath Ltd., Basingstoke, UK] 39g L^{-1} distilled H_2O), containing Penicillin G (Sigma, Poole, UK) at 50 units ml^{-1} and Streptomycin sulphate (Sigma) at 100 units ml^{-1} to prevent bacterial contamination. Antibiotic solutions were filter sterilised and added to the PDA after autoclaving. Sporulating cultures were obtained by incubation of 4d old dark-grown colonies under near UV light (TLD36W/08, Phillips, Holland) for a further 5-7d. Isolates were deemed pure by microscopic observations and were subcultured onto PDA without antibiotics. Single spore isolates were subsequently generated by harvesting spores in SDW and diluting to a final spore concentration of 1 x $10^2 ml^{-1}$. One hundred microlitres of this suspension were spread over the surface of a PDA plate, and incubated in the dark for 4d. Colonies growing from a single spore were then subcultured as before.

The growth rates of the 9 isolates were recorded over a 5d period. Isolates were subcultured by transfer of a 5mm agar plug taken from the leading edge of the colony, onto PDA and maintained in the dark. The diameter of the colony was measured at the widest part, after 24, 48, 72, 96 and 120h. The size of the initial plug was subtracted from the measurement. Data presented are mean values ± SE, n = 5.

2.2.2.1.2 Storage

The following methods were employed to investigate storage of the fungus. Ten millilitres of sterile distilled water (SDW) were pipetted onto the surface of the sporulating cultures and the spores dislodged using a sterile spreader (Technical Service Consultants Ltd., Heywood, UK). Mycelial debris was removed by filtration through a sterile 20μm nylon sieve (Wilson Sieves, Nottingham, UK). The

suspension was transferred to a sterile centrifuge tube and the spores pelleted by centrifugation (MSE Chilspin, Fischer Scientific, Loughborough, UK) at 600 x g for 10min. The supernatant was discarded and the spores resuspended in the cryopreservative fluid from a "protect" bacterial preserver vial (Technical Service Consultants Ltd.). The spore suspension was returned to the vial, which was capped and inverted to disperse the spores amongst the beads. Excess fluid was discarded and the vials stored at -20°C. After 2 weeks, cultures were retrieved from the vials by placing a single bead into the centre of a PDA plate. Plates were incubated as before.

Small agar blocks (approx. $5mm^3$) of non-sporulating and sporulating mycelia were cut from the growing edge of the fungal colony, placed in sterile universal bottles containing 15ml SDW and stored at room temperature. Blocks were periodically removed and placed onto PDA plates, which were incubated as before.

Preservation in silica gel (Smith & Onions, 1983) was undertaken. Glass universal bottles were filled one third full with medium grain, plain mesh 6-16 non-indicating silica gel (Fischer Scientific) and sterilised at 180°C for 3h. Bottles were cooled, placed in a tray of water and stored at -20°C for 2h. Ten millilitres of sterile 5% (w/v) reconstituted skimmed milk (Oxoid) were pipetted onto the surface of the sporulating cultures and the spores dislodged using a sterile spreader. Mycelial debris was removed by filtration through a sterile 20µm sieve. The spore suspension was added to the cooled gel to three-quarters wet it (approx. 1ml) and the bottle agitated to distribute the suspension evenly. The bottles were stored with loose caps at room temperature for 14d until the silica gel crystals had dried and separated. The caps were screwed tightly down and the bottles stored at room temperature, in an airtight container over medium grain, plain mesh 6-16 indicator

silica gel (Fischer Scientific Ltd.) to absorb moisture. Strains were retrieved by placing a few crystals onto PDA plates, which were incubated as before.

2.2.2.1.3 Determination of isolate sensitivity to carbendazim

Each isolate (BC1-BC9) was tested for sensitivity to the fungicide carbendazim. Carbendazim (Bavistin™, BASF, Germany) was incorporated into PDA after autoclaving at 0 (control), 1, 10 and 100ppm. For each isolate, four plates at each concentration were inoculated with a 5mm agar plug from the leading edge of the fungal colony. Cultures were kept at 20°C for 4d and the colony diameters measured. The size of the initial plug was subtracted from the measurement. Data presented are mean values, n = 4 from a single occasion.

2.2.2.2 *Phytophthora infestans*

Cultures of *P. infestans* (phenylamide sensitive and resistant single spore isolates) were supplied by AgrEvo, Chesterford Park, UK and grown on rye meal agar (g L^{-1} distilled H_2O: rye meal [Holland & Barrett] 12.5, glucose 1.25, $CaCO_3$ 10, agar #3 [Oxoid] 20, V8® juice [Campbell Grocery Products Ltd., King's Lynn, UK] 50ml). Sporangia were produced by cultures grown in the dark for 14d. Cultures were maintained by serial subculture and stored as agar plugs under water (as described for *B. cinerea*).

2.2.2.3 *Stagonospora nodorum*

Cultures of a wheat adapted wild-type strain of *S. nodorum* (BS171 single spore derivatives) were supplied by AgrEvo, Chesterford Park, UK and grown on

CzV8 medium (Cooke & Jones, 1970). Sporulating cultures were obtained by incubation of 5d old dark-grown colonies under near UV light for a further 8-10d. Cultures were maintained by serial subculture and stored as agar plugs under water (as described for *B. cinerea*), or as spore suspensions in 5% (w/v) reconstituted sterile skimmed milk (Oxoid) at -20°C.

2.2.3 Production of mycelia in liquid culture

2.2.3.1 *Botrytis cinerea*

Botrytis cinerea spores were harvested by pipetting 10ml of glucose growth medium (GGM, [Zimmerman, unpublished data] g L^{-1} distilled H_2O: glucose **2**, $NaNO_3$ **2**, KH_2PO_4 **0.4**, $CaCl_2$ **0.4**, NaCl **0.4**, $MgSO_4$ **0.2** and mg L^{-1}: $FeSO_4.4H_2O$ **12.5**, $ZnSO_4.7H_2O$ **10.0**, $CuSO_4.5H_2O$ **2.0**, $MnSO_4.4H_2O$ **2.0**, $NaMo_4.2H_2O$ **2.5**) onto the surface of a 7d sporulating culture, scraping gently with a sterile spreader to dislodge the spores and filtering through a 20µm sieve to remove mycelial debris.

The concentration of the resulting spore suspension was determined using a haemocytometer counting chamber (Improved Neubauer) and adjusted to different concentrations between 1 x 10^5 and 1 x 10^6 spores ml^{-1}. Four hundred millilitres of spore suspension were incubated in 1L Erlenmeyer flasks at 20°C for 48h at 140rpm in a cooled orbital incubator with a glass-panelled lid (9R250, Gallenkamp Plc., Uxbridge, UK). Mycelia were harvested by centrifugation at 3000 x g and washed with 0.8M KCl buffered to pH 5.8 with a 10mM phosphate buffer (Phos-KCl).

2.2.3.2 *Phytophthora infestans*

Phytophthora infestans spores were harvested by pipetting 10ml of sterile pea broth medium (300g Sainsbury's economy frozen peas boiled for 10 minutes in 500ml distilled H_2O, strained through muslin and made up to 1L with distilled H_2O) onto the surface of a 14d old sporulating culture, scraping gently with a sterile spreader to dislodge the spores and filtering through a 35μm sieve to remove mycelial debris. The concentration of the resulting suspension was determined using a haemocytometer and adjusted to 1-5 x 10^4 spores ml^{-1}. Four hundred millilitres of this suspension were incubated in 1L Erlenmeyer flasks at 20°C for 48h at 120rpm in a cooled orbital incubator (as before). Mycelia were harvested by filtration using a 35μm sieve and washed with KC osmoticum (Judelson *et al.*, 1991 [0.64M KCl, 0.2M $CaCl_2$).

2.2.3.3 *Stagonospora nodorum*

Stagonospora nodorum spores were harvested by pipetting 10 ml of liquid complete medium (Newton & Caten, 1988) onto the surface of a 14d old sporulating culture, scraping gently with a sterile spreader to dislodge the spores and filtering through a 30μm sieve to remove mycelial debris. The concentration of the resulting suspension was determined using a haemocytometer counting chamber and adjusted to 1 x 10^6 spores ml^{-1}. Four hundred millilitres of this suspension were incubated in 1L Erlenmeyer flasks at 25°C for 24h at 190rpm in a cooled orbital incubator (as before). Mycelia were harvested by centrifugation (MSE Chilspin) at 1000 x g and washed with 600 mM $MgSO_4$.

2.2.4 Isolation and purification of protoplasts

2.2.4.1 *Botrytis cinerea*

Using a sterile spatula, *Botrytis cinerea* mycelia were resuspended in Phos-KCl buffer containing 5mg ml^{-1} lysing enzymes from *Trichoderma harzianum* (Sigma, Poole, UK). After incubation at room temperature for 2h with gentle shaking on an orbital incubator, the protoplasts were harvested by filtration through a 56μm sieve, separated from the enzyme solution by centrifugation (MSE Mistral 2000R, Sanyo Gallenkamp Plc., Uxbridge, UK) at 800 x g for 10min, washed twice and resuspended by gentle pipetting in Phos-KCl. Purified protoplasts were stored at 4°C.

2.2.4.2 *Phytophthora infestans*

Phytophthora infestans protoplasts were isolated essentially as described by Judelson *et al.*, (1991). Mycelia were resuspended in KC osmoticum containing 5mg ml^{-1} lysing enzymes (as before) and 2.5mg ml^{-1} cellulase (cellulysin ®, CalBiochem, Beeston, UK). After incubation at room temperature for 1h with gentle shaking on an orbital incubator, the protoplasts were harvested by filtration through a 35μm sieve, separated from the enzyme solution by centrifugation (MSE Mistral) at 600 x g for 10min, washed twice, resuspended in KC and stored at 4°C (Treatment A). For a comparison, a lytic enzyme solution consisting of 5 mg ml^{-1} lysing enzymes (as before) in KC with no additional cellulase was also used. All other parameters remained constant (Treatment B).

2.2.4.3 *Stagonospora nodorum*

Stagonospora nodorum protoplasts were isolated essentially as described by Cooley *et al.*, (1988). Mycelia were resuspended in 1.2M $MgSO_4$ buffered to pH 5.8 with 10mM phosphate buffer, containing 2.5mg ml^{-1} lysing enzymes (Sigma, as before). After incubation at 30°C for 2h, the suspension was transferred to sterile centrifuge tubes (in 5ml aliquots), overlaid with an equal volume of 600mM sorbitol, 100mM Tris HCl, pH 7.0 and centrifuged (MSE Chilspin) at 4000 x g for 25min. Protoplasts banded at the interface, whilst debris was pelleted. Protoplasts were transferred to a fresh centrifuge tube, mixed with an equal volume of 1M sorbitol, 10mM Tris HCl, pH 7.5 and centrifuged at 1500 x g for 10min. Pelleted protoplasts were resuspended in STC buffer (1.2M sorbitol, 10mM $CaCl_2$, 10mM Tris HCl, pH 7.5) and stored at 4°C.

Yields were calculated for all 3 species microscopically, using a haemocytometer. Measurements of the protoplasts and spores (necessary to determine the pore size of the sieves used) were made using a calibrated ocular micrometer.

2.2.5 Protoplast viability

The use of fluorescein diacetate to determine cell viability is extensively discussed in Chapter 4. Fluorescein diacetate (FDA, Sigma) was used at 0.01% (w/v aq.) in the relevant stabilised buffer for each species to determine protoplast viability. After incubation of equal volumes of FDA stain and protoplast suspension for 2min in the dark, protoplasts were examined under an Axioskop microscope (filter set 24), Zeiss, West Germany. Only viable protoplasts are capable of cleaving FDA to fluorescein, producing a bright green fluorescence visible under blue light.

Percentage viability was determined by counting the number of protoplasts visible under normal light, switching the microscope to blue light and counting the number of viable protoplasts in the same field. At least 100 protoplasts were counted on each occasion.

2.2.6 Respiration studies

Respiration rates were determined polarographically using a Hansatech oxygen electrode apparatus (Hansatech Instruments Ltd., King's Lynn, UK). An oxygen electrode is a type of electrochemical cell, in which a current is generated proportional to the amount of oxygen in a solution (Walker, 1985). The electrochemical reactions occurring in the electrode can be represented diagramatically (Figure 2.1).

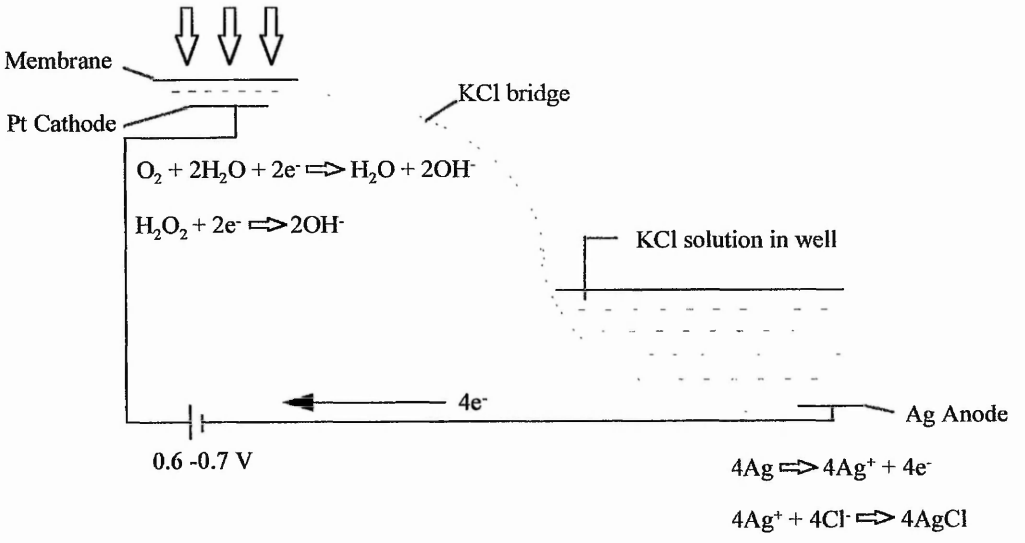


Figure 2.1 Electrode reactions (after Walker, 1985)

The electrode was prepared for use, essentially as described by Walker (1990). One drop of electrolyte (saturated KCl) was placed onto the platinum cathode of the electrode disc. A $2cm^2$ piece of spacer (cigarette paper) was placed on top of the electrolyte and a $2cm^2$ piece of Teflon membrane placed carefully on top of the spacer. The spacer and membrane were firmly secured using a rubber O ring. Excess spacer and membrane were carefully trimmed away and the well surrounding the cathode filled with electrolyte. An example of the electrode apparatus can be seen in Figure 2.2.

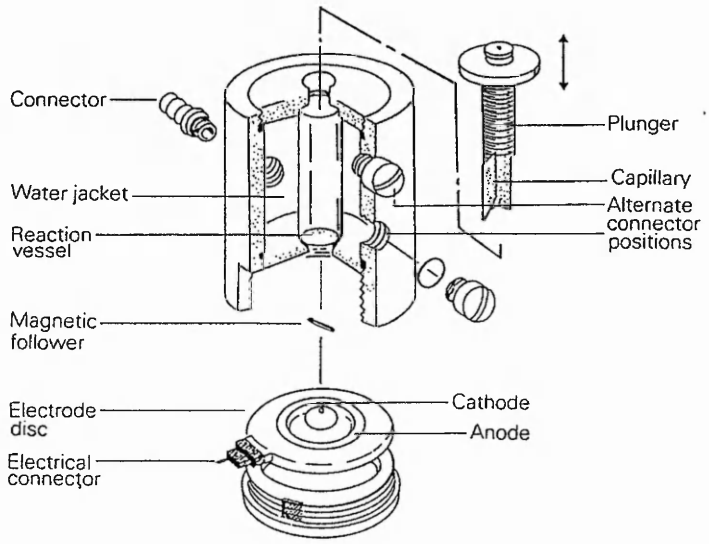


Figure 2.2 An oxygen electrode unit (Walker, 1985)

The electrode disc was secured into position at the base of the constant temperature chamber and placed onto the magnetic stirrer. The water supply was connected to a water jacket to allow a controlled temperature of 25°C. The electrode disc was connected to the control base, itself connected a chart recorder (both set to 10mV). The chart recorder was set to "signal" and the control base to "variable". Zero was adjusted on the control base to correspond with zero on the chart recorder. The output switch was then set to "variable" and the baseline

reading adjusted to zero. The chart recorder was set to zero using the pen offset control. The baseline switch was then set to "cancel", causing an increase in signal. The output on the control base was adjusted to 9mV, and the pen set to this level using the calibration control knob.

To calibrate the system, 1ml of aerated water was pipetted into the constant temperature chamber containing a small (4mm) stirrer bead. The solubility of oxygen in water at the set temperature (25°C) is a known value (0.253 μmol ml^{-1}). The pen was adjusted to 9 mV, representing 100% saturation with oxygen. Oxygen was then removed from the sample by the addition of a small quantity of sodium dithionite, causing the pen to fall to a steady level representative of zero oxygen. A suitable rate for the chart recorder was chosen, usually 10mm min^{-1}. The number of divisions on the chart paper between 0% and 100% saturation were counted and used in the calculation of respiration rates.

Prior to incubation in the electrode chamber, protoplasts were kept under aerobic conditions (gentle shaking in small Erlenmeyer flasks in relevant stabilised buffer). Protoplasts were transferred to the electrode chamber and allowed to equilibrate for 15min. After this time glucose (in relevant stabilised buffer) was added to the chamber at a final concentration of 100mM. A screw lid with a narrow aperture was inserted to reduce the surface area of the sample in direct contact with the air and the oxygen uptake measured over a suitable time period. Results were expressed as nmol oxygen used $hour^{-1}$ per protoplast, e.g.,

The system was calibrated and the number of divisions corresponding to 100% O_2 saturation determined, e.g., let 84 divisions = 100%. The O_2 uptake by the protoplast sample is determined over a suitable period of time (15min) e.g., 5 divisions in 15 minutes. The saturation value of O_2 in water at 25°C is 0.253 μmol ml^{-1}. The concentration of protoplasts was predetermined, e.g., = 2×10^5 ml^{-1}.

$\frac{0.253}{84}$ = 3 nmol O_2 in 1 division

3 x 5 divisions = 15 nmol O_2 in 15 min

$\frac{15}{15}$ = 1 nmol O_2 min-1

1 x 60 = 6 nmol O_2 hr^{-1} per sample

$\frac{6}{2 \times 10^5}$ = 3 x 10^{-4} µmol O_2 hr^{-1} per protoplast

3 x 10^{-4} x 1000 = 0.3 pmol O_2 hr^{-1} per protoplast

2.3 Results and discussion

2.3.1 Characterisation of *B. cinerea* isolates

2.3.1.1 Growth

The growth rates of the 9 isolates were recorded over a 5d period (Figures 2.3a, 2.3b, 2.3c). Data presented are colony diameter mean values ± SE, n = 5. Isolates 1,4 and 5 were the fastest growing and exhibited similar growth patterns. It would seem likely that these isolates are from the same origin. Isolates 6, 7 and 8 also grew at similar rates and were likely to be from the same origin. Isolate 2

Figure 2.3a Growth rates of *B. cinerea* isolates 1, 2 & 3 (results are mean values ± SE).

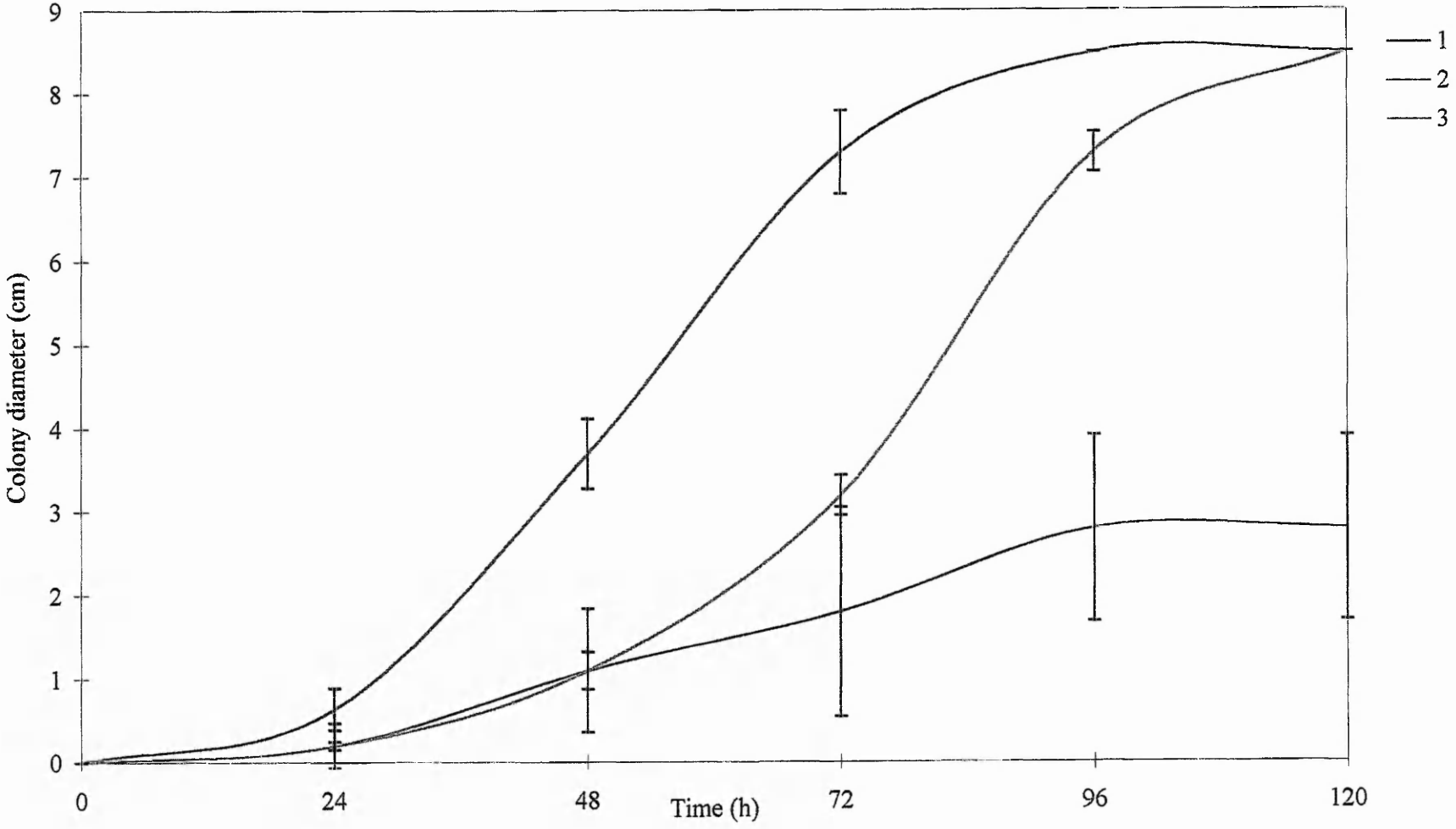


Figure 2.3b Growth rates of *B. cinerea* isolates 4, 5 & 6 (results are mean values ± SE).

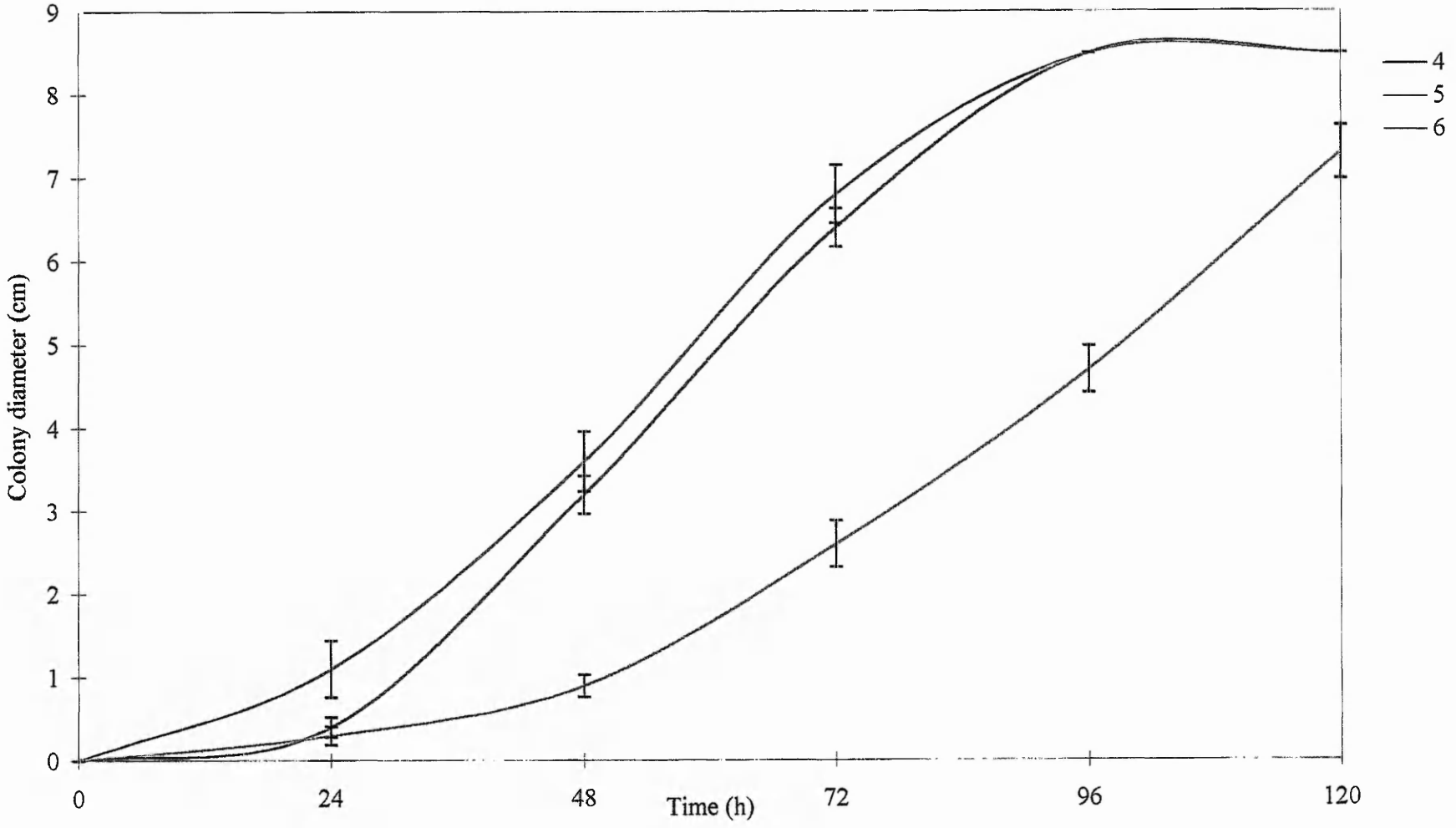
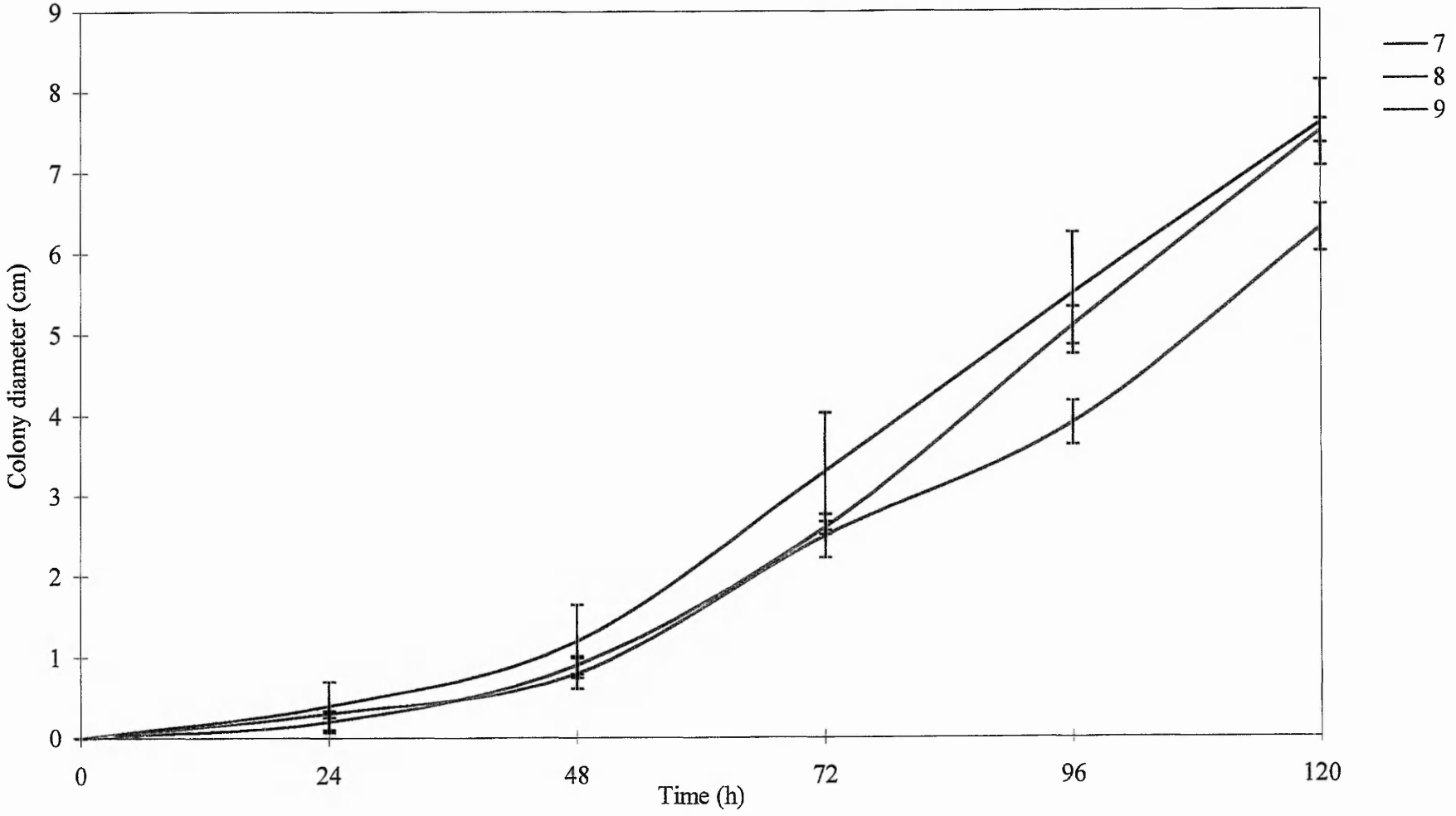


Figure 2.3c Growth rates of *B. cinerea* isolates 7, 8 & 9 (results are mean values ± SE).



was the slowest growing, and also showed the most variation. Isolate 1 was chosen for future studies as it grew quickly and sporulated well.

2.3.1.2 Storage

Isolates were recovered from all 3 storage methods over a 36 month period. Silica gel storage was the most time consuming and also the least satisfactory, with some crystals failing to re-establish fungal colonies after inoculation onto PDA. Agar plugs stored under water were able to continue growing when transferred to fresh PDA, however this method was prone to bacterial contamination. Spores stored in the bacterial cryopreservation vials were able to re-establish fungal colonies after transferral of a bead to PDA. No bacterial contamination was encountered and colonies were successfully re-established on every occasion. Bacterial cryopreservation vials were found to be the choice method of storage of *B. cinerea* spores.

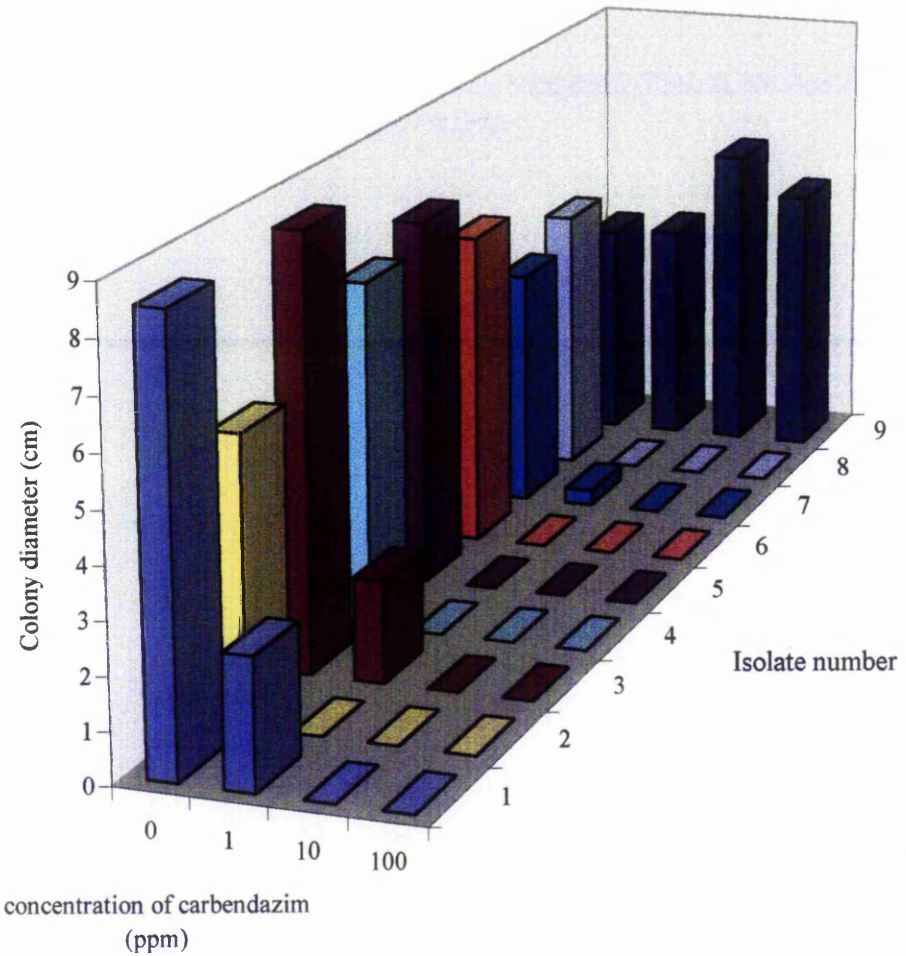
2.3.1.3 Determination of isolate sensitivity to carbendazim

Each isolate (BC1-BC9) was tested for sensitivity to the fungicide carbendazim. Figure 2.4 illustrates that isolates 1-8 were sensitive to carbendazim, at concentrations as low as 1ppm. Isolate 9 showed no sensitivity to the fungicide, even at 100ppm, and was therefore resistant to carbendazim.

2.3.2 Production of mycelia in liquid culture

Spores of *P. infestans* and *S. nodorum* germinated and grew well in liquid culture, as described in the relevant literature. Spores of *B. cinerea* were

Figure 2.4 Sensitivity of 9 *B. cinerea* isolates to the fungicide carbendazim. (Results are mean values, SE ranged from 1.8 to 13.0%)



incubated in GGM, at concentrations ranging from 1 x 10^5 ml^{-1} to 1 x 10^7 ml^{-1}. At lower concentrations spores germinated and produced large quantities of pale-grey mycelium. At concentrations in excess of approx. 5 x 10^6 ml^{-1}, spores germinated but did not grow well, often producing smaller quantities of dark-grey mycelium. This phenomenon, known as self-inhibition, occurs when spore concentrations are too high, resulting in competition for nutrients and space leading to poor growth. Protoplast isolation from this dark mycelium proved unsuccessful, possibly due to increased pigmentation which can interfere with enzyme action (Peberdy, 1979). Spore concentrations below 1 x 10^6 ml^{-1} were used for further experiments.

2.3.3 Protoplast isolation and purification

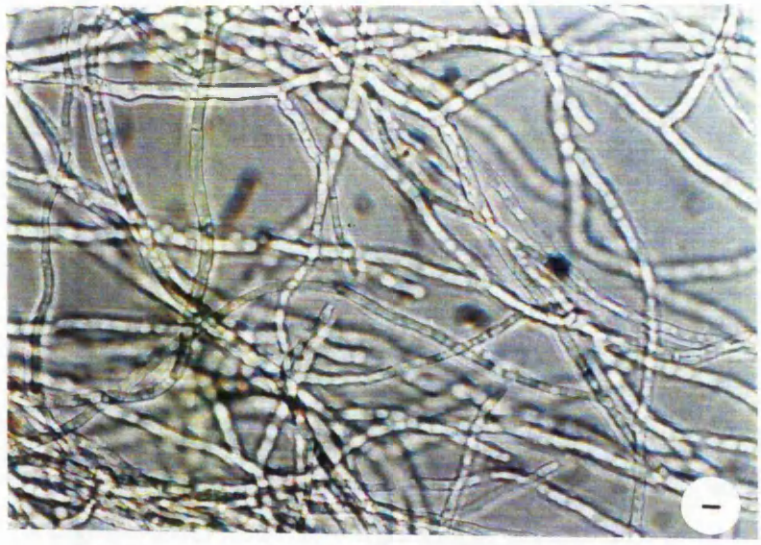
Protoplasts were successfully isolated from all 3 species (Table 2.1). Figure 2.5 shows protoplast isolation from *B. cinerea* mycelia.

Species	Mean yield protoplasts g^{-1} fresh weight ± SE		n
B. cinerea	4.16 ± 0.70 x 10^7		119
P. infestans	1.79 ± 0.33 x 10^7	(Treatment A)	33
P. infestans	2.00 ± 0.15 x 10^5	(Treatment B)	10
S. nodorum	1.39 ± 0.23 x 10^7		46

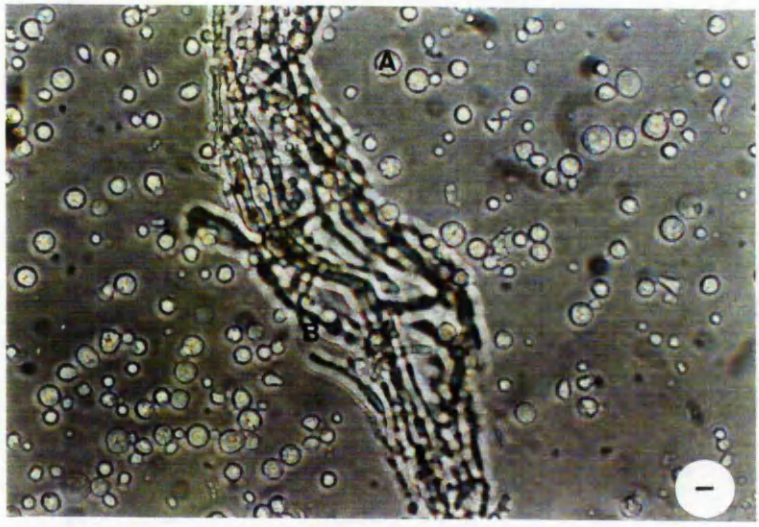
Table 2.1 Summary of protoplast yields obtained over a 36 month period of experimentation. Data are mean values ± SE, n = no. of isolations.

Figure 2.5 Isolation of *B. cinerea* protoplasts

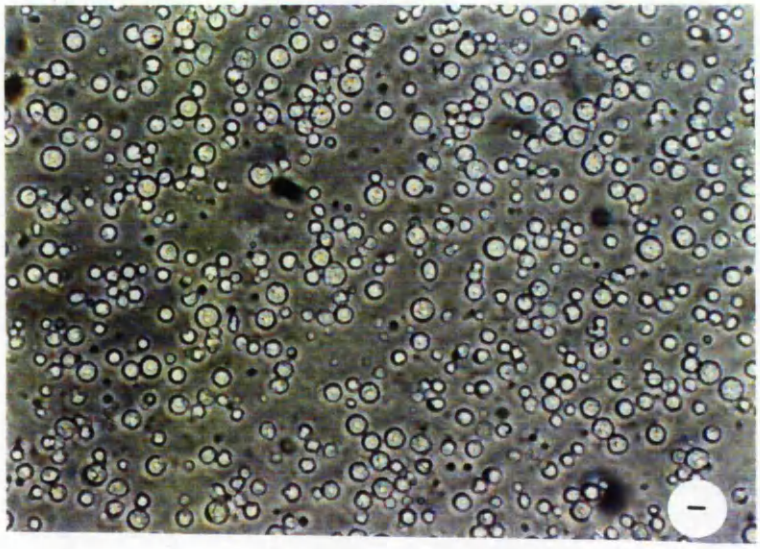
Mycelia were resuspended in stabilized phosphate buffer containing lysing enzymes.



After 2h incubation, the mycelial wall had been largely degraded by the enzymes, generating both protoplasts (a) and mycelial fragments (b).



The protoplasts were separated from the mycelial fragments by filtration through a sieve, and the enzyme solution removed by centrifugation.



bar = 20μm

These results are comparable to published data for *B. cinerea* (Shirane & Hatta, 1986; Braun & Heisler, 1990), *P. infestans* (Kinghorn *et al.*, 1991; Judelson & Michelmore, 1991) and *S. nodorum* (Cooley *et al.*, 1988), and demonstrate that these methods are a reliable and reproducible means of isolating protoplasts.

Higher yields of protoplasts were obtained from *P. infestans* using Treatment A, although the extent of mycelial debris was also much higher. This can be attributed to the increased concentration of cellulase in the lytic enzyme solution, causing greater fragmentation of the cellulose-containing cell wall. Cellulose is only found in a limited number of fungi, mainly the Oomycetes, where it is a typical cell wall constituent (Bartnicki-Garcia, 1968).

Increased mycelial debris is undesirable in the protoplast samples, which should be as clean as possible to avoid misleading results. However, attempts to separate the *P. infestans* protoplasts from the debris by (a) repeated passage of the sample through a sieve and subsequent centrifugation, (b) sedimentation through sugar gradients and (c) separation using Sephadex columns, proved unsuccessful, as the mycelial fragments were of a similar size and density to the protoplasts. Isolation of protoplasts in $MgSO_4$ and attempts to collect protoplasts between solutions of different density were also unsuccessful. The decision whether or not to include additional cellulase in the isolation mixture would need to take into consideration the purity of the protoplast samples required for each experiment.

Stagonospora nodorum protoplasts were separated from mycelial debris by centrifugation at 4000 x g, where they formed a distinct band at the interface of the different solutions. This phenomenon was first recognised by de Vries and Wessels (1972) working on the release of protoplasts from *Schizophyllum commune*. It was discovered that protoplasts released in 0.6M $MgSO_4$ often contained one large vacuole and were able to float. This effect was not seen when the protoplasts were isolated in other osmotic stabilisers. Work by Isaac (1978)

confirmed this property of $MgSO_4$, which could not be induced by other sulphates or other magnesium salts. Releasing protoplasts in $MgSO_4$ is therefore a good way of obtaining clean samples, free from debris although it may not be suitable for all fungi, as demonstrated here.

2.3.4 Protoplast viability

FDA staining revealed high protoplast viability in all cases, on average 96% for *B. cinerea*, 93% for *P. infestans* and 97% for *S. nodorum*. Protoplasts stored at 4°C retained viability for in excess of 7d, however freshly isolated protoplasts were used for future experiments, as it was expected that their metabolic capabilities were closer to that of the intact fungus than protoplasts which had been stored at 4°C. FDA stained protoplasts of *B. cinerea* are shown in figure 4.2 (Chapter 4).

2.3.5 Respiration studies

Respiration rates were calculated for protoplasts from all 3 species (Table 2.2). It is unrealistic to compare the respiration rates for each species, as these will be influenced by many factors, such as protoplast size and robustness. Respiration rates were fairly consistent for *B. cinerea* and *S. nodorum* protoplasts, but showed a high degree of variation for *P. infestans* protoplasts. This might reflect the increased mycelial debris present in the *P. infestans* samples, and would need to be investigated prior to further use.

Species	Mean consumption O_2 per protoplast (pmol h^{-1}) ± SE
B. cinerea	0.17 ± 0.008
P. infestans	8.2 ± 3.9 (Treatment A)
S. nodorum	0.027 ± 0.004

Table 2.2 Protoplast respiration rates. Data are mean values ± SE, n = 10.

Determining the normal metabolic status of protoplasts, as has been achieved here, is a pre-requisite for studying the metabolic capabilities of chemically treated protoplasts. Oxygen electrodes were subsequently used to establish EC50 values for a range of fungicides, and the results compared to enzyme, whole-cell and *in planta* data (Chapter 6) to allow protoplast research to be placed into the wider context of agrochemical discovery.

To facilitate a more in-depth approach to future experiments, it was necessary to choose one species to continue studying. Protoplast production from *B. cinerea* proved easier and more consistent than from the other two species, for this reason it was chosen for all future work.

2.4 Conclusions

- Field isolates of *B. cinerea* were characterised with respect to growth, storage and sensitivity to the fungicide carbendazim.

- Protoplasts were successfully isolated from *B. cinerea*, *P. infestans* and *S. nodorum*, with yields comparable to published data.

- The viability and metabolic capabilities of the isolated protoplasts were assessed using fluorescent microscopy and polarography techniques.

Chapter Three: Interactions of surfactants with *B. cinerea* protoplasts

3.1 Introduction

Adjuvants are substances other than water, which have no pesticidal properties alone, that are mixed with formulated pesticides to improve their efficiency (Stock, 1996). Adjuvants may act to improve pesticide efficiency in a number of different ways including increasing uptake into the plant and pathogen (de Ruiter & Meinen, 1996), better delivery of the active compound to the target site, and optimising the physical properties of the active ingredient in pesticide formulations (Caux, Weinberger & Szabo, 1993). Adjuvant-increased foliar absorption may be influenced by many factors including drop spreading, drop drying time, permeability of the cuticle and cell membrane (de Ruiter & Meinen, 1996). Although much is known about the action of adjuvants on plant absorption of pesticides, less is understood about possible mechanisms within the pathogen. Such information would be extremely valuable for the development of new compounds which could promote increased absorption of fungicides into the pathogen.

Surfactants are a common group of adjuvants used in the agrochemical industry. The interaction of surfactants with both lipid bilayers and biological membranes has been well studied (see the review by Helenius & Simons, 1975). When small amounts of surfactants are added to water, a monolayer forms at the air/water interface, with monomers also dissolving in solution. When the monomer concentration is increased beyond a critical value, known as the critical micellar concentration (CMC), micelles form by monomer association (Figure 3.1).

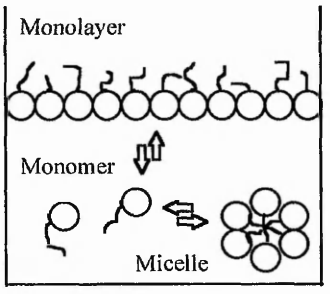


Figure 3.1 Equilibrium of surfactant between monomeric, monolayer and micellar forms (after Helenius & Simons, 1975).

The effects of detergents on biological membranes can be viewed at 3 levels, binding to the membrane, solubilization of the membrane and separation of lipids and proteins. At low surfactant to membrane ratios, changes to the membrane may result in an increase in permeability and altered membrane-bound enzyme activities (Helenius & Simons, 1975). This is followed by leakage of macromolecules and finally membrane disintegration, where the extent of membrane solubilization is related to the amount of detergent bound to it. Membrane concentrations are therefore important and should be considered when presenting results (Helenius & Simons, 1975). A series of alcohol ethoxylate surfactants made by Lankro (Akcros Chemicals, Eccles, UK) were used in this study. The surfactants have the following general structure:

$$CH_3(CH_2)x(CH_2OCH_2)nOH$$

Where: n = the number of ethylene oxide groups (in this series 8, referred to as E8) &

x = the point at which the carbon chain length varies in 2 units, from C8 to C16

The surfactants are 100% linear. The CMC values were provided for each compound, calculated using molar values from Rosen, 1978 (Table 3.1).

Surfactant	Molecular weight	CMC g L^{-1}
C8E8	482	4.338
C10E8	510	0.51
C12E8	540	0.054
C14E8	566	0.005094
C16E8	594	0.001247

Table 3.1 Properties of the alcohol ethoxylate series used in this study

As evident in Table 3.1, the true solubility decreases with increasing carbon chain length, those surfactants with a longer carbon chain will form micelles in solution at lower concentrations. The aim of this study was to investigate the effects of different concentrations of these surfactants on protoplasts of *B. cinerea* and to ascertain the preferential carbon chain length for protoplast interaction.

The approach used involved incubation of the protoplasts with the surfactants for a given time period within an oxygen electrode chamber and measurement of respiration rates, followed by microscopic examination of the protoplasts. As respiration is a measure of protoplast physiology, any perturbation of this process is a valuable marker of surfactant interaction. Additionally, if the surfactants act on the protoplast membrane as described in the literature (de Ruiter & Meinen, 1996, found an increased uptake of herbicide into plant protoplasts in the presence of surfactants), then an increased uptake of fungicides that act by inhibition of electron transport may be detectable in the oxygen electrode system with the addition of surfactant.

3.2 Materials and methods

3.2.1 CMC studies

As the detergency of surfactants is related to the CMC, this concentration was chosen as a starting point to investigate surfactant/membrane interactions. Concentrations of the Lankro surfactants (obtained from Dr P. Holloway, IACR-LARS, Bristol, UK), equivalent to one tenth of the CMC and ten times the CMC value were studied for comparison. From the series, surfactants with carbon chain lengths of 8, 12 and 16 were chosen as representative of short, medium and long carbon chain molecules. Surfactant solutions were made in Phos-KCl at 10 times the final concentration required and diluted 1 in 10 in the oxygen electrode chamber. *Botrytis cinerea* protoplasts were isolated and prepared for the oxygen electrode (1×10^6 ml^{-1}), as described previously in Chapter 2.

An initial (control) respiration rate was established for each protoplast sample. After 15min surfactants were gently added to the chamber using a Hamilton syringe, (as far as possible avoiding the introduction of air bubbles) to final concentrations of 0.1 CMC, CMC or 10 x CMC. Respiration rates were measured for a further 20min. A separate protoplast sample was incubated for 35min (the total time of the experiment) under the same conditions, after 15min buffer with no surfactant was added using a Hamilton syringe and the respiration rate measured for a further 20 min. This was necessary in order to ascertain if any differences observed could be attributed to the addition of the surfactant, or to the time period of the incubation. Small samples were routinely removed from the electrode and examined microscopically. Results are expressed as mean pmol O_2 h^{-1} per protoplast ± SE, data are duplicates from 4 or 5 separate occasions and have been compared using a 2 way ANOVA. Treatment means were separated using Tukey's HSD criterion.

3.2.2 Molarity studies

Following the CMC experiments and in view of the surfactant properties (Table 3.1 demonstrates that at concentrations ≡ CMC, C8 is present at a far greater concentration than C16), investigations based on a molarity series were undertaken, in order to determine if the results observed using the CMC solutions were related to the carbon chain length of the surfactants, or were due to the amount of surfactant present. Surfactants C8 and C16 were used as representative of short and long carbon chain molecules. The experiments were carried out as detailed above, with the exception that surfactants were used at final concentrations of 2,4,6,8 and 10mM. Results are expressed as mean pmol O_2 h^{-1} per protoplast ± SE, data are from 2 separate occasions, n = 4 or 5 and have been compared using a 2 way ANOVA. Treatment means were separated using Tukey's HSD criterion.

3.3 Results and discussion

3.3.1 CMC studies

Table 3.2 shows the respiration rates of *B. cinerea* protoplasts incubated with each surfactant at three concentrations, compared to control data. Protoplasts rapidly ruptured upon addition of C8 surfactant at and above the CMC concentration, and C12 surfactant at 10 times the CMC (Table 3.2). Protoplasts did not rupture upon addition of C16 surfactant at any concentration tested. There was no significant difference in the mean respiration rates of protoplasts incubated with surfactants compared to the controls, at those concentrations that did not cause protoplasts to rupture (Table 3.2, Tukey's HSD criterion). This shows that over the time course

investigated, pre-rupture levels of surfactants were not significantly interfering with cellular respiration.

	Mean respiration rate (pmol O_2 h^{-1} x 10^{-2} per protoplast) ± SE, n = 8 or 10			
Surfactant	Control	0.1 x CMC	CMC	10 x CMC
C8	2.08 ± 0.55 [a]	2.97 ± 0.26 [a]	0 (ruptured) [b]	0 (ruptured) [b]
C12	2.13 ± 0.32 [a]	3.04 ± 0.37 [a]	2.54 ± 0.30 [a]	0 (ruptured) [b]
C16	2.67 ± 0.36 [a]	2.45 ± 0.38 [a]	2.28 ± 0.40 [a]	2.26 ± 0.42 [a]

Table 3.2 Mean respiration rates of *B. cinerea* protoplasts (1 x 10^6 ml^{-1}) incubated with alcohol ethoxylate surfactants C8, C12 & C16. Data are duplicates from 4 or 5 separate occasions (n = 8 or 10) ±SE. Two-way analysis of variance found a significant surfactant-type (C8, C12 or C16) effect ($F = 15.91$, $p<0.01$), a significant concentration effect ($F = 25.45$, $p<0.01$) and a significant interaction between these variables ($F = 9.33$, $p<0.01$). Means sharing the same letter were not significantly different from each other ($p<0.01$, Tukey's HSD criterion).

The results indicated a possible carbon chain length effect, with the shorter molecules proving more damaging to the protoplast membrane than the larger ones. However, as the surfactants used were based on CMC values, the C8 surfactant was present at a much higher molarity than the C12 and C16 surfactants, i.e. the CMC value of C8 = 4.338 g L^{-1}, whereas the CMC value of C16 = 000127 g L^{-1}. In order to determine if the results observed were related to the carbon chain length of the surfactants, or were due to the concentration of surfactant present in the solutions, a second experiment based on molarity was undertaken.

3.3.2 Molarity studies

The C8 surfactant caused protoplasts to rupture at a concentration equivalent to the CMC, but not at 0.1 CMC. These figures equate to 10mM and 1mM, respectively. A molarity-based series between these values was investigated for the C8 and C16 surfactants. At 2mM (Figure 3.2), neither C8 or C16 surfactants caused the protoplasts to rupture. At 4mM and above, the C8 surfactant caused the protoplasts to rupture whereas the C16 surfactant did not (Figure 3.3), indicating that the effect of the surfactants on the protoplast membranes was related to carbon chain length and not simply to the quantity of surfactant present. Two-way analysis of variance found a significant surfactant-type (C8 or C16) effect ($F = 4.72$, $p<0.05$), and a significant concentration effect ($F = 7.13$, $p<0.05$) but no significant interaction between these variables ($F = 0.77$, $p>0.05$). Tukey's HSD criterion multiple comparison test found no significant difference ($p>0.05$) between the respiration rates of protoplasts incubated with pre-rupture levels of surfactant compared to the controls, consolidating the data obtained from the CMC series of experiments.

The smaller C8 molecule is able to cause membrane solubilization to occur, whereas the larger C16 molecule cannot at the concentrations tested. It is possible that the C8 surfactant is able to partition into and cross the protoplast membrane, where it accumulates causing membrane damage and ultimately, disintegration. The larger C16 molecule may be unable to partition into or cross the membrane, and is thus incapable of causing protoplast rupture.

These experiments have identified C8 to be the preferential carbon chain length for protoplast interaction. In addition the concentration of surfactant which causes membrane solubilization and hence protoplast rupture has been identified as 4mM for protoplasts at a concentration of 1×10^6 ml^{-1}. The results demonstrated that the length of the carbon chain rather than the ability of the surfactants to form micelles in

Figure 3.2 Mean respiration rates of *B. cinerea* protoplasts incubated with 2mM C8 and C16 surfactants. (n = 5, SE 6-18%) Two way AVOVA found a significant ($p<0.05$) surfactant-type effect ($F=4.72$) and a significant concentration effect ($F=7.13$).

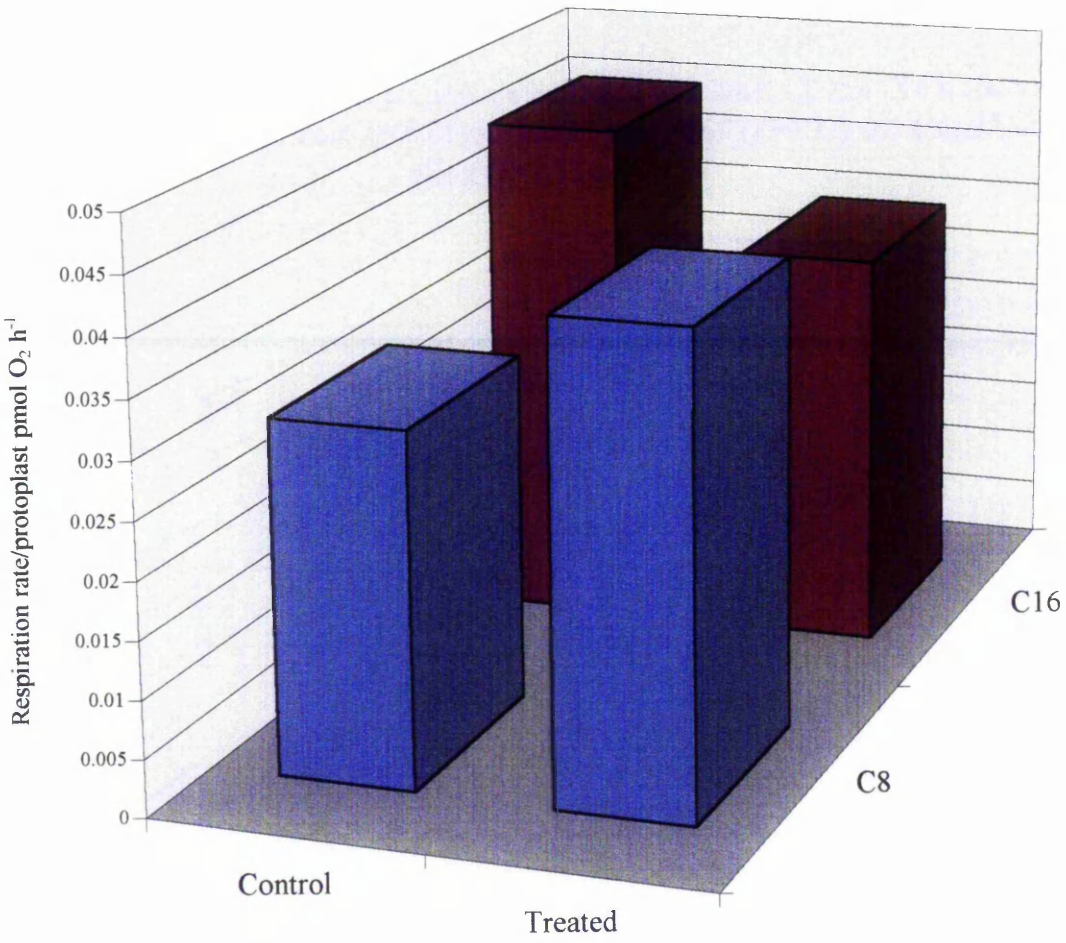
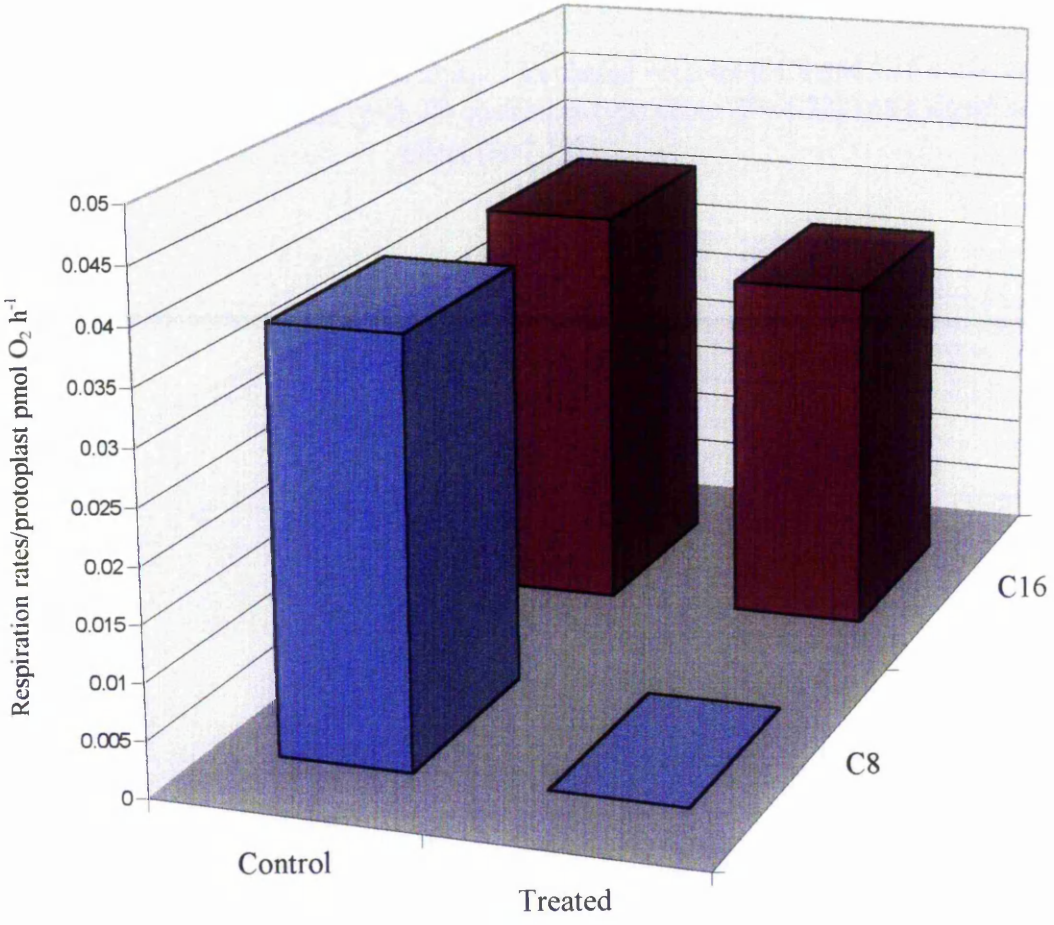


Figure 3.3 Mean respiration rates of *B. cinerea* protoplasts incubated with 4mM C8 and C16 surfactants. (n = 5, SE 6-16%) Two way AVOVA found a significant ($p<0.05$) surfactant-type effect ($F= 4.72$) and a significant concentration effect ($F=7.13$).



solution, was the predominant driving force leading to membrane solubilization. When protoplasts remained intact, there was no significant change in respiration rates. This work is a prerequisite to studying the effects of adding pre-rupture levels of C8 surfactant to protoplasts treated with fungicides. Fungicides that act by inhibiting the electron transport chain (for example, the β-methoxyacrylates) can be used in the oxygen electrodes to generate EC50 values for the isolated protoplasts. If, as postulated, the pre-rupture level C8 surfactant is able to partition into the protoplast membrane and influence transport processes, this will be reflected in the EC50 values of control versus treated protoplasts (Chapter 6).

3.4 Conclusions

- The effect of alcohol ethoxylate surfactants C8, C12 and C16 on protoplasts of *B. cinerea* was examined. The ability of the surfactants to form micelles in solution was shown not to be the predominant driving force leading to membrane disruption.

- A carbon chain length effect was discovered, with the smaller compounds (C8, C12) able to solubilize the protoplast membrane, whilst the larger compound (C16) was unable to cause protoplasts to rupture at the concentrations tested.

- The surfactant with the preferential carbon chain length for protoplast penetration, was found to be C8, which caused membrane solubilization at final concentrations of 4mM, but not at 2mM when incubated with 1 x 10^6 ml^{-1} protoplasts.

- Respiration rates of surfactant treated protoplasts which remained intact, were not significantly different to the untreated controls.

Chapter Four: The use of fluorescein diacetate to determine protoplast viability

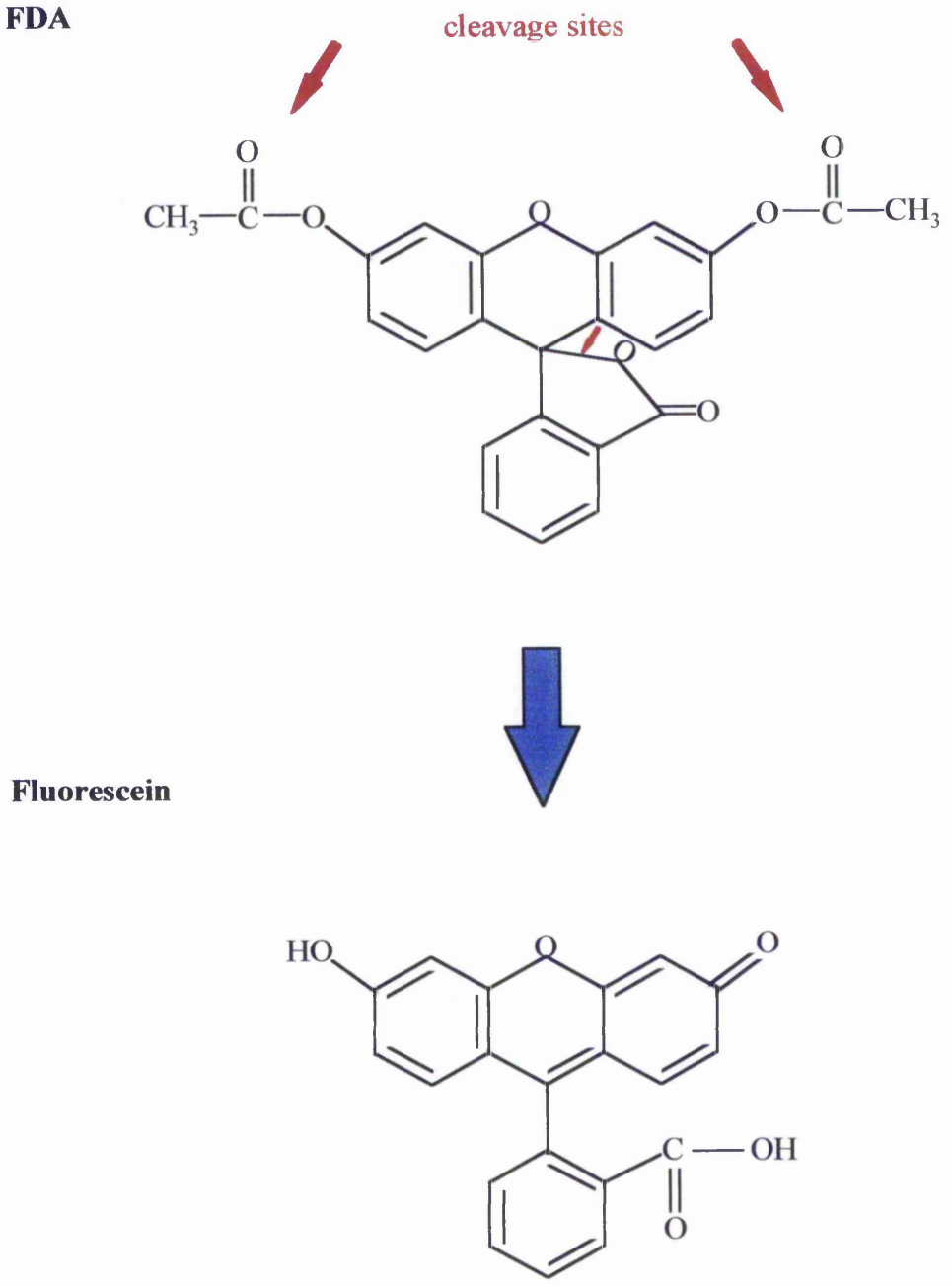
4.1 Introduction

Fluorochromes are dyes that fluoresce when excited by ultraviolet (UV) or visible light (Butt, Hoch, Staples & Leger, 1989). The range of fluorescent probes available makes fluorescent microscopy an increasingly valuable tool, with many applications in modern research (see Handbook of Fluorescent Probes and Research Chemicals, Haughland, 1996). One such application is the determination of cell viability, which allows live cells to be distinguished from dead cells within a population using fluorescent dyes either alone or in combination. Cell permeant fluorogenic esterase substrates act as viability probes and are capable of determining both enzyme activity and membrane integrity of living cells (Haughland, 1996).

Fluorescein diacetate (FDA) has been used to determine cell viability in a wide range of organisms including mycobacteria, fungi and plants (Butt *et al.*, 1989) and has more recently been used in combination with ethidium bromide for live/dead assays (Jayapal, Sharmila, Selvibal, Thyagarajan, Shanmugasundaram & Subramanian, 1991). The non-polar FDA molecule crosses the plasma membrane of the cell and its ester bonds are hydrolysed by non-specific esterases in the cytoplasm to release free fluorescein (Figure 4.1) The polar fluorescein molecule is unable to cross the plasma membrane or tonoplast and therefore accumulates in the cell, which fluoresces bright green when viewed under blue light (Butt *et al.*, 1989).

Dead cells and cells with damaged membranes do not retain the fluorescein molecule and can be seen either by counter-staining with dyes such as propidium iodide and ethidium bromide, which stain red and have been extensively used to detect dead and dying cells (Haughland, 1996), or by switching the microscope between

Figure 4.1 The structure of FDA and fluorescein molecules. The non-polar FDA molecule is cleaved by non-specific cell esterases to form the polar fluorescein molecule, which is retained by the cell and fluoresces bright green under blue light.



visible and blue light. The latter demands that all cells viewed under a normal light field be reassessed under a blue light field, allowing percentage viability to be determined, whereas the former employs a double blue/green light exposure technique to allow simultaneous recording of fluorescence from live and dead cells (Butt *et al*, 1989). Figure 4.2 shows viable protoplasts of *B. cinerea*, stained with 0.01% (w/v) FDA.

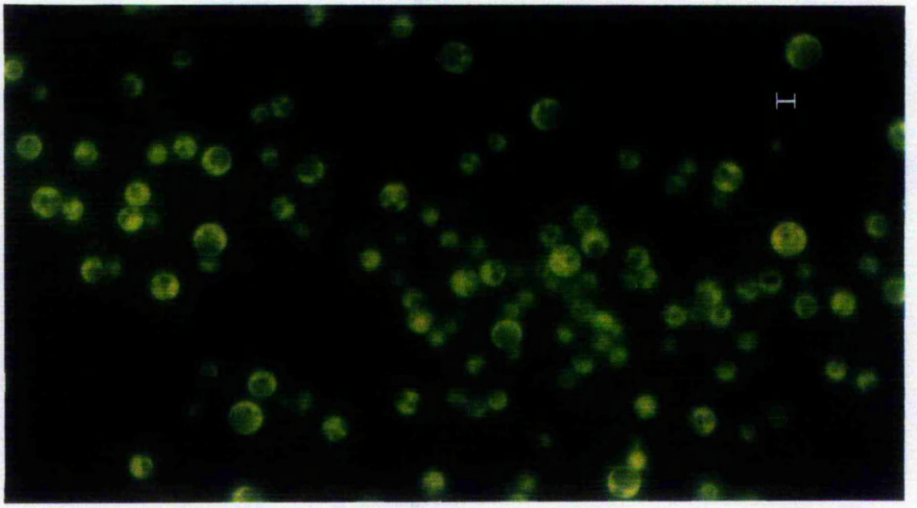


Figure 4.2 Viable protoplasts of *B. cinerea* (1×10^7 ml^{-1}) after staining with 0.01% (w/v) FDA, viewed with an Axioskop microscope filter set 24, (Zeiss, West Germany). Bar = 10μM.

FDA has previously been used to determine viability in plant, fungal and marine red alga protoplasts (Huang, Cornejo, Bush & Jones, 1986; Richards, 1994; Sivan, Vanmoppes & Arad, 1992) and is a useful indicator of percentage viability within a population of cells (Chapter 2). In 1991, the development of a sensitive and convenient fluorometric assay for the *in vitro* evaluation of anticytomegalovirus agents (Neytus, Snoeck, Schols, Himpens & DeClercqe, 1991) allowed easy distinction by fluorimetry of infected and non-infected human cell cultures. After infection, cells

showed an enhanced esterase activity and therefore an increased FDA-derived fluorescence. Results were similar to those obtained from the more time consuming microscopic evaluation. Similar assays have been successfully used to quantify fluorescent intensity in mammalian cells (Maeda, Tanaka, Koga, Zhang, Sasaki, Kimura & Nomoto, 1993), plant cells and protoplasts (Aoyagi & Tanaka, 1994) and to determine microbial activity in wood (Bjurman, 1993).

The development of an assay allowing quantification of the fluorescence from FDA-treated fungal protoplasts using a fluorescence spectrophotometer, may provide a means of rapidly screening protoplasts treated with potential toxophores for viability and generate useful information regarding protoplast sensitivity to other compounds such as organic solvents.

4.2 Materials and Methods

Protoplasts from *B. cinerea* were isolated as described previously in Chapter 2. A Perkin-Elmer 650-40 Fluorescent Spectrophotometer and 150 Xenon power supply were used, with the excitation and emission wavelengths set at 490nm and 525nm respectively. In order to develop an assay, it was necessary to investigate and optimise a number of experimental parameters, including FDA concentration, incubation period prior to detection of fluorescence and protoplast concentration. It was also necessary to measure any background fluorescence generated from either the buffer, the protoplasts or the FDA stain itself and correct the data for this. The sensitivity of the assay was then investigated by challenging the protoplasts with treatments expected to significantly alter their viability, for example by heating to 50°C for 1h, prior to addition of FDA. Finally, the assay was employed to determine protoplast tolerance to organic solvents and known fungicides.

4.2.1 FDA Concentration

FDA is conventionally dissolved in acetone and diluted in water, buffer or culture medium to a final working concentration of approximately 0.01% w/v (Butt *et al*, 1989). In this study, 0.01% (w/v) FDA (Sigma) was successfully used to examine protoplast viability microscopically (Chapter 2) and was therefore chosen as a starting point for the assay. As a comparison, a final concentration of 0.001% FDA was also used. This equates to 240μM and 24μM FDA, respectively. Subsequent experiments used FDA at final concentrations 100, 10, 1 and 0.1μM. Stocks of 100, 10, 1 and 0.1mM FDA in acetone were stored at -20°C and diluted with Phos-KCl buffer (final concentration acetone 0.1%) Fresh dilutions from the stock were made on each occasion.

4.2.2 Incubation period prior to fluorescence detection

The extent of FDA derived fluorescence detected by the spectrophotometer was considered likely to alter over time as fluorescein accumulated within intact, viable protoplasts. It was therefore necessary to investigate this parameter in order to choose a suitable time period for incubation of the protoplasts with the stain prior to measurement of the fluorescent product. FDA was added to the protoplasts in individual 3ml polystyrene fluorimeter cuvettes (Sigma) which were inverted to mix and placed into the spectrophotometer with the light source covered. The samples were kept in the dark for all but 5 seconds prior to the reading being taken, in order to prevent fading of the fluorescence. Readings were taken every minute for 15min. This time course was undertaken for the different FDA concentrations tested.

4.2.3 Protoplast concentration

The above FDA concentration and time-course experiments were carried out using protoplasts at 2 densities, 5 x 10^5 ml^{-1} and 1 x 10^6 ml^{-1}. Results are mean values ± SE, from 2 separate occasions, n = 4 & 6 for each time point respectively. A second experiment using FDA at 10μM over a time-course of 10min was undertaken using protoplasts at 1 x 10^6 ml^{-1}, 1 x 10^5 ml^{-1}, 1 x 10^4 ml^{-1} and 1 x 10^3 ml^{-1}. Protoplast densities were determined by haemocytometry. All samples within individual experiments were generated by diluting a single concentrated protoplast stock with Phos-KCl buffer. Results are mean values ± SE from a single occasion, n = 4 for each time point.

4.2.4 Background fluorescence

In order to account for any background fluorescence generated from either the protoplasts, the buffer or the FDA stain, the following readings were also taken:

1. FDA stain only (diluted in buffer) (B)
2. Protoplasts (diluted in buffer), prior to addition of the FDA stain (C)
3. Buffer only (D)

In all cases, multiple readings were taken and a mean value calculated. These values were then used in the following formula:

A -B - C + D

Where A is the reading of the protoplasts after the addition of the FDA stain at time *x*, and B, C and D are as outlined above. This formula subtracts the background fluorescence generated from the FDA stain (B) and the protoplasts (C) and adds on the value generated from the buffer (D), which has been subtracted twice, as buffer is present both with the protoplasts (C) and the FDA stain (B). It is this corrected value that has been presented in the results.

4.2.5 Assay sensitivity

In order to determine the sensitivity of the assay, protoplasts were subjected to different treatments expected to significantly alter their viability, prior to addition of the FDA stain. FDA was used at a final concentration of 10μM and incubated with the protoplasts for 10min prior to taking the reading. Protoplasts from the same concentrated stock were diluted in Phos-KCl buffer and used in all treatments:

1. Incubation in 0.1N HCL for 2h.
2. Incubation with 0.01M C8 surfactant (see Chapter 3) for 2h.
3. Incubation at 50°C for 1 or 2h.
4. Incubation at 70°C for 1 or 2h.

Untreated protoplasts were used as a control. Buffer without protoplasts was treated as per the protoplast samples and used in the background calculations. Results are mean values ± SE from 2 separate occasions, n = 4 for each data set.

4.2.6 Tolerance to organic solvents

Following optimisation of the assay parameters, protoplast tolerance to various organic solvents was determined. Protoplasts were resuspended in Phos-KCl containing 0.01, 0.1, 1 or 10% (v/v aq.) methanol, ethanol, DMSO or acetone to a

final concentration of 1 x 10^6 ml^{-1} and incubated for 3h prior to the addition of 10μM FDA and fluorescence detection. Results are expressed as % increase or decrease in protoplast viability compared to controls, data are duplicates from 3 separate occasions and have been compared using a 2 way ANOVA. Treatment means were separated using Tukey's HSD criterion.

4.2.7 Tolerance to azoxystrobin and pyrimethanil fungicides

Azoxystrobin (manufactured by Zeneca, supplied by AgrEvo) was dissolved in DMSO and added to 1 x 10^6 ml^{-1} protoplasts in Phos-KCl to final concentrations of 1, 10 or 100μM and 0.1% (v/v aq.) DMSO. Pyrimethanil (manufactured and supplied by AgrEvo) was dissolved in DMSO and added to 1 x 10^6 ml^{-1} protoplasts in Phos-KCl to a final concentration of 10μM, 0.1% DMSO. Samples were incubated for 30min or 60min prior to the addition of 10μM FDA and fluorescence detection. Results are expressed as mean % decrease in protoplast viability compared to controls. Data are from 3 separate occasions, n = 15 for each data set and have been compared using a 2 way ANOVA. Treatment means were separated using Tukey's HSD criterion.

4.3 Results and discussion

4.3.1 FDA concentration

The initial feasibility experiment using 240μM and 24μM FDA over a 15min time-course showed good detection of the FDA-derived fluorescein, which increased over time. No difference was seen between the 2 concentrations (data not shown). Subsequently, FDA concentrations of 0.1, 1, 10 and 100μM were tested. The protoplasts incubated with 10μM and 100μM FDA produced similar quantities of

fluorescent product, whilst the protoplasts incubated with 1μM and 0.1μM FDA produced lower quantities of fluorescent product (Figures 4.3 and 4.4), indicating that at 10μM and above the FDA is in excess over the time course investigated. 10μM was therefore chosen for future work.

4.3.2 Incubation period prior to fluorescence detection

The levels of fluorescence increased over the 15min time-course as the protoplasts cleaved the FDA and the fluorescein accumulated within the viable intact cells (Figures 4.3 and 4.4). In order that the fluorescence generated was within the detection range of the spectrophotometer (samples left for an hour were found to exceed this range), and yet reached a reasonably high level to allow for any reductions to be detected accurately, a 10min incubation period was chosen for future work.

It has been suggested that despite its extensive successful use as a viability indicator, the fluorescein produced by FDA hydrolysis rapidly leaks from cells (Haughland, 1996). Evidence from an experiment where the relative cellular fluorescence of a human lymphoid cell line loaded with 7 different intracellular marker dyes was assessed over a 2.5h time period, found that high levels of fluorescein derived from both FDA and carboxyfluorescein diacetate (CFDA) had leaked out of the cells over this time. However, it was unlikely that much leakage would occur over the 10min time period chosen for this assay , indeed no evidence of any such leakage was encountered here.

4.3.3 Protoplast concentration

Figures 4.3 and 4.4 show the results of the assay using 1×10^6 ml^{-1} and 5×10^5 ml^{-1} protoplasts, respectively. Increased fluorescence was seen at higher protoplast

Figure 4.3 Variation in RFU with time, after addition of different concentrations of FDA to protoplasts of *B. cinerea*, 1×10^6 ml $^{-1}$. (Results are mean values ± SE, n = 6)

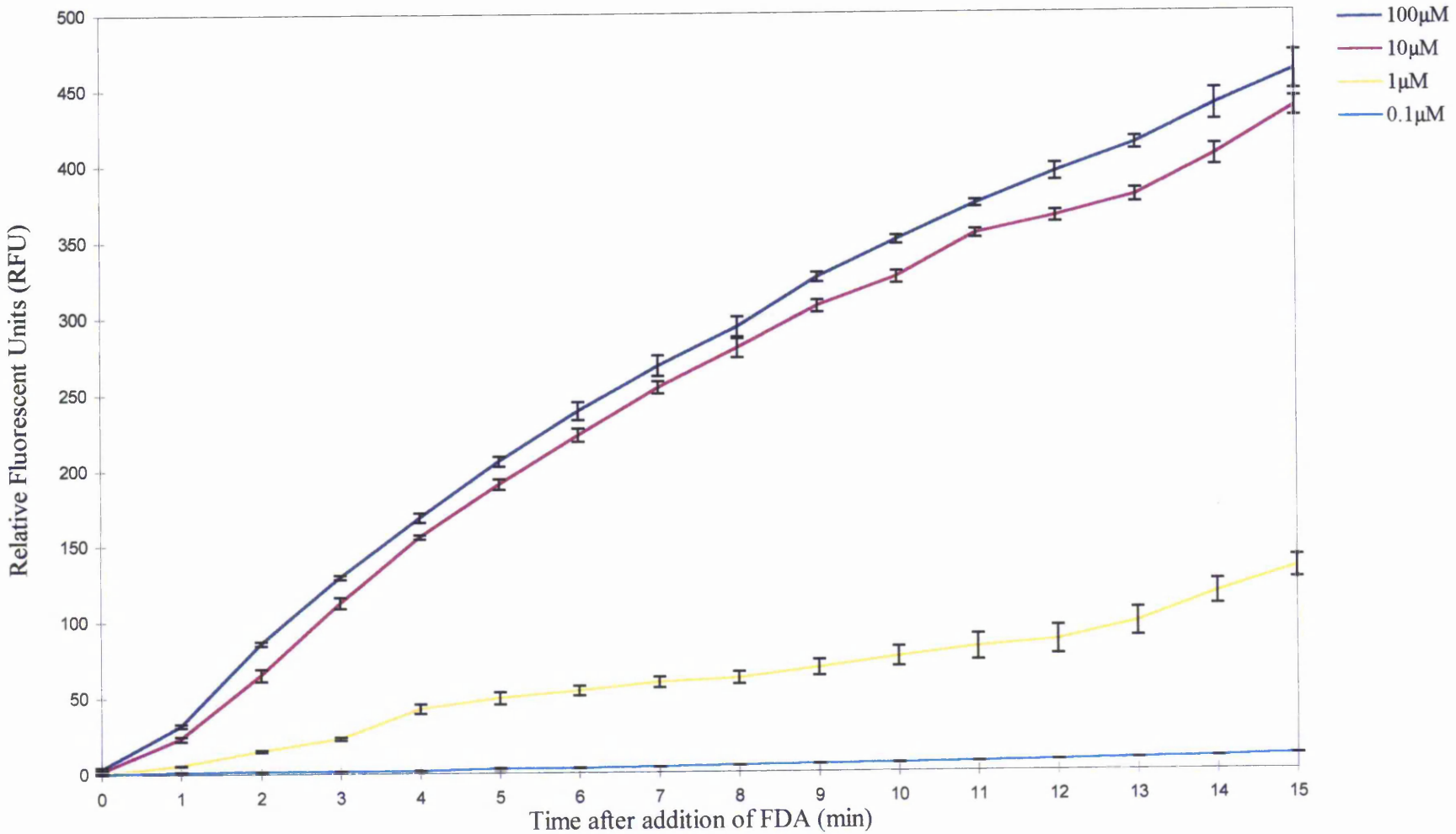
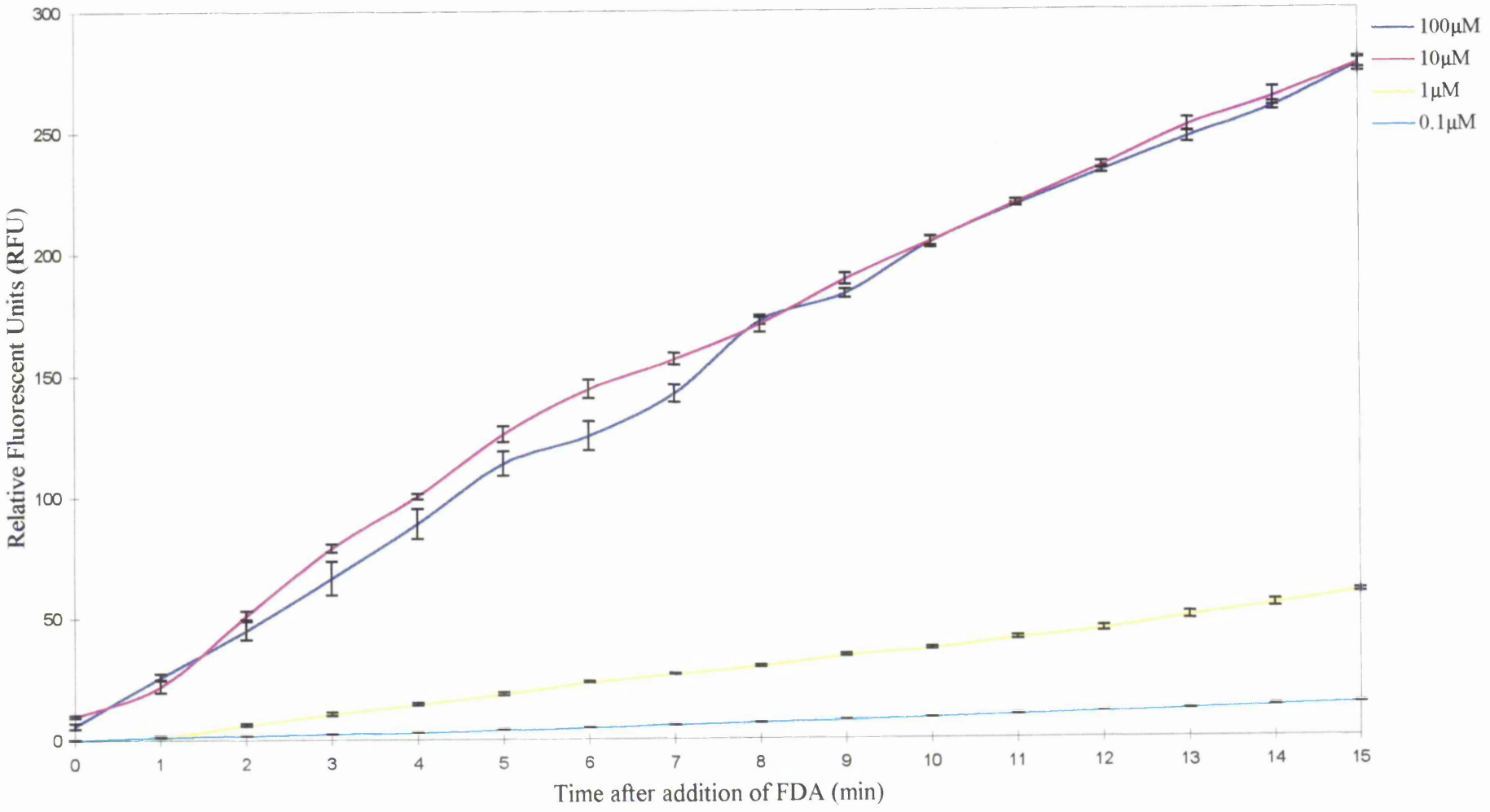


Figure 4.4 Variation of RFU with time, after addition of different concentrations of FDA to protoplasts of *B. cinerea*, 5 x 10^5 ml^{-1}. (Results are mean values ± SE, n = 4)



concentrations, with the 1 x 10^6 ml^{-1} sample exhibiting almost twice the fluorescence of the 5 x 10^5 ml^{-1} sample (350 & 204 RFU following 10min incubation with 100μM FDA respectively). Figure 4.5 shows the results of the assay using 1 x 10^3, 1 x 10^4, 1 x 10^5 and 1 x 10^6 ml^{-1} protoplasts. A significant ($p<0.01$, $r_s = 0.97$) positive correlation was found between protoplast concentration and fluorescence intensity, indicating that the assay accurately measures protoplast viability. For future studies protoplast concentrations were chosen within the range 5 x 10^5 - 1 x 10^6 ml^{-1}, where suitably high levels of fluorescence were generated within the detection scale of the fluorimeter.

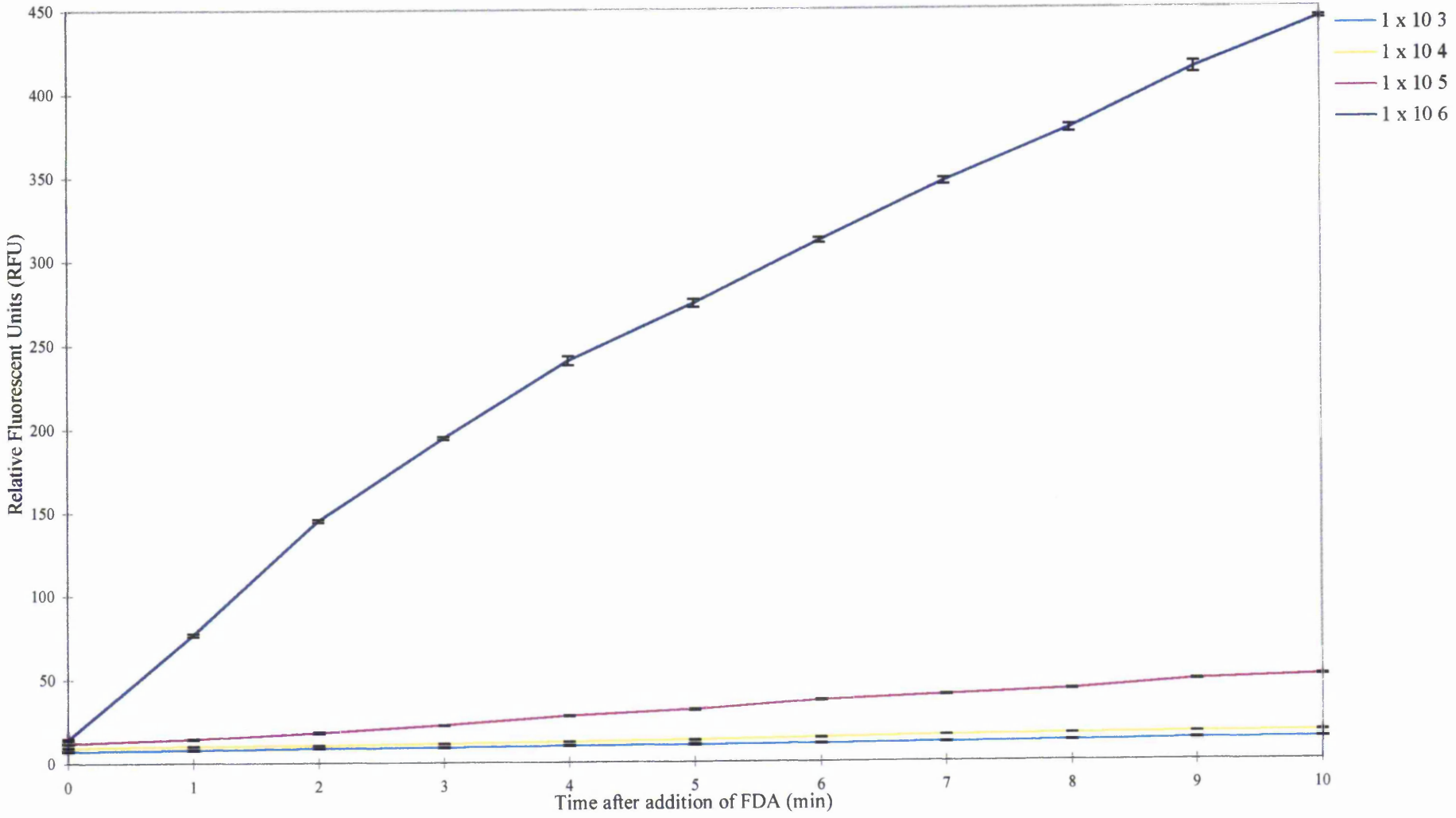
4.3.4 Background fluorescence

Extremely low levels of background fluorescence were generated by the protoplasts, the FDA stain and the buffer. These could be accounted for using a simple mathematical equation (as detailed in section 4.2.4) for example, using the data from Table 4.1:

Time	Relative Fluorescent Units (RFU)		
(min)	Protoplasts	FDA stain	Buffer
0	2.14	1.16	0.61
5	2.12	1.06	0.60
10	2.16	1.05	0.59
15	2.13	1.03	0.57

Table 4.1 Fluorescence (relative fluorescent units) detected in protoplasts, FDA stain and buffer over a 15min time-course. Results are from a single occasion, where the FDA stain = 10μM, and protoplasts = 5 x 10^5 ml^{-1}.

Figure 4.5 Variation of RFU with time, after addition of 10μM FDA to varying concentrations of *B. cinerea* protoplasts. (Results are mean values ± SE, n = 4)



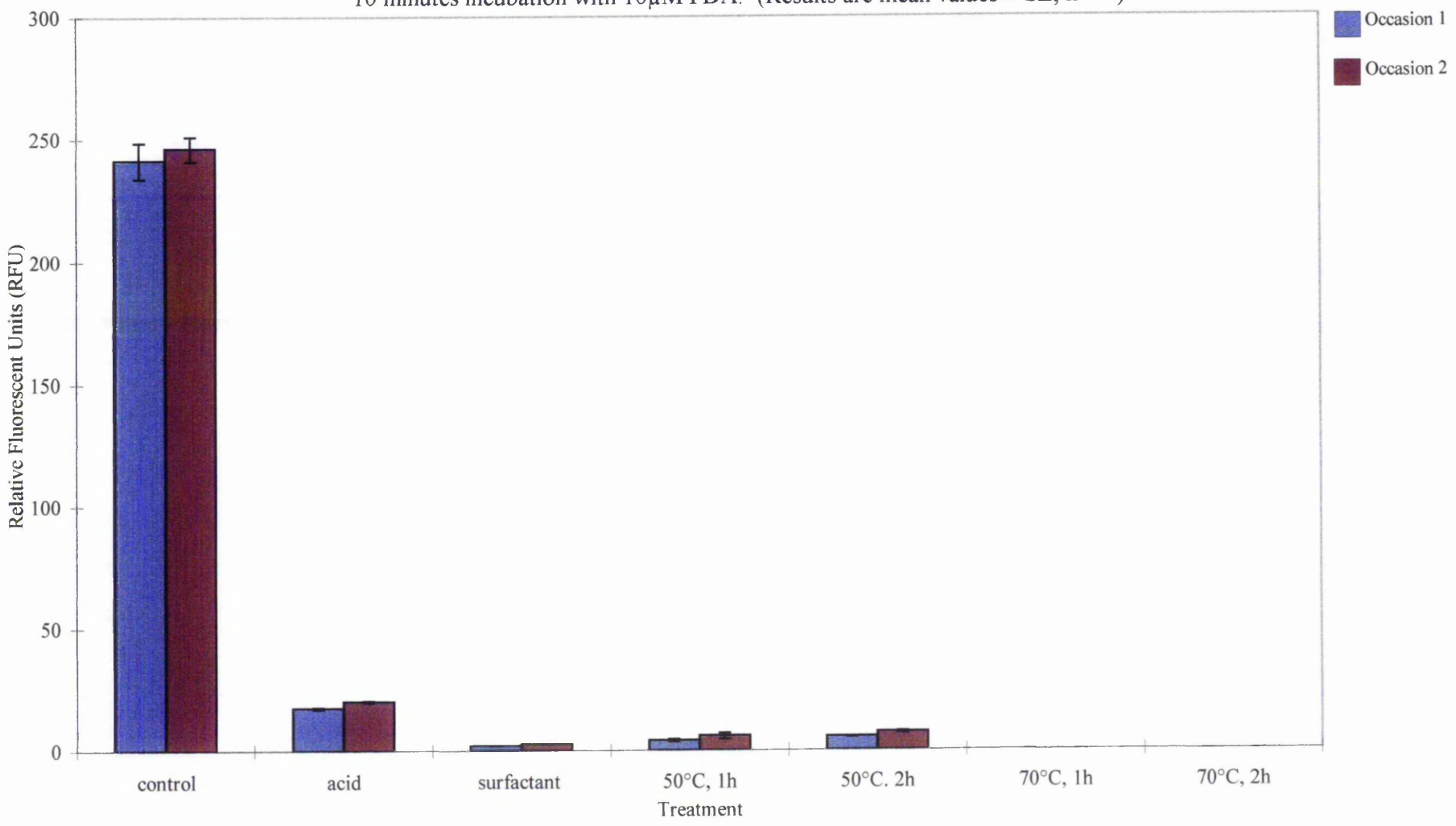
After 10min, the reading from the protoplasts incubated with 10μM FDA (A) was 314 RFU, from the FDA stain (B) was 1.05 RFU, from the protoplasts with no stain (C) was 2.16 RFU, and from the buffer only (D) was 0.59 RFU. Therefore, using the equation, A - B - (C +D) the RFU produced from the protoplast sample A, can be corrected for background to give a final reading of 311 RFU.

Neither the protoplasts, the FDA stain nor the buffer showed an increase in fluorescence over the time periods tested (Table 4.1), thus allowing such an increase to be attributed to esterase activity of the protoplasts cleaving the FDA to the fluorescent product. The investigation of assay parameters enabled optimal conditions to be determined. Subsequently, these optimal parameters were employed to investigate the effects of acid, surfactant and heat treatment upon the protoplasts and the assay.

4.3.5 Assay sensitivity

Protoplasts treated with either 0.1N HCl, 0.01M C8 surfactant or heated to 50°C for 1 and 2h, were only able to generate or retain a low amount of FDA-derived fluorescein compared to the control (untreated) protoplasts (Figure 4.6). Protoplasts heated to 70°C for both 1 and 2h were unable to generate or retain any FDA-derived fluorescein. It is likely that this can be attributed to both a lack of esterase activity and poor membrane integrity, as the treatments denatured cellular enzymes preventing FDA cleavage and disrupted membranes preventing retention of any fluorescent product. Microscopic examination confirmed that the protoplast membranes exhibited an abnormal morphology, appearing 'crinkled' rather than smooth, which is likely to be indicative of membrane damage. The addition of 0.01M C8 surfactant to the protoplasts caused them to rupture (as previously determined, see Chapter 3), resulting in the production of extremely low levels of fluorescence.

Figure 4.6 Effect of 0.1N HCl, 0.01M surfactant and heat treatment on protoplasts of *B. cinerea*. RFU measured after 10 minutes incubation with 10µM FDA. (Results are mean values ± SE, n = 4)



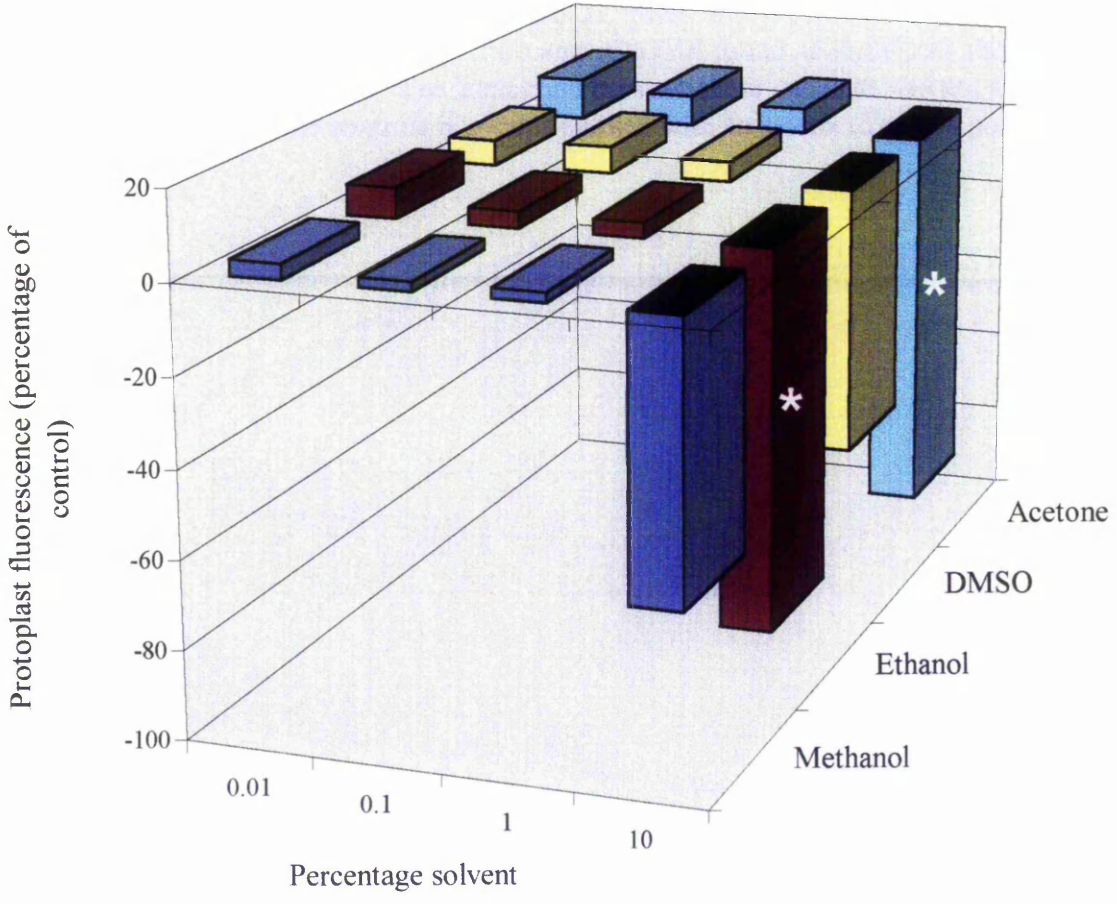
4.3.6 Tolerance to organic solvents

Following incubation with organic solvents up to and including 1% (v/v aq.), protoplasts continued to fluoresce at levels slightly above the control protoplasts (Figure 4.7). This increase in fluorescence may represent increased cell enzyme activity, suggesting that over the 3h time course the fungus was able to utilise the organic solvents as a carbon source and therefore exhibit increased enzyme activity compared to the control protoplasts. These data may also reflect changes in uptake or solubility of the FDA stain, which may be increased in the presence of organic solvents. The increase in fluorescence found following incubation with solvents up to and including 1% was not significantly different to the control protoplasts (determined by Tukey's post analysis multiple comparison test).

Protoplasts incubated in 10% solvent showed a large decrease in fluorescence compared to the controls. This was most marked following incubation in acetone, where protoplast fluorescence was reduced by 91%, followed by ethanol (87 %), methanol (62%) and DMSO (62%). Tukey's post analysis multiple comparison test found that at 10%, ethanol and acetone significantly reduced protoplast viability compared to the controls. The reduction in viability seen following incubation in 10% methanol or DMSO was not significantly different from the controls. It is probable that 10% solvent is sufficient to cause some solubilization of lipids from the protoplast cell membrane, resulting eventually in decreased integrity, cessation of metabolic activities and ultimately cell death. However it is unlikely that a solvent should ever be used at such high concentrations in a biological or biochemical assay.

It is interesting to note that DMSO was tolerated better than some of the other solvents. It has been suggested that DMSO interacts less with biological systems than other organic solvents (J. Pittis [AgrEvo] - pers. comm.) and it is for this reason that

Figure 4.7 Percentage increase or decrease in fluorescence of *B.cinerea* protoplasts incubated with 0.01, 0.1, 1 or 10% methanol, ethanol, DMSO or acetone for 3h, compared to control data (mean values, n = 6). Two way ANOVA found no significant solvent-type effect ($F = 1.49$, $p>0.05$), but a significant concentration effect ($F = 89.6$, $p<0.01$). The interaction between these variables was not significant ($F = 0.55$, $p>0.05$). Protoplast viability was significantly lower than the controls in groups marked with an asterix (Tukey's post analysis multiple comparison test).



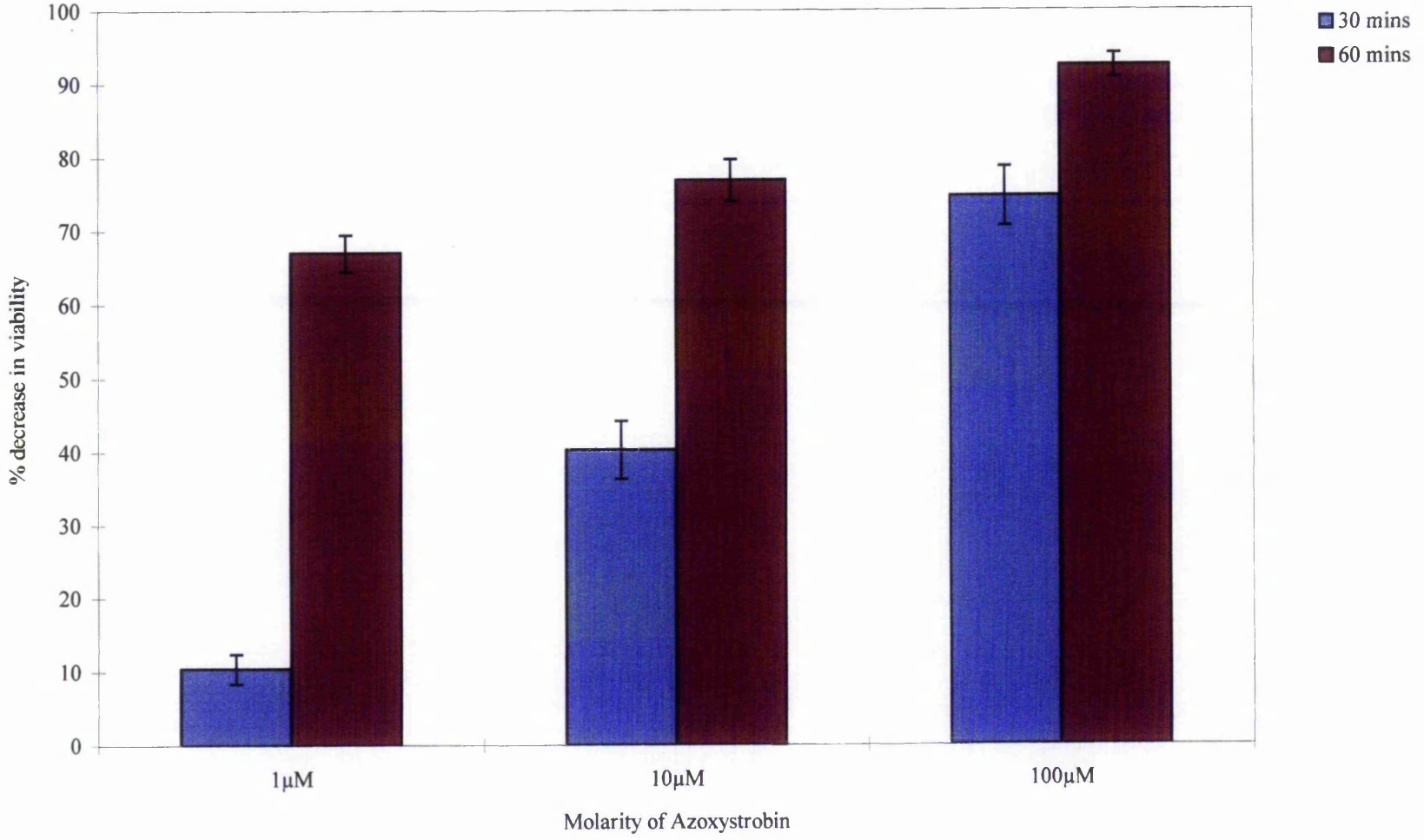
DMSO has become the solvent of choice for biochemical assays in the agrochemical industry. Conversely, DMSO is not used for assays *in planta*, where it can be quite damaging to plant tissues (J. Pillmoor [AgrEvo] - pers. comm.). Where an organic solvent is required in future protoplast work, DMSO will be used at concentrations not exceeding 1%. This assay has been used to determine protoplast tolerance to different organic solvents. Although data concerning the tolerance of intact fungi to such solvents was available (J. Pittis [AgrEvo] - pers.comm.), little data thus far existed with regard to protoplasts. It is a pre-requisite of using organic solvents in protoplast assays, that the effects of the solvents upon the protoplasts should be ascertained.

4.3.7 Tolerance to azoxystrobin and pyrimethanil fungicides

Azoxystrobin inhibits the mitochondrial electron transport chain (see Chapter 6 for details). The mode of action of the anilinopyrimidine fungicide pyrimethanil is less well defined. Explanations include inhibition of fungal methionine biosynthesis (Fritz, Lanen, Colas & Leroux, 1997) and inhibition of fungal enzyme secretion (Milling *et al.*, 1995). These fungicides were chosen to investigate the effectiveness of using the developed assay as a fungicide screen, as they exhibit very different modes of action. Following incubation with azoxystrobin, protoplasts showed a decrease in fluorescence compared to the controls (Figure 4.8). This ranged from 10% (1μM, 30 min) to 92% (100μM, 60 min). Two way ANOVA found significant differences between means based on time ($F = 23.7$, $p<0.01$) and concentration ($F = 36.3$, $p<0.01$). The interaction between these variables was also significant ($F = 4.22$, $p<0.01$).

Tukey's post analysis multiple comparison testing showed that the viability of protoplasts incubated with 1μM azoxystrobin for 30min, was not significantly different from the control. The viability of protoplasts incubated with 1μM compound for

Figure 4.8 Mean ±SE% decrease in viability of *B. cinerea* protoplasts incubated 1, 10 & 100μM azoxystrobin for 30 & 60 min, compared to control data (n=15). Two way ANOVA found significant ($p<0.01$) time ($F=23.7$) and concentration ($F=36.3$) effects.



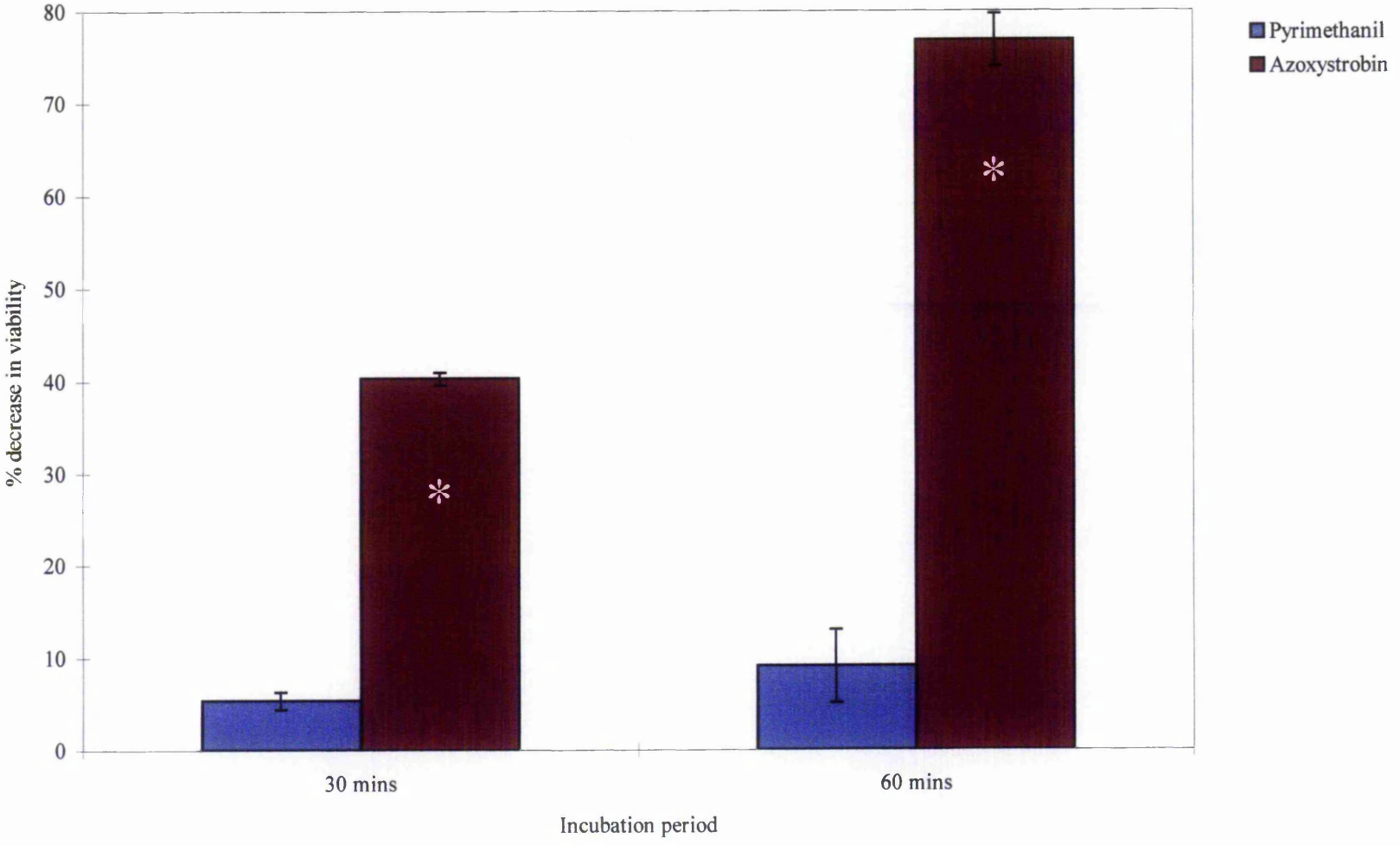
60min was significantly lower than the control protoplasts, highlighting the time/concentration effect. Where higher concentrations of compound were used (10μM and 100μM), differences between protoplasts incubated for 30 min and those incubated for 60 min were less pronounced than at 1μM, although at 10μM the difference between protoplast viability at 30min and 60min was significant. After 60min incubation with any of the fungicide concentrations tested, protoplast viability was significantly reduced.

This assay measured cell viability as indicated by the ability of non-specific cell esterases to cleave the FDA molecule. As azoxystrobin reaches the target site and inhibits respiration, the normal biochemical processes of the cell are prevented by the lack of ATP, ultimately causing cell death. Alterations in the cell caused by the interaction of azoxystrobin with the electron transport chain (for example, altered pH) prevent the esterase enzymes from cleaving the FDA molecule into the fluorescent product, and a corresponding drop in fluorescence is detected. The assay is therefore suitable for measuring direct reductions in viability of the cell.

One of the limitations of this assay is that it is unlikely to recognise the fungicidal properties of compounds that do not immediately affect the metabolism or viability of the fungal cell, *i.e.,* those which are not instantly lethal. Pyrimethanil was chosen as one such compound. Following incubation with pyrimethanil, protoplasts showed much smaller decreases in fluorescence, 5% after 30 min and just 9% after 60 min incubation (Figure 4.9). By contrast, after 60min incubation with 10μM azoxystrobin under the same conditions, a 77% decrease in fluorescence was observed. Tukey's post analysis of variance found the decreases in fluorescence associated with azoxystrobin incubation were significant, whilst the decrease in protoplast viability following incubation with pyrimethanil was not significant.

This assay does not measure interaction between the pathogen and the host, or growth of the pathogen. Therefore it cannot detect fungicidal activity like that

Figure 4.9 Mean ±SE % decrease in viability of *B. cinerea* protoplasts incubated with 10μM azoxystrobin or pyrimethanil for 30 & 60 min, compared to control data (n =15). Treatments with * significantly reduced protoplast viability (Tukey's HSD)



exhibited by pyrimethanil, where inhibition of the secretion of fungal enzymes relevant for pathogenicity and growth may prevent pathogen infection. In a screening situation therefore, this compound would not be recognised as having fungicidal properties - although it is a potent inhibitor in the field. However, this criticism can be applied to any other biochemical assays which screen for activity against specific targets (see Chapter 7).

4.4 Conclusions

- Fluorescein diacetate has been successfully used to determine the viability of *B. cinerea* protoplasts using a fluorescent spectrophotometer.

- FDA-derived fluorescence showed a positive correlation with protoplast concentration and increased in a linear fashion over the time period investigated.

- Optimal parameters were determined to be 5 x 10^5 ml^{-1} - 1 x 10^6 ml^{-1} protoplasts incubated with 10μM FDA for a 10min time period.

- Low levels of background fluorescence were detected from the protoplasts, buffer and FDA stain, which could be accounted for using simple mathematical formulae.

- Protoplasts subjected to treatments expected to significantly reduce their viability, showed a corresponding reduction in FDA-derived fluorescence.

- The optimised assay was used to determine protoplast sensitivity to various organic solvents. Protoplasts were found to tolerate acetone, ethanol, methanol and DMSO at concentrations up to and including 1% (v/v aq.).

- DMSO and methanol did not significantly reduce protoplast viability, even at 10%. DMSO was chosen for use in subsequent experiments.

- The effects of 2 commercially available fungicides on protoplast fluorescence were investigated. Incubation with azoxystrobin was found to significantly reduce FDA derived fluorescence at 10 and 100μM (at 30 and 60min) and at 1μM (60min only).

- Incubation with pyrimethanil did not cause a significant reduction in protoplast viability, which could be linked to the mode of action of this compound.

Chapter Five: The uptake and incorporation of radiolabelled compounds into *B. cinerea* protoplasts

5.1 Introduction

Fungal protoplasts have been used in conjunction with radioactive compounds to investigate cell wall regeneration and reversion (de Vries & Wessels, 1975), protein and nucleic acid synthesis (Isaac, 1978), protein secretion (Sorensen, Wallis & Peberdy, 1996) and heat shock response (Newbury & Peberdy, 1996). Macromolecule biosynthesis can be studied by measuring the incorporation of radiolabelled precursors into products. For example, de Vries and Wessels (1975) studied the incorporation of ^{14}C-glucose into 3 cell-wall components of *Schizophyllum commune*, α-1-3-glucan, β-1-3, β-1-6-glucan and chitin; and the incorporation of ^{14}C-leucine and adenine into protein and nucleic acid respectively. Isaac (1978) also studied the incorporation of ^{14}C-leucine and ^{14}C-adenine as measurements of protein and nucleic acid synthesis in *Aspergillus nidulans*. The method employed for these measurements was essentially as follows:

1. Protoplasts were incubated in stabilised regeneration medium, with ^{14}C-leucine at constant temperature with gentle shaking.
2. Protoplasts were pelleted by centrifugation and washed twice with cold regeneration medium. Supernatants were pooled and monitored for radioactivity.
3. Protoplast pellets were resuspended in ice-cold 5% (w/v) trichloroacetic acid (TCA) and stored at 0-4°C for 1h.
4. Samples were centrifuged, supernatants collected and monitored for radioactivity (cold TCA extract).

5. Precipitates were extracted with 5% TCA at 100°C for 15min and cooled in ice. Residues were collected on glass fibre discs.
6. Discs were washed with TCA, dried and monitored for radioactivity.

Isaac (1978) found that the hot TCA fraction (which represented incorporation of ^{14}C-adenine into nucleic acids) only accounted for 2-5% of the total incorporated radioactivity, and concluded that ^{14}C-leucine was preferentially incorporated into proteins. De Vries and Wessels (1975) investigated the effectiveness of cycloheximide to inhibit protein synthesis and found that a concentration as low as 1.8×10^{-7}M was sufficient to achieve almost complete inhibition of leucine incorporation.

These investigations aimed to establish reproducible methods for studying the uptake and incorporation of radiolabelled compounds into *B. cinerea* protoplasts, with reference to compound-based research within the agrochemical industry. Initially, the incorporation of amino acids into proteins is reported, followed by the uptake of an experimental fungicide (AG1). Subsequently the uptake of a range of compounds chosen with regards to their log P values was investigated.

5.2 Materials and methods

5.2.1 Radiolabelled amino acids

A protein hydrolysate mixture of 16 amino acids (Amersham, Little Chalfont, UK) with a high specific activity (53.2 mCi/milliatom carbon) was chosen to investigate protein synthesis. Typically glycine, alanine, valine, leucine, serine and glutamic acid occur in proteins at a little over 6%, whilst the remaining amino acids occur at below 6% (Voet & Voet, 1995). The use of a mixture of radiolabelled amino acids should therefore result in higher incorporation into protein and increased

detection, compared to the use of a single amino acid. A similar high specific activity mixture of amino acids was used by Newbury & Peberdy (1996) to investigate the heat shock response in *A. nidulans* protoplasts, at a final concentration of 0.18MBq ml^{-1}, whilst Isaac (1978) used ^{14}C-leucine at a concentration of 0.125μCi ml^{-1} (4.625KBq ml^{-1}) to investigate protein synthesis in *A. nidulans*. In addition Miura, Kamakura, Maeno, Nagata, Hayashi & Yamaguchi (1994) used radiolabelled precursors (including two amino acids) at a final concentration of 3.7KBq ml^{-1}, to investigate macromolecule biosynthesis in germlings of *B. cinerea*. Based on this information, the protein hydrolysate mixture was used at a concentration of 37KBq ml^{-1} for initial experiments.

5.2.2 Incubation apparatus

Experimental design was based on the methods of Isaac (1978). Four oxygen electrode chambers (Rank Brothers, Cambridge, UK) were connected in series to a circulating water bath to allow the experiments to be carried out at a controlled temperature (20°C). The electrode chambers were mounted on magnetic stirrers attached to a control base to allow stirring. The electrodes were not set up for oxygen monitoring, instead 4ml scintillation vial inserts (miniature polyethylene hang in vials, Canberra Packard Ltd., Pangbourne, UK) were placed into the electrode chambers to act as reaction vessels for the radioactive samples, (Figure 5.1).

5.2.3 Filter system

A Whatman VectaSpin Micro 0.2μM anopore filter (Whatman International Ltd, Maidstone, UK) was used to retain the synthesised proteins but not the unbound amino acids. These consist of a filter insert holding 400μl and an outer tube with a capacity of 1ml with the insert in. Filtration was centrifugally driven (Figure 5.2).

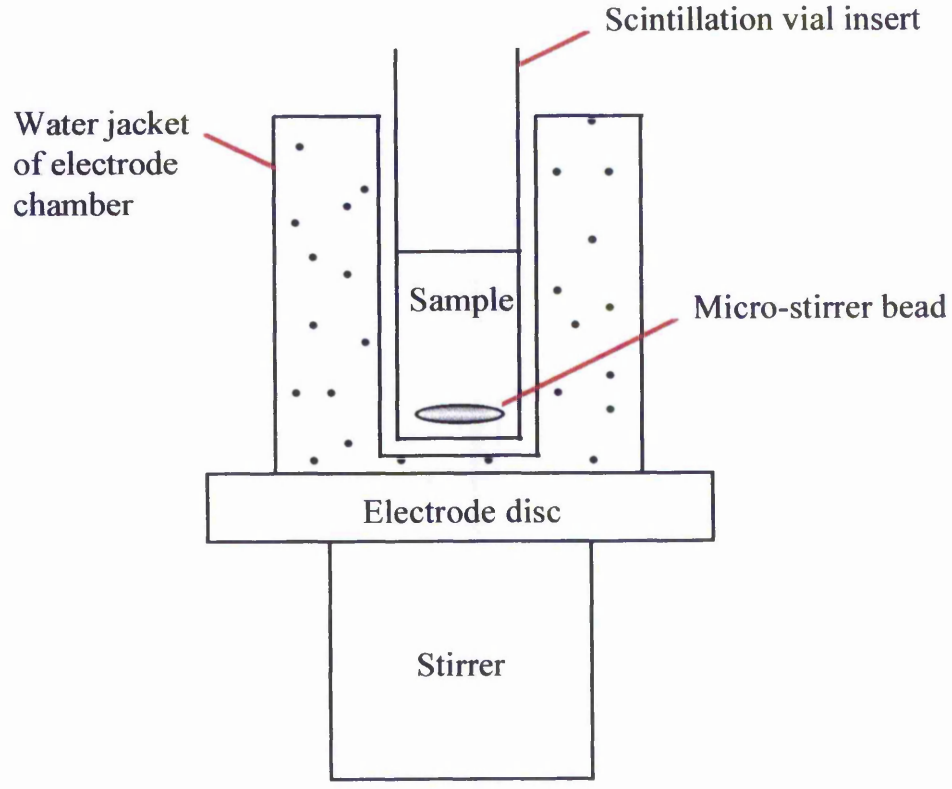


Figure 5.1 Apparatus for radiolabel studies

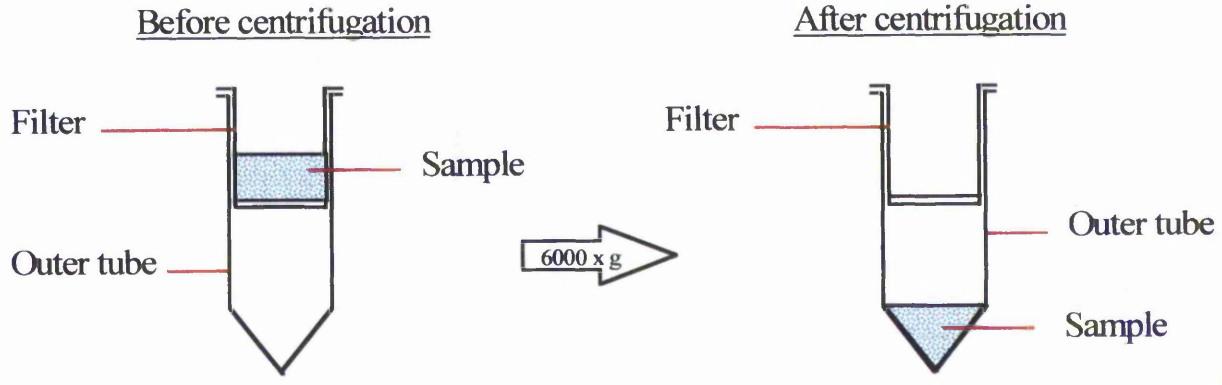


Figure 5.2 Filtration using Whatman VectaSpin Microfilters

An initial experiment was undertaken to ascertain what percentage of the ^{14}C-amino acids placed into the insert passed though the filter and to determine the level of washing required to aid this process.

Radiolabelled protein hydrolysate was diluted in Phos-KCl buffer to a concentration of 37KBq ml^{-1}. The filter insert was loaded with 200µl of the protein hydrolysate, capped and micro-centrifuged (IEC Micromax®) at 6000 x g for 1min, after which time the sample had passed though the filter into the outer tube. Four hundred microlitres of Phos-KCl buffer were loaded onto the filter and the tube was micro-centrifuged at 6000 x g for 1min. This washing step was repeated for a second time, after which the outer tube contained all 1ml of the filtrate. The filter insert was removed from the outer tube using forceps and placed into a scintillation vial (miniature polyethylene hang in vials, Canberra Packard Ltd., Pangbourne, UK), covered with 4ml scintillation fluid (Starscint LCS cocktail, Canberra Packard Ltd.) capped and mixed well. One hundred microlitres of the 1ml filtrate were transferred into a plastic scintillation vial (as before), covered with 4ml scintillant, capped and mixed well. The scintillation counter (Intertechnique, Lablogic, Sheffield, UK) was set to count each sample for 4min.

5.2.4 Incorporation of ^{14}C-amino acids into proteins by *B. cinerea* protoplasts

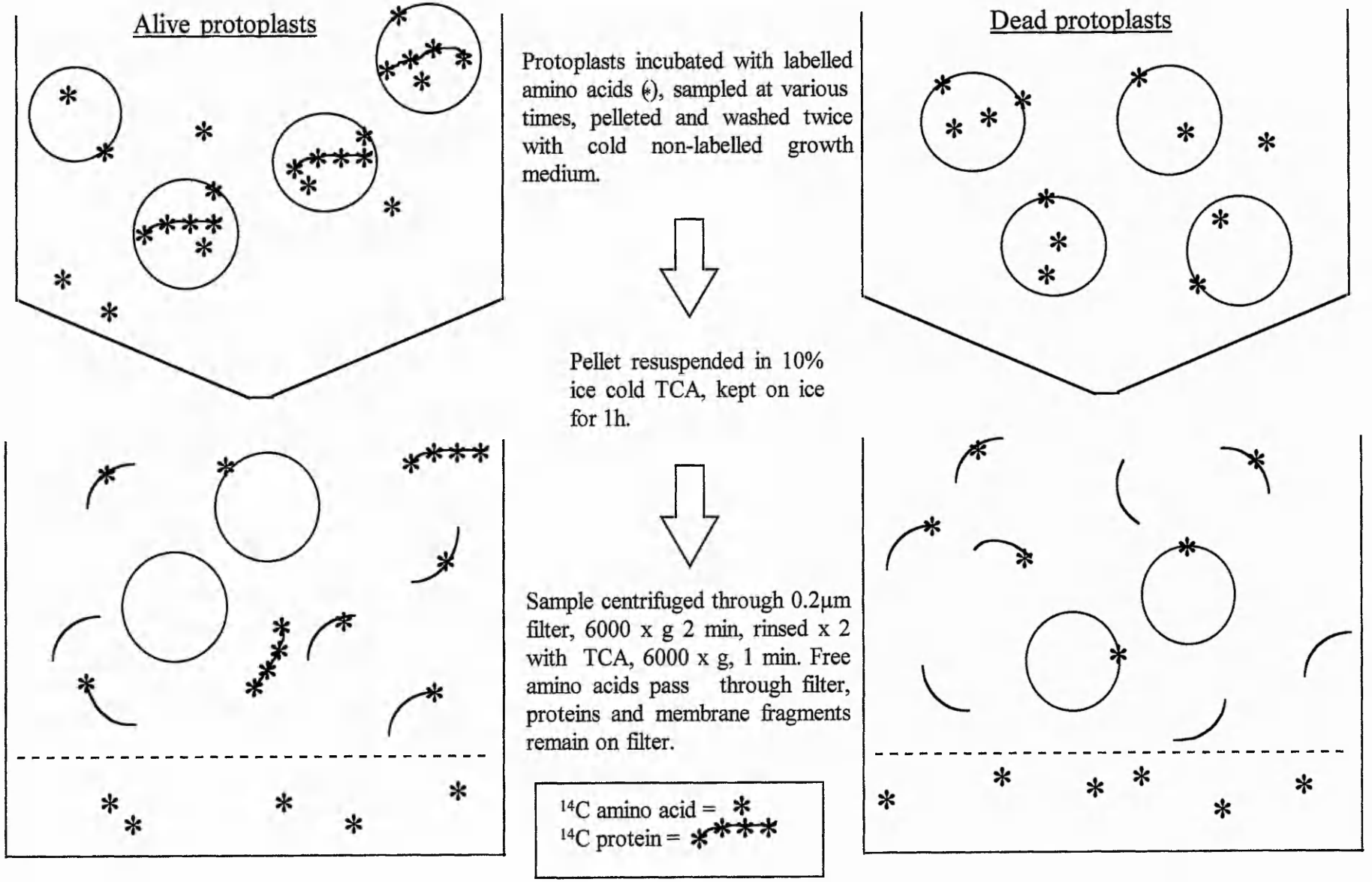
Protoplasts were isolated as previously described and resuspended either in glucose growth medium (GGM) or 2% (w/v) malt extract broth (MEB, Sigma) both stabilised with 0.8M Phos-KCl. Subsequently protoplasts were isolated from *B. cinerea* mycelia grown in 2% MEB, in the same way as mycelia grown in GGM. These protoplasts were resuspended in Phos-KCl stabilised MEB. Non-viable protoplasts were generated by heating samples to 70°C for 1h (Chapter 4). Protoplasts were examined microscopically using FDA and heat-treated protoplasts were found to be

non-viable compared to the untreated protoplasts, which exhibited a bright green fluorescence. The incubation of non-viable protoplasts with ^{14}C-amino acids was carried out in order to ascertain what proportion of radioactivity detected in the protoplast pellet could be attributed to non-specific adsorption to the protoplast membrane (Figure 5.3). Control chambers contained either growth medium and protoplasts with no ^{14}C-amino acids, or growth medium and ^{14}C-amino acids with no protoplasts.

One thousand eight hundred microlitres of the various protoplast samples were placed into the inserts of the oxygen electrodes, which were stirring at a constant rate. Two hundred microlitres of ^{14}C-amino acids (diluted in Phos-KCl to 370KBq ml^{-1}) were added to the electrode chamber to a final concentration of 37KBq ml^{-1} and the time noted. At hourly intervals (>4h), 200µl aliquots were removed from each sample in duplicate and placed into Eppendorf tubes. The samples were micro-centrifuged at 6000 x g for 2min to pellet the protoplasts. The supernatant (containing the unbound amino acids) was removed and retained. The pellet was resuspended by vortexing, (Maxi Mix II Thermolyne type 37600) in 900µl stabilised GGM or MBE and micro-centrifuged at 6000 x g for 2min. The supernatant was removed and added to the initial supernatant. This washing process was repeated a second time. The 2ml supernatant/washings were retained and a portion monitored for radioactivity. The pellet was resuspended in 400µl ice-cold 10% (w/v) TCA and kept on ice for 1h. After this time the TCA/protoplast mixture was loaded into a 0.2µm Whatman filter tube and micro-centrifuged at 6000 x g for 2 min. The filter was washed with 300µl ice-cold TCA and micro-centrifuged at 6000 x g for 1.25min. This washing process was repeated a second time, giving a total volume of 1ml filtrate which was retained.

The filter insert was removed from the outer tube using forceps and placed into a scintillation vial insert, covered with 4ml scintillation fluid, capped and mixed well. The 2ml supernatant and the 1ml filtrate containing any unbound amino acids were

Figure 5.3 Schematic representation of the use of non-viable protoplasts to determine ^{14}C amino acid adsorption to the membrane



thoroughly mixed and 100µl aliquots from each were placed into separate scintillation vial inserts, covered with 4ml scintillation fluid, capped and mixed well prior to scintillation counting (as before). Results are expressed as mean dpm per million protoplasts ± SE, where n = 4 from 2 separate occasions.

5.2.5 Association of the experimental fungicide AG1, with *B. cinerea* protoplasts

The uptake of a ^{14}C radiolabelled experimental fungicide AG1 which acts by inhibition of the fungal electron transport chain, was investigated in protoplasts of *B. cinerea*. Protoplasts were isolated as described previously and resuspended in GGM buffered with Phos-KCl to a final concentration of 3×10^7 ml^{-1}. Non-viable protoplasts were generated as before. AG1 was added to the protoplasts in the experimental chambers at final concentrations of 10µM or 1µM, specific activity 111KBq and 11.1KBq respectively.

At 1 and 2h after the addition of the fungicide, 1ml aliquots were removed from the samples (4ml total) in duplicate and placed in Eppendorf tubes. The samples were micro-centrifuged at 9000 x g for 3min and the pelleted protoplasts washed twice with 1ml buffered GGM. The supernatants were pooled and retained. The protoplast pellet was resuspended in 50µl GGM by vortexing. Fifty microlitres of C8 surfactant (see Chapter 3) were added to the second replicate of each sample, to a final concentration of 10mM. The protoplasts were transferred to scintillation insert vials, covered with 3ml scintillation fluid, capped and thoroughly mixed. One millilitre of the supernatant/washings was transferred to scintillation insert vials, covered with 3ml scintillation fluid, capped and thoroughly mixed. All samples were monitored for radioactivity by liquid scintillation spectrometry as before. Results are duplicates from a single experiment, expressed as mean dpm per protoplast pellet.

5.2.6 Association of ^{14}C radiolabelled methyl-glucose, propamocarb, pyrimethanil, ethofumesate, endosulfan and carbendazim with *B. cinerea* protoplasts

Protoplasts were isolated as previously described and resuspended in Phos-KCl to a final concentration of 5 x 10^7 ml^{-1}. Non-viable protoplasts were generated by heating the samples at 70°C for 1h. Incubation apparatus was set up as in section 5.2.2. Six ^{14}C radiolabelled compounds where chosen for study with reference to their physio-chemical properties. The compounds represented a range of log P values and were dissolved in DMSO, except where supplied in a different solvent (see Table 5.1). With the exception of methyl-glucose, all compounds were supplied by AgrEvo.

Subsequent to optimisation of the assay parameters with methyl-glucose (section 5.2.6.1), the following protocol was used. Compounds were added to the protoplasts in the chamber to a final concentration of 10µM, 0.1% (v/v aq.) solvent. Protoplasts were incubated at 20°C with stirring and 200µl samples removed after 30min, 60min and 120min. Protoplasts were pelleted by micro-centrifugation at low speed and washed twice with 200µl Phos-KCl. Supernatants were pooled and monitored for radioactivity. The protoplast pellet was resuspended in 200µl Phos-KCl by gentle vortexing and monitored for radioactivity. All samples were mixed well with 4ml Ultima Gold Scintillation fluid (Packard) prior to scintillation counting. Results are expressed as mean nmol compound associated with the protoplast pellet ± SE where n = 12 - 15 from 3 separate occasions.

5.2.6.1 Assay optimisation using methyl-glucose

Several assay parameters were optimised using ^{14}C methyl-glucose including; incubation period, the effect of adding surfactant to the samples prior to scintillation

counting, and the method of generating non-viable protoplasts. Initially 10μM methyl-glucose was added to viable and non-viable (heat generated) protoplasts and samples taken after 1, 2 & 3h to investigate a suitable incubation period for the assay. C8 Surfactant was added to samples at a final concentration of 10mM prior to scintillation counting, to ascertain its potential use in the increased detection of radioactivity. Results have been expressed as mean ^{14}C methyl-glucose per protoplast sample ± SE, n = 9 -10 from 2 separate occasions.

In a separate experiment, non-viable protoplasts were generated by heating (as before) and by the addition of 100μM sodium azide (Sigma) to the protoplast samples 45min before the start of the assay. Results are expressed as mean ^{14}C methyl-glucose per protoplast sample ± SE, n = 5 from a single occasion. Means have been compared by two way ANOVA and separated using Tukey's HSD criterion. Following these initial experiments, the optimised assay was repeated with all 6 compounds (5.2.6).

Compound	Log P Value	Type of Xenobiotic	Solvent
Propamocarb	-1	Fungicide	HCl/H_2O
Methyl-glucose	-1	Non-xenobiotic	Ethanol
Carbendazim	1	Fungicide	DMSO
Ethofumesate	2.7	Herbicide	DMSO
Pyrimethanil	2.8	Fungicide	DMSO
Endosulfan	4.7	Insecticide	DMSO

Table 5.1 Properties of the chosen compounds

5.3 Results and discussion

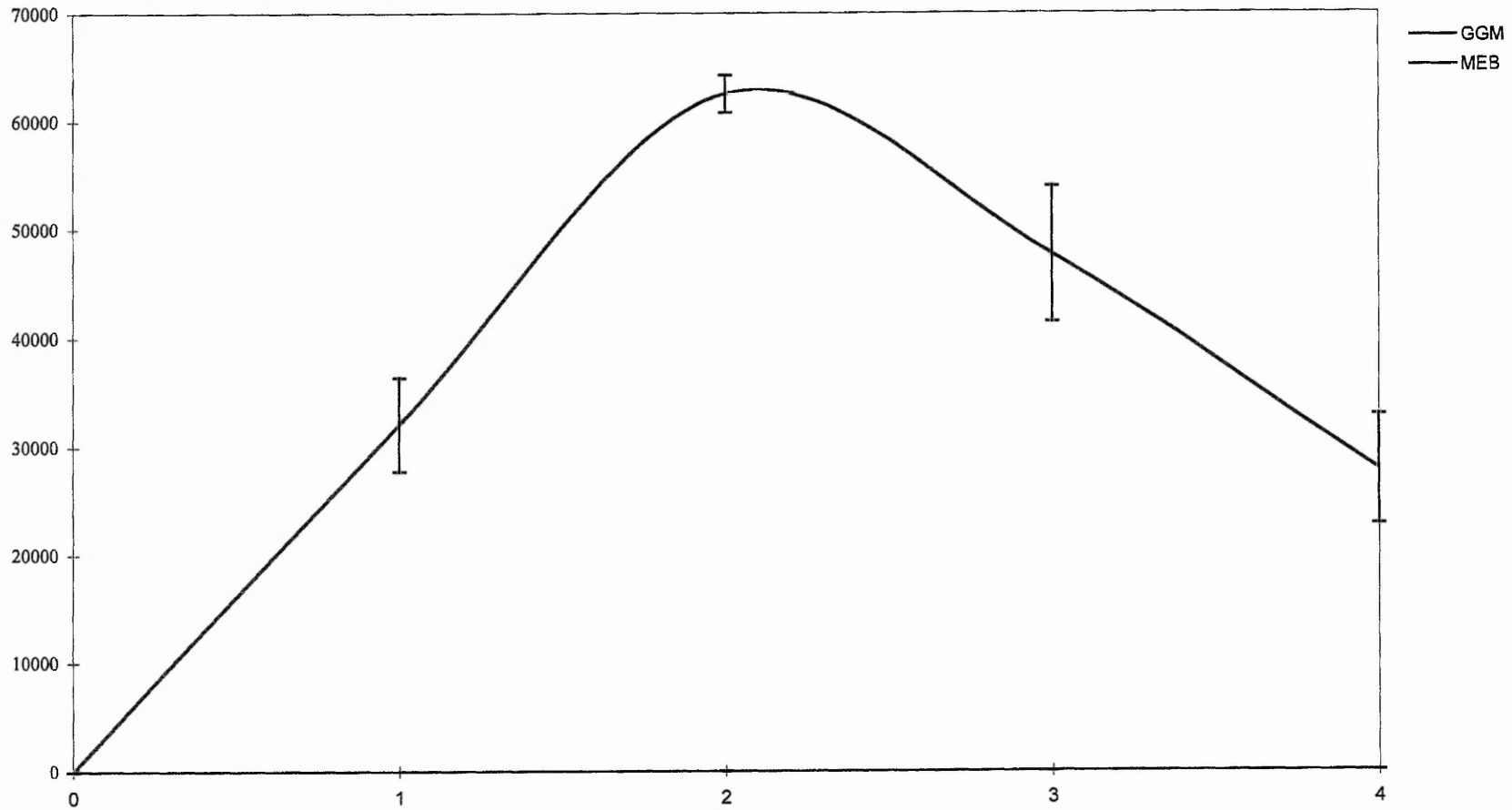
5.3.1 Filter system

When 7.4KBq ^{14}C-amino acids were filtered though the Whatman VectaSpin micro 0.2µm anopore filter and washed twice with 400µl buffered growth medium, an average of 2549 ± 173 dpm were detected on the filter (n = 6), equating to less than 1% of the total dpm used. Washing the filter with 800µl of buffer was therefore sufficient to remove the majority of unbound amino acids, those remaining may be accounted for by subtracting the dpm counted on the control (no protoplast) filter from the experimental data.

5.3.2 Incorporation of ^{14}C-amino acids into proteins by *B. cinerea* protoplasts

The incorporation of amino acids into proteins was measured over a 4h time-course. The data was corrected for any radioactivity generated by unbound amino acids adsorbed to the filter, and for background radioactivity from the protoplasts and growth medium, as determined from the control data. More amino acids were incorporated into protoplasts when glucose growth medium (GGM) was used rather than malt extract broth (MEB, Figure 5.4) This may reflect the fact that glucose is more readily utilised by the protoplasts than the more complex malt extract, which may start to show an increase in amino acid incorporation if sampled over a longer time period, reflecting a longer lag phase. However, the protoplasts used in this experiment were isolated from mycelia grown in GGM, which might explain why protoplasts resuspended in GGM were able to utilise the amino acids. An experiment was

Figure 5.4 Uptake of ^{14}C radiolabelled amino acids into *B. cinerea* protoplasts incubated in GGM or MEB. (Results are mean values ± SE, n = 4)

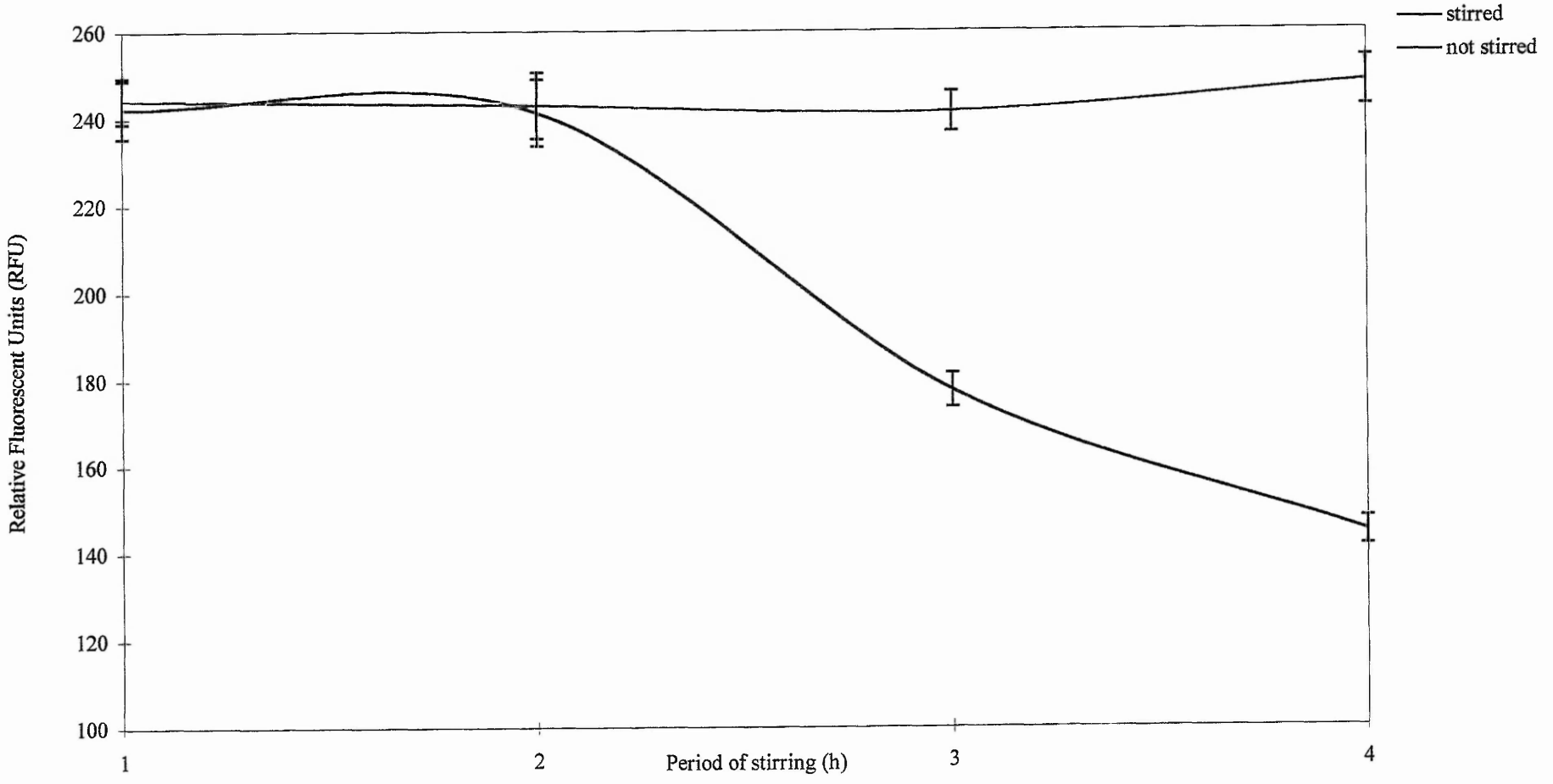


undertaken to investigate if mycelia grown in MEB produced protoplasts that were able to utilise the amino acids when resuspended in MEB. No increase in amino acid uptake was seen under these conditions (data not shown), suggesting that it is the composition of the regeneration media (i.e., glucose based or malt extract based) that influenced uptake/incorporation of amino acids into the protoplast, rather than any predisposed ability to utilise the substrates present during the production of the initial "parent" mycelia.

The protoplasts incubated in GGM showed an increase in amino acid incorporation for 2h after addition of the radiolabel, after which lower values were recorded (Figure 5.4). The extent of amino acid incorporation into protein may be expected to continue increasing (or at least remain constant) over this time period, as the protoplasts begin to resynthesise cell wall components and regenerate to hyphal form. A decrease of radiolabel within the protoplasts suggests that they are releasing amino acids into the surrounding medium and may not simply reflect less uptake, as those amino acids already incorporated into proteins would still be detected by scintillation counting, resulting in a constant detection of radioactivity rather than the decrease observed.

An explanation was that the continual stirring of the protoplasts over 4h caused some protoplasts to rupture, hence releasing the amino acids and proteins into the surrounding medium and resulting in detection of less radioactivity from fewer intact protoplasts. This hypothesis was tested using non-radioactive protoplast samples (set up as before), which were tested for viability every hour using the FDA assay (Chapter 4). After 3 & 4 hours of continual stirring, protoplast viability declined as determined by the assay (Figure 5.5) and microscopic evaluation of protoplast numbers confirmed that a reduction occurred over the time course investigated. This would also mean that fewer protoplasts were available for uptake of amino acids and protein synthesis after 2h of stirring. A gentler stirring system would be required for investigating

Figure 5. 5 Viability of *B. cinerea* protoplasts following stirring over a 4h period. (Results are mean values ± SE, n = 8)

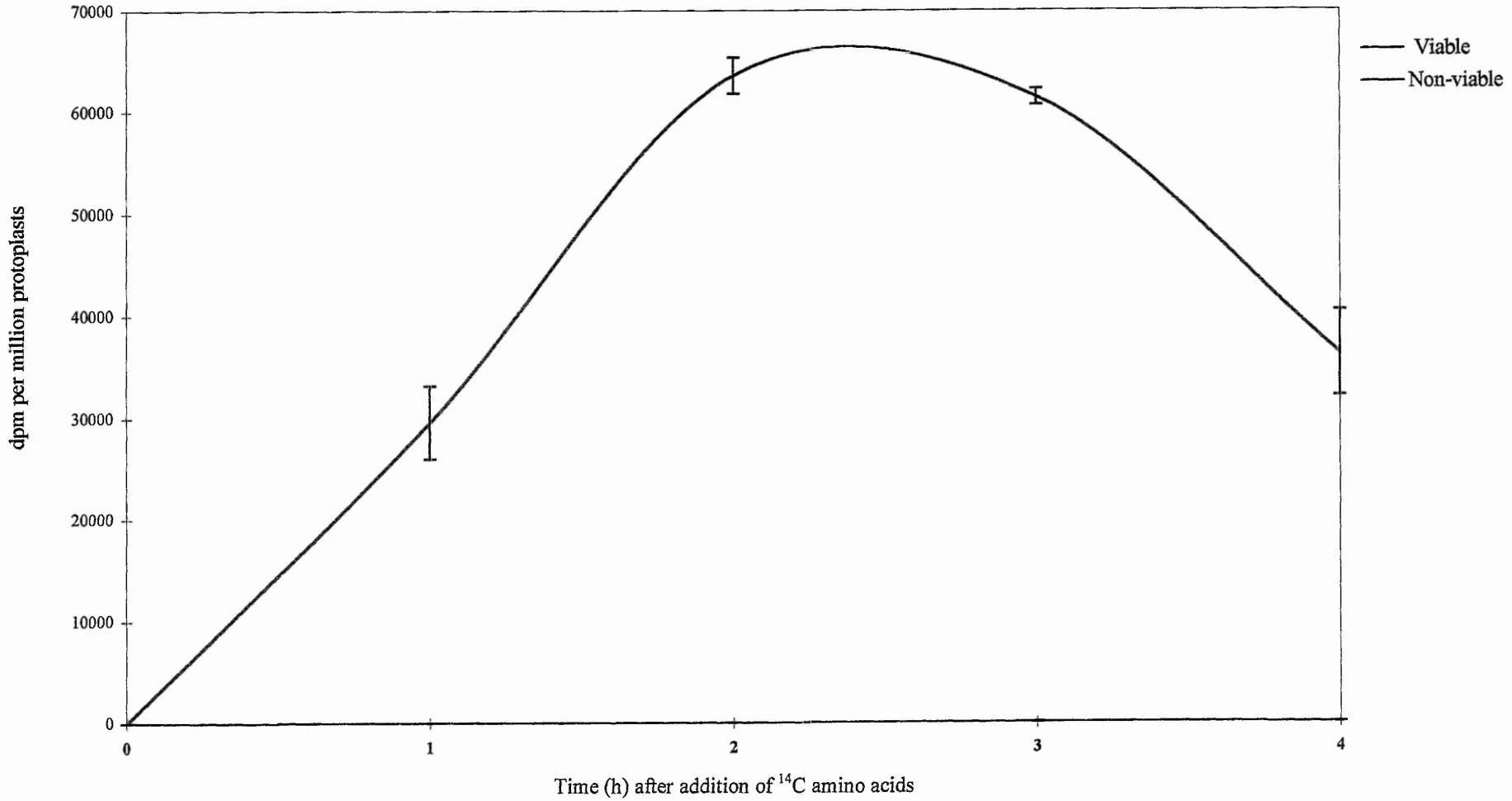


uptake over longer time periods. However, as this was not possible with the available equipment, subsequent experiments investigated uptake over a 2h time period only.

The incorporation of ^{14}C-amino acids into viable and non-viable protoplasts was investigated, with the aim of ascertaining what proportion of the radioactivity detected in the protoplast pellet could be attributed to non-specific adsorption of amino acids to the protoplast membrane. Non-viable protoplasts should not be capable of protein synthesis and so, following treatment with TCA and subsequent filtration, only amino acids bound to the membrane should remain on the filter (as predicted in Figure 5.3). Figure 5.6 shows the incorporation of ^{14}C-amino acids into viable and non-viable protoplasts incubated in GGM. The amount of radioactivity detected in the non-viable protoplasts was extremely low compared to the viable protoplasts. The amount of amino acids bound to protoplast membranes must therefore be negligible in this instance.

Investigating inhibition of protein synthesis in this assay, using fungicides and standard inhibitors which are known to work in this manner (for example cycloheximide), would provide further evidence to consolidate the data obtained. Establishing similar assays for other pathways of macromolecular biosyntheses, for example cell wall or lipid synthesis, would allow a range of such assays to be employed where the specific mode of action of a fungicide was unknown, in an attempt to identify it at the biochemical level. However, an extensive set of assays would need to be developed, which would not prove suitable for screening large numbers of compounds due to the high cost of both radioactive compounds and protoplasting enzymes. Techniques such as these may have a role in confirming rather than determining the mode of action of a compound, or in structure optimisation procedures for promising molecules.

Figure 5.6 Uptake of ^{14}C radiolabelled amino acids into viable and non-viable protoplasts of *B. cinerea* incubated in GGM. (Results are mean values ± SE, n = 2)



5.3.3 Association of the experimental fungicide AG1, with *B. cinerea* protoplasts

The uptake of AG1 into viable and non-viable protoplasts was investigated by the addition of this radiolabelled fungicide to the protoplasts at 10μM and 1μM (concentrations known to have an inhibitory effect, based on EC50 values of an electron transport inhibition assay using *B. cinerea* germlings - AgrEvo, unpublished data). Protoplasts were not subjected to a TCA protein precipitation and filtration step, as the experiment was not investigating protein synthesis, but attempting to ascertain the total amount of fungicide taken up into the protoplasts. Surfactant was added to one replicate of each sample to establish whether increased detection of radioactivity was apparent. Samples were taken after 1 and 2h incubation with the fungicide.

More fungicide was found to associate with the non-viable than the viable protoplasts in all cases (Figures 5.7 and 5.8). This was most marked after 2h incubation with 1μM compound, where 47% more compound was associated the non-viable protoplasts than the viable protoplasts. This may indicate that the fungicide is somehow excluded from the viable protoplasts, or may reflect increased permeability of the non-viable protoplasts to the compound (due to the heat treatment used to render the protoplasts non-viable). Clearly further work is required to understand these results.

Approximately 82% of the fungicide was detected in the protoplast pellet, whilst 18% remained in the supernatant. There are several alternative explanations for this observation. Firstly; that once inside the protoplast, the fungicide molecule was sequestered possibly by binding to the active site (or remains thereof) or by non-specific binding, thus reducing the pool of free fungicide molecules within the protoplast. This may account for the high percentage of fungicide within the protoplasts, with equilibrium existing between the pool of free fungicide and the

Figure 5.7 Uptake of a radiolabelled fungicide "AG1" (10μM) into viable and non-viable protoplasts of *B. cinerea*, with or without surfactant.

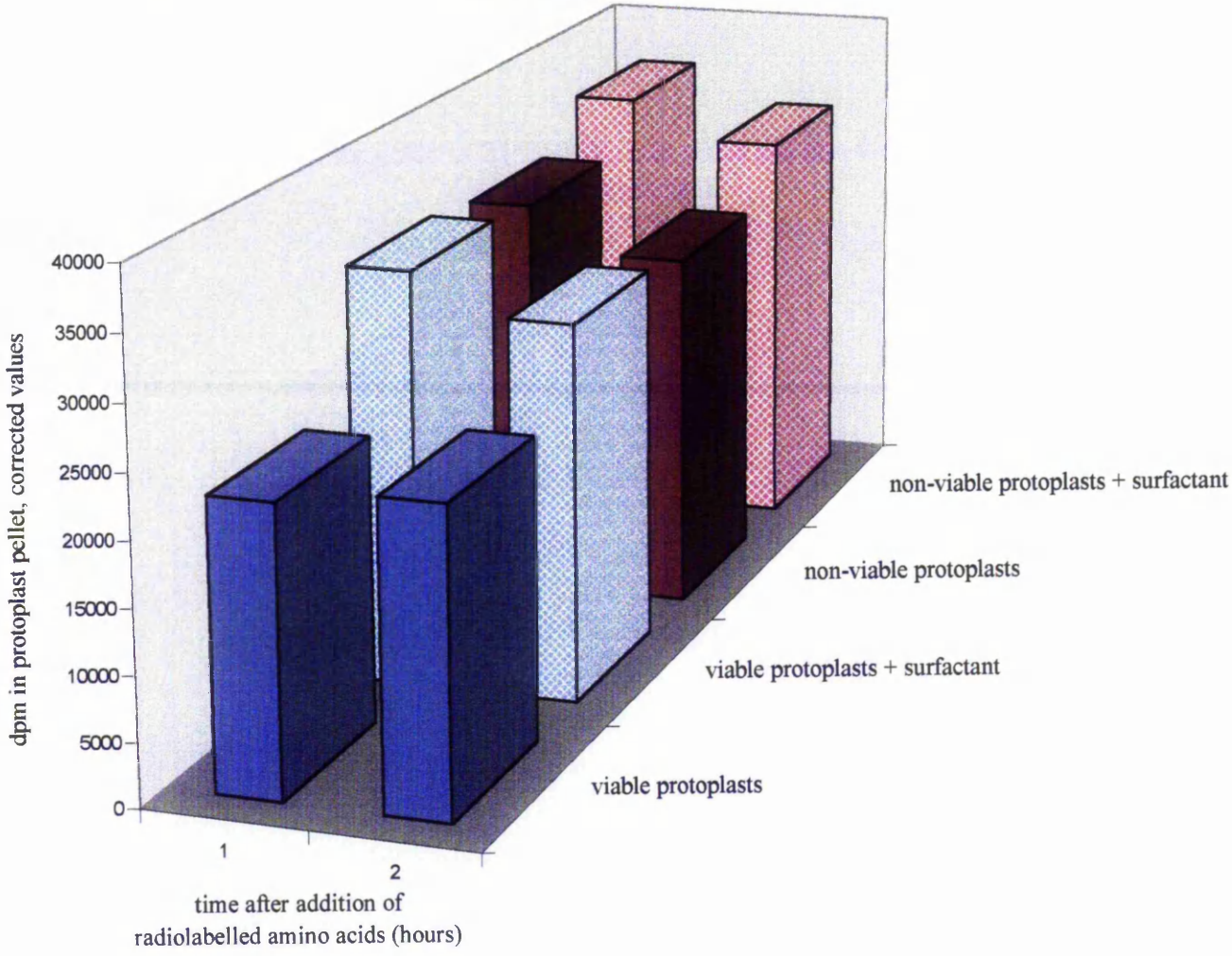
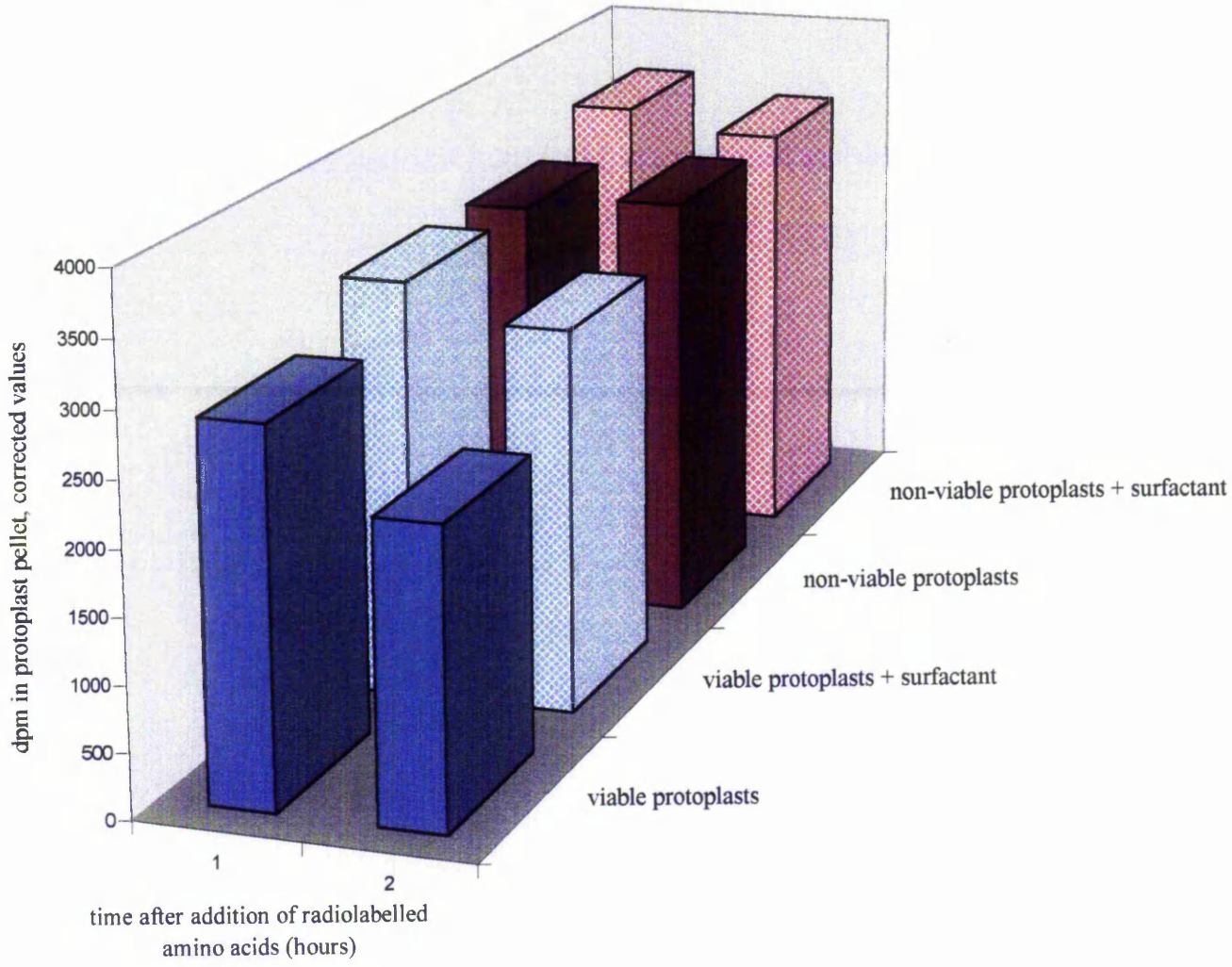


Figure 5.8 Uptake of a radiolabelled fungicide "AG1" (1μM) into viable and non-viable protoplasts of *B. cinerea,* with or without surfactant



surrounding medium. However, the amount of fungicide that could be expected to bind to and occupy all of the available active sites would be relatively small, resulting in a smaller deviation from equilibrium than that observed. Therefore a more likely theory involves high adsorption of the compound to the cell membrane. Knowledge of the physio-chemical properties of AG1 supports this hypothesis, as the compound is lipophilic (log P 4.2) and would therefore form strong associations with the lipids in the protoplast membrane. To examine this further, experiments using six radiolabelled compounds with different log P values, and hence differing states of lipophilicity/lipophobicity were undertaken, and are discussed in section 5.3.4.

The addition of surfactant to the protoplast pellet prior to the monitoring of radioactivity, was found to increase detection in all instances (Figures 5.7 and 5.8). Potentially the surfactant solubilised at least some of the protoplast membranes, allowing detection of more of the radioactive molecules within the protoplasts, or alternatively helped the compound mix more effectively with the scintillant. Unlike the protein synthesis assay, the design of this experiment does not allow the location of the radiolabelled compound to be determined. It is therefore more accurate to refer to the "uptake" of the compound as an "association" with the protoplast, as uptake implies that the compound has been taken fully into the cell, where association allows for the possibility of membrane adsorption. This is discussed further in section 5.3.4.

5.3.4 Association of ^{14}C radiolabelled methyl-glucose, propamocarb, pyrimethanil, ethofumesate, endosulfan and carbendazim with *B. cinerea* protoplasts

5.3.4.1 Assay optimisation using methyl-glucose

Protoplasts were sampled after 1, 2 & 3h to investigate a suitable incubation period for the assay. The amount of radioactivity associated with the viable protoplast samples increased until 2h and showed a decrease after 3h incubation (Figure 5.9). This consolidates the findings of the ^{14}C amino acid uptake assay, that after 3h constant stirring protoplasts are presumed to be rupturing. Samples were therefore taken after 30, 60 and 120min in the optimised assay to avoid this problem.

C8 Surfactant was added to samples at a final concentration of 10mM prior to scintillation counting, to ascertain its use in the detection of radioactivity. Unlike in the previous experiment with the AG1 fungicide, there was no increase in the detection of radioactivity (Figure 5.9), inferring that the methods employed adequately detected the radioactivity in the protoplast samples. This was confirmed by calculating the total radioactivity detected in the protoplast samples and supernatant and comparing this to the total radioactivity introduced into the assay, which were found to be equal. This is probably reflecting both the relatively aqueous nature of the protoplast samples, which are not clumping together in the scintillation fluid to create quenching effects, and the hydrophilicity of methylglucose allowing it to get freely into the scintillant. Surfactants were not therefore used in the final assay.

Non-viable protoplasts were generated by heating (as before) and by the addition of sodium azide to the protoplast samples. Both sets of protoplasts exhibited similar low levels of association with the ^{14}C methyl-glucose compared to the viable protoplasts (Figure 5.10). Two way analysis of variance and subsequent Tukey's multiple comparison analysis found no significant difference in compound association with protoplasts treated with azide and those rendered non-viable by heat treatment. Generating non-viable protoplasts by heat-treatment rather than by sodium azide

Figure 5.9 Uptake of ^{14}C methylglucose into viable and non-viable (heat treated) *B. cinerea* protoplasts, radioactivity detected in the presence and absence of surfactant. (Results are mean values ± SE, n = 9 -10)

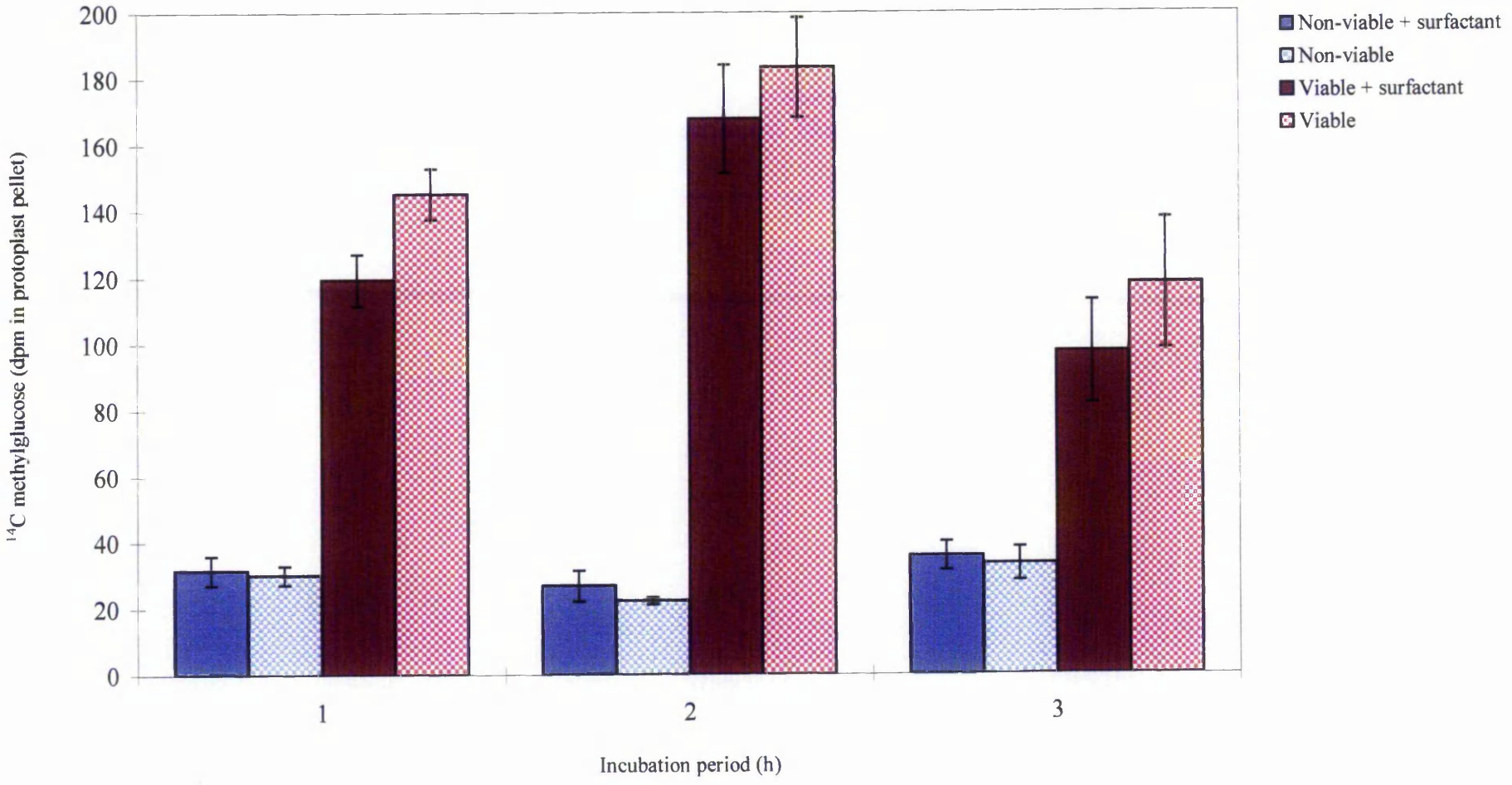
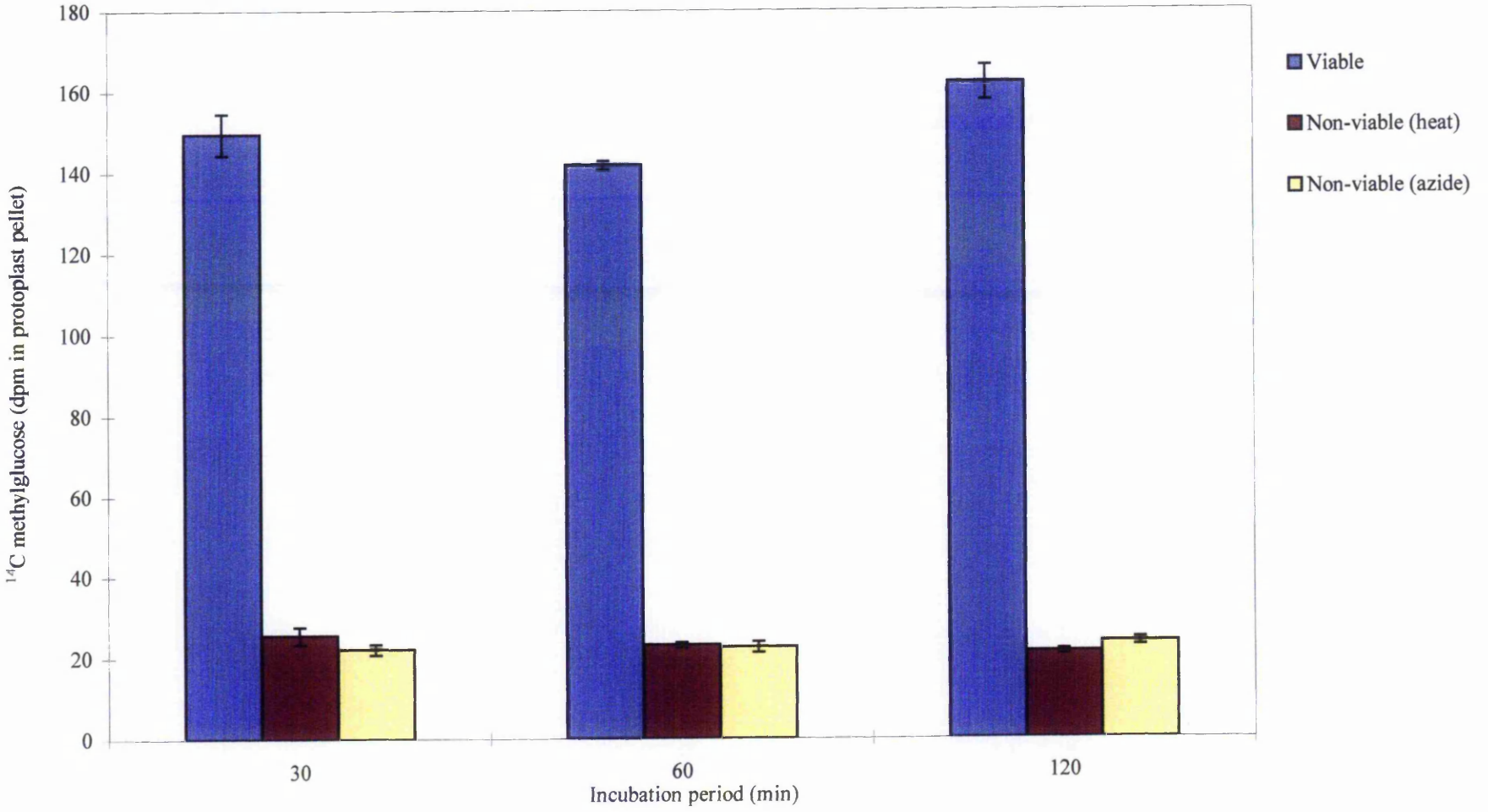


Figure 5.10 Association of ^{14}C methylglucose with viable and non-viable *B. cinerea* protoplasts. (means ± SE, n =5)
Two way ANOVA/Tukey's HSD found no significant difference between heat and azide treated protoplasts.



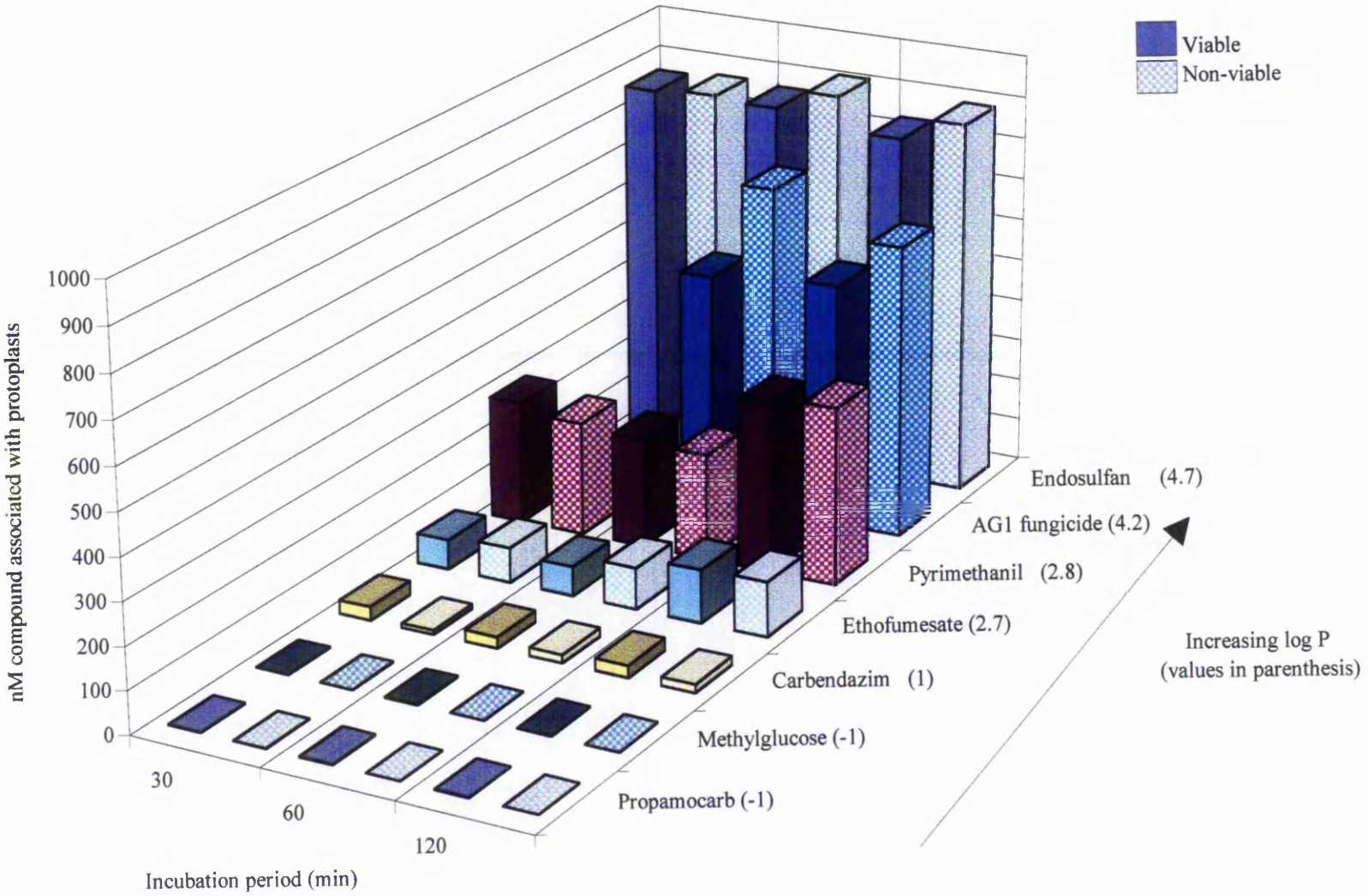
treatment was chosen for the assay, so as to avoid the introduction of additional compounds into the experimental system.

Significantly more methyl-glucose was associated with the viable protoplasts than the non-viable samples ($F = 2672.6$, $p<0.01$, for viable/non-viable effect). This may indicate an active transport mechanism is involved in the uptake of methyl-glucose into the protoplast, or may reflect a lack of retention of the molecule by the non-viable cells because of membrane damage. Protoplasts rendered non-viable by heat treatment exhibited a lack of membrane integrity, as demonstrated by the FDA assay (Chapter 4), however protoplasts metabolically poisoned with sodium azide should retain their structural integrity. As no significant difference was found in the quantities of methyl-glucose associated with both non-viable protoplast populations, the uptake of this compound must require at least some cellular biochemical function. This may be linked to recognition of the glucose-part of the molecule, although further work would be required to confirm this.

5.3.4.2 Association of ^{14}C radiolabelled methyl-glucose, propamocarb, pyrimethanil, ethofumesate, endosulfan and carbendazim with *B. cinerea* protoplasts

This experiment was designed to test the hypothesis that; the higher the log P value of a compound, and therefore the more lipophilic in nature, the greater the association with a population of protoplasts. The hypothesis was confirmed (Figure 5.11), establishing that the amount of compound associated with the protoplast samples increased with log P value. The amount of compound associated with the protoplasts reflects the lipophilic or lipophobic nature of the molecule, inferring that high quantities of lipophilic compound are bound to the protoplast membrane, or taken up and subsequently trapped.

Figure 5.11 Association of ^{14}C radiolabelled compounds with viable and non-viable *B. cinerea* protoplasts. (Results are mean values, n = 12 - 15, SE ranged from 1.6 % to 29 %)



As previously mentioned, the design of this experiment does not allow the exact location of the compound to be determined. To find out what proportion of the compound was membrane bound, and how much had crossed the membrane into the cytoplasm; it would be necessary to employ cell fractionation techniques or EM autoradiography, allowing the precise cellular location of the compound to be confirmed. Tracing the destination of fungicides may be useful for structure optimisation processes and mode-of-action determination, however care would need to be taken with data interpretation as the compound may dissociate rapidly from the target site during the cell fractionation process.

It is interesting to note that the standard error of these experiments was less than 10% for propamocarb, methyl-glucose, ethofumesate and endosulfan; but more than 10% (on average 15%, increasing to 29% in one instance) for carbendazim and pyrimethanil. These two compounds are fungicides recommended for the control of *B. cinerea*, whereas the others (with the exception of propamocarb) are not. Resistance to carbendazim is common in *B. cinerea*, however the isolate used in these experiments was found to be carbendazim sensitive (Chapter 2, Figure 2.4). This increase in variability may reflect changes in the metabolic and/or physiological competence of the protoplasts induced by the fungicides, similar variation might be expected if the assay were used to look at other fungicides effective against the pathogen. Indeed, high variability in an otherwise reliable system might be useful as an indicator of fungicidal activity in a screening situation, although clearly more evidence would be required to justify such an inference.

The data presented here can be regarded as a "standard curve" for non-specific association between the compound and the protoplast; that is to say association which occurs passively. Differences between the viable and non-viable protoplast populations allow this conclusion to be drawn, as, with the exception of the methyl-glucose data (potentially different due to the glucose part of the molecule), there were

few differences between the viable and non-viable data. If an active transport process were involved in the uptake or exclusion of these molecules, differences between the viable and non-viable samples would have been observed, as occurred with AG1. This "standard curve" could potentially be used as a reference system to look for active transport of radiolabelled molecules - either if large discrepancies were found between viable and non-viable protoplasts, or if association were not synonymous with log P values.

This work has shown that it is possible to study radiolabelled compounds in a protoplast system, although the assay would need to be extended to determine the precise cellular location of test compounds to be valuable in mode-of-action investigations. These experiments offer a promising alternative to using mycelia for radiolabel work, in which the study of compound uptake is further complicated by the presence of the cell wall and associated mucilage. It also provides a more aqueous system for detecting radioactivity, in which there are few problems associated with quenching, a serious consideration for all radioisotope experimental work.

5.4 Conclusions

- Radiolabelled amino acids were incorporated into proteins within viable protoplasts, but not within non-viable protoplasts.

- A suitable assay for the detection of amino acid uptake was developed and optimised. A preferential growth medium which stimulated protoplast regeneration and hence protein synthesis was determined.

- Protoplast association with the radiolabelled experimental fungicide "AG1" was found to be higher in non-viable protoplasts. A high percentage of the compound

was found to be associated with the protoplast samples, probably linked to the lipophilicity of the compound.

- An assay was developed and optimised to investigate the association of a range of compounds with *B. cinerea* protoplasts. Compounds were chosen with reference to log p values.

- A relationship between compound log P value and association with a protoplast population was discovered; association of the compound with the protoplasts increased with increasing log P values.

- A "standard curve" was generated by the assay, which could potentially provide a useful marker to study structure optimisation and delivery of experimental compounds.

Chapter Six: The value of protoplast systems in fungicide screening

6.1 Introduction

Screening test compounds against a representative set of economically important and taxonomically diverse plant pathogens *in planta*, has been the primary means of assessing potential fungicides for many years in the agrochemical industry. Such empirical screening procedures are based on protecting (or occasionally curing) plants from subsequent fungal infection and offer some distinct advantages to *in vitro* testing. By inference, compounds that show good activity against pathogens on plants are likely to be bio-stable, photo-stable and good inhibitors of the target site (Knight, Anthony, Brady, Greenland, Heany, Murray, Powell, Schulz, Spinks, Worthington & Youle, 1997). Compounds that work by interfering with plant-pathogen interactions are more likely to be identified as potential fungicides using *in planta* rather than *in vitro* screening. Additionally, compounds may interact with the plant to produce a more active molecule, or may activate plant defence systems (Lucas, 1998). It is also possible to screen compounds for activity against obligate biotroph fungi on plants, which are often difficult or impossible to culture for use in *in vitro* assays.

There are however, several disadvantages to using *in planta* primary screens to detect compound activity. Potential fungicides may be overlooked if they do not show activity. Frequently experimental compounds exhibit a discrepancy between *in vitro* and *in planta* activity. Compounds which are potent inhibitors of the molecular target may offer little or no protection to the plant when applied as a fungicide and challenged with a pathogen. The reasons for this are not always clear, but may include problems with compound biostability, uptake, transport or metabolism (Dancer, Schulz, Peine & Wright, 1998). Such compounds should not be disregarded as a

starting point for the synthesis of more stable analogues, or structure-optimisation processes.

I*n planta* screens are time and space consuming when compared to *in vitro* methods, which can generate data in a matter of hours rather than weeks (Dancer *et al.*, 1998) This is becoming increasingly relevant with the recent advances in compound synthesis. Over the past five years, developments in combinatorial chemistry and automation systems have resulted in a rapid increase in the number of new experimental compounds produced in the agrochemical industry (Corran, Renwick & Dunbar, 1998) with the potential to produce millions, rather than thousands of compounds. This, coupled with our increasing knowledge of genomic information, has been the driving force behind the development of *in vitro* biochemical screening from a structure optimisation process to a lead finding tool (Dancer *et al.*, 1998). High throughput biochemical screening (HTBS) as this process has been termed, is able to accommodate large numbers of test compounds through the use of microtitre plate test assays (Dancer *et al.*, 1998).

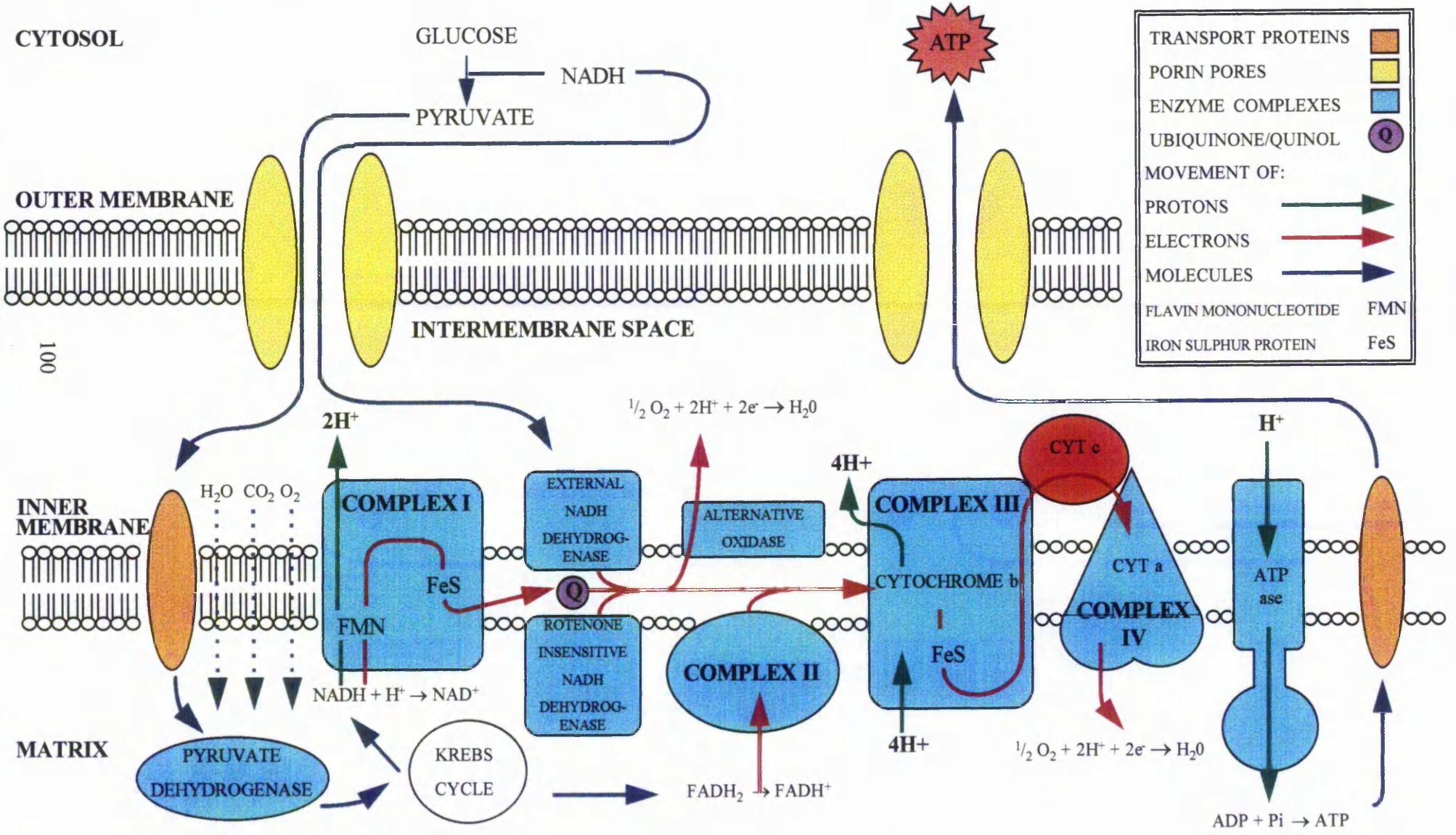
The development of biochemical screening assays begins with defining and validating inhibitor targets, before progressing to developing screening systems suitable for identifying specific inhibitors of the target (Koeller, 1998). Recent advances in molecular biology have created a move away from using more conventional targets identified by discovering the mode of action of a biologically active compound, towards exploiting biochemical processes which represent novel sites of inhibition (Dancer *et. al.*, 1998). Suitable targets include processes essential to fungal pathogenicity, growth or development (Lucas, 1998), that are ideally fungus-specific and critical to pathogens across different taxonomic groups (Corran *et al.*, 1998). One obvious benefit of this approach is the synthesis of compounds with a novel mode of action, which would be less likely to show cross-resistance with commercially available fungicides (Corran *et al.*, 1998).

In vitro HTBS therefore relies on predetermining the basic mode of action of a potential fungicide. Screens must be developed to accommodate many different targets in order that enough potential fungicides can be identified. An obvious disadvantage of this technique is that a fungicidal compound will not be detected if it targets an area not represented in the screening process. So just as compounds with potentially good fungicidal activity may be missed through biological screening, they will certainly be overlooked by biochemical screening. Therefore, just as the best strategy for disease control is achieved by implementing an integrated pest management system, so the best strategy for fungicide development involves an integrated multi-disciplinary approach involving chemistry, formulation, biochemistry, biology and molecular biology.

Work presented here demonstrates some of the techniques used across disciplines to screen experimental compounds for activity, and examines niches that may exist for protoplast research. Thirteen AgrEvo-synthesised experimental compounds belonging to chemical classes known to act by the inhibition of the mitochondrial electron transport chain were chosen for use in this study. Azoxystrobin (Zeneca) and kresoxim-methyl (BASF) were also included as commercially available fungicides with the same mode of action. Figure 6.1 represents events occurring in the electron transport chain of fungal mitochondria.

The processes of electron transport and oxidative phosphorylation are coupled, such that as electrons from NADH or $FADH_2$ (generated by the Krebs cycle in the mitochondrial matrix) are passed along a chain of protein complexes to molecular oxygen and the formation of H_2O, a proton gradient is generated across the inner membrane which drives the rephosphorylation of ADP to ATP, as protons flow back across the membrane through the ATPase protein complex. The structure of the mitochondrion is specialised to allow these processes to occur. The outer membrane contains porin, a protein which creates pores to allow the diffusion of molecules

Figure 6.1 Schematic representation of electron transport and oxidative phosphorylation within a mitochondrion



between the cytosol and the intermembrane space. The inner mitochondrial membrane contains a high percentage of protein and is only permeable to H_2O, CO_2 and O_2, other molecules and ions can only traverse the membrane through specific transporter proteins. This allows the H^+ electrochemical gradient generated by the oxidoreductase complexes across the inner membrane to be maintained.

Inhibition of electron transfer or oxidative phosphorylation will result in the cessation of energy production within the cell and ultimately cause cell death. As these processes are well characterised at the molecular and biochemical level, they are good targets for inhibition. The highly conserved nature of respiratory electron transport genes across human, plant and fungal systems makes it desirable to discover areas novel to fungi to act as targets for inhibitors, and to comprehensively assess potential effects in non-target organisms. Often however, compounds which have the potential to harm non-target organisms do not do so in practice - for example kresoxim-methyl, which acts by inhibiting cytochrome c reductase (complex III) is quickly metabolised in rats and some plant species (The Pesticide Manual, 1996).

Compounds that inhibit mitochondrial electron transport and oxidative phosphorylation have been known for many years (Hollingworth & Ahammadsahib, 1995). Interest in their potential use as pesticides has been renewed following the recent introduction of β-methoxyacrylate fungicides onto the market. The overlay of Figure 6.1 outlines the target sites of a range of standard inhibitors and fungicides. Most respiratory inhibitors work by preventing electron transfer within or between the oxidoreductase complexes, for example cyanide reacts with the ferric form of the cytochrome oxidase complex to prevent electron flow. Some compounds are able to carry protons across the inner mitochondrial membrane, dissipating the proton gradient and preventing the proton-pumping ATPase (complex V) from phosphorylating ADP to produce ATP. Electron transport proceeds normally, but is uncoupled from

oxidative phosphorylation; consequently these compounds (for example dinitrophenol) are referred to as respiratory uncouplers.

Electron transport and oxidative phosphorylation are well characterised both biochemically and at the molecular level, with known inhibitors for each step in the process. Theoretically this allows new compounds to be designed to "fit" the target site, although as such a rationally designed fungicide has yet to enjoy commercial success.

6.2 Materials and methods

6.2.1 Biological screening of test fungicides against *B. cinerea*

For 8 of the 15 experimental compounds (1, 2, 3, 4, 7, 8, 14, 15) data regarding their effectiveness as protectant fungicides when applied to tomato plants and challenged with *B. cinerea* was available (AgrEvo - unpublished data). For those compounds where no such data were available (5, 6, 9, 10, 11, 12, 13), the following experiment was undertaken.

6.2.1.1 Fungicide application

First in the Field cultivar tomato seeds were sown in John Innes #1 compost and grown under glasshouse conditions (20°C, 14/10h light/dark) for 3-4 weeks. Seedlings were sprayed to run-off with the test compounds dissolved in 60% distilled H_2O, 40% acetone and 0.25% (v/v) Tween 20 to final concentrations of 100, 50, 10 and 1 ppm using a hand-held sprayer (Binks Bullows model 2900, UK). Iprodione was used as a standard fungicide, effective against *B. cinerea* at 200, 100, 50 and 5 ppm. Control plants were sprayed with the H_2O/acetone/Tween 20 mixture only.

Untreated control plants were also included. For non-volatile compounds, 1 replicate of each treatment was randomly placed onto large metal trays with 3 control plants and 2 untreated plants per tray and left to dry for 24h prior to inoculation with the pathogen. For the volatile compound (11) all replicates of a single treatment were placed onto a tray in a block with 1 control and 1 untreated plant. A block of 3 control and 3 untreated plants were also included as a single block. Plants sprayed with volatile compounds were inoculated within 1h of compound application.

6.2.1.2 Pathogen inoculation

After the appropriate drying times, the plants were inoculated with the pathogen. Spores from a 21d culture of *B. cinerea* grown on PDA were harvested in glucose acidic buffer (0.1M glucose buffered to pH 6 with 30mM KH_2PO_4/Na_2HPO_4), filtered through muslin and the concentration adjusted to 5×10^5 ml^{-1}. The spore suspension was sprayed onto the plants until droplet formation using a coarse inoculating gun. Clear polythene covers were placed over the whole tray for non-volatile compounds and each replicate block for volatile compounds. Two litres of warm water were poured into the bottom of each tray to increase the relative humidity. Plants were incubated at 18°C with 14h light (single Philips TLD 58W/35 lamp) /10h dark for 5-7d prior to the assessment of disease.

6.2.1.3 Disease assessment

Disease severity was scored on a 1 - 10 scale, where 0 = no disease, 1 = 10% disease infection, 2 = 20 % infection, etc. Disease control offered by the fungicides has been expressed as a percentage of the control plants. Results are mean values from 3 replicates of a single experiment.

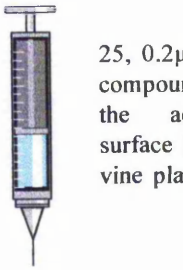
6.2.2 Biostability screening of test fungicides.

Compounds 1 and 2 were chosen since results from the agar plate screening (6.3.3) and germling experiments (6.3.4) did not suggest problems with uptake or fungal metabolism of these compounds, leaving poor biostability as a potential explanation of their good activity at the target site and poor activity *in planta.* Compound 14 (azoxystrobin) was chosen as a fungicide which should not exhibit biostability problems. A further compound known to remain stable under the test conditions was also included as a control. Experimental procedures are summarised in Figure 6.2.

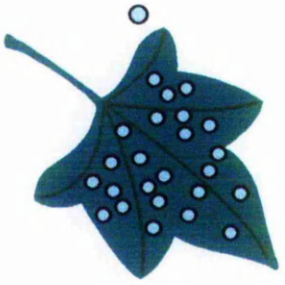
6.2.2.1 Fungicide application

Riesling vine seeds (Hoechst vineyard - Germany) were sown in John Innes #1 compost and grown under glasshouse conditions (23°C, 14h photoperiod) for 6 weeks. Using a micro-applicator and automatic dispenser (Burkard Scientific Pax 100) fitted with a 50µl SGE syringe, the upper 2 leaves of each seedling were spotted 25 times each with 0.2µl of the test compounds plus a standard (dissolved in 85% (v/v) acetone, 0.5% (v/v) dialkyl alkylphosphonates) to a final concentration of 2500ppm. Fifty spots of each compound were also dispensed into 1ml glass vials before and after application to the plants, representing the total quantity of compound applied to each replicate for the purposes of subsequent analysis. The plants were incubated at 23°C with a 13h photoperiod (Philips MCFE 65-80W/35 bulb) for 24h and 7d.

Figure 6.2 Summary of the methods used to investigate compound biostability

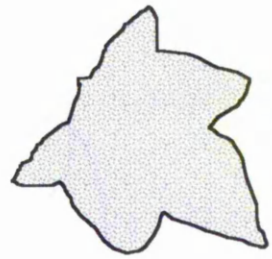


25, 0.2µl droplets of compound applied to the adaxial leaf surface (2 leaves per vine plant).



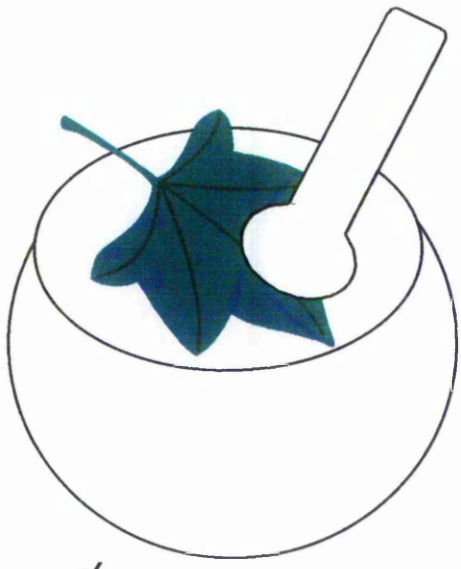
Plants incubated at 22°C with a 13h photoperiod for 24h & 7d.

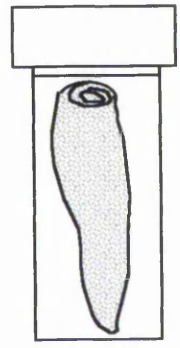
7% (w/v) cellulose acetate in acetone applied to the leaves with a wooden applicator.



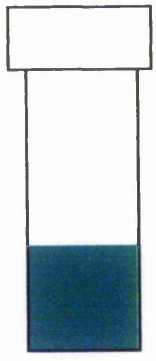
Dry cellulose acetate peeled from leaves and placed in a capped vial.

Treated leaves detached and stored at -20°C prior to grinding in a pestle and mortar with liquid nitrogen. Powder resuspended in methanol and centrifuged at 2800 x g for 15 minutes to pellet debris.





Cellulose strips extracted in 2ml methanol overnight.



Samples evaporated to dryness and resuspended in 250µl 95% methanol. Samples filtered through 0.2µm anapore by centrifugation at 15 000 rpm for 15 minutes, transferred to HPLC vials with a crimped cap and analysed.

Extract evaporated to dryness, resuspended in a known volume of 95% methanol and subjected to HPLC analysis.

6.2.2.2 Biostability measurements

Following incubation, the presence of the applied compound either on the leaf surface or the within the leaf tissue was determined as follows:

Leaf surface

Cellulose acetate 7% (w/v) in acetone was applied to the upper leaf surface using a wooden applicator and allowed to dry. The dried cellulose acetate was carefully peeled away from the leaf and placed in a capped glass vial (2 leaf peels/plant/vial). The cellulose acetate peels were covered with 2ml methanol and left for 24h to facilitate extraction. Following this incubation, the methanol was removed to a 2ml glass vial using a Pasteur pipette. The samples were evaporated to dryness in a Gyrovap (version D3KJ, V.A.Howe & Co. LTD, London, UK.), resuspended in 250µl 95% methanol, transferred to crimped-capped HPLC vials and subjected to HPLC analysis.

Leaf tissue

Following the removal of the cellulose acetate, the treated leaves were detached from the plant at the petiole and stored at -20°C. Once frozen, the leaves were transferred to a mortar, covered with liquid nitrogen and ground to a fine powder using a pestle. The powder was resuspended in 4ml methanol and centrifuged at 2800 x g for 15min to pellet the debris. The supernatant was evaporated to dryness as before and the samples resuspended in 250µl 95% (v/v) methanol. To remove any further debris the samples were filtered by centrifugation through a 0.2µm anapore filter (Whatman) at 15 000 x g for 15 minutes, prior to HPLC analysis.

6.2.2.3 HPLC analysis

HPLC analysis was carried out by Fiona Fletcher-Brown at AgrEvo (Chesterford Park Research Station, Essex, UK) to determine the location of the parent compound at 24h and 7d post application. Results are expressed as percentage of applied compound located on the leaf surface or within the foliage, n = 4 from a single occasion.

6.2.3 Agar plate screening of test fungicides against *B. cinerea*

Test compounds were dissolved in DMSO and incorporated into 5cm PDA plates at final concentrations of 0.25, 2.5, 25 & 250 ppm (final concentration DMSO = 0.1% v/v). Control plates contained DMSO only. Ten millilitres of sterile distilled water was pipetted onto the surface of a 14d sporulating *B. cinerea* culture and the spores dislodged using a sterile spreader. Mycelial debris was removed by filtration through a 20µm sieve and the remaining spore suspension adjusted to a final concentration of 1×10^6 ml^{-1}. Plates were inoculated centrally with 5µl of the spore suspension and incubated at 20°C in a UV incubator for one week. After this time colony diameters were measured and percentage inhibition of growth compared to the controls calculated. Results are triplicates, except * which are duplicates (insufficient compound available) from 2 separate occasions. There was an insufficient quantity of compound 11 to carry out the plate test at 250ppm.

6.2.4 Screening of test compounds against respiration by *B. cinerea* mycelia and protoplasts in the oxygen electrode

6.2.4.1 Germlings

Liquid cultures of *B. cinerea* were grown as for protoplast isolation (section 2.2.3.1). Harvested mycelia were resuspended in fresh GGM at a ratio of 1g mycelia: 10ml GGM. One millilitre aliquots of this suspension were placed into the oxygen electrode chamber (set up for monitoring as before, Chapter 2) and aerated by rapid pipetting. The rate of oxygen consumption was recorded for approx. 5min prior to the addition of the inhibitors. Inhibitors were dissolved in DMSO and added to the chamber through the opening in the lid using a 1µl Hamilton syringe to a final concentration of 10µM and 0.1% (v/v) DMSO. The rate of oxygen consumption was recorded for a further 10min. Measurements of oxygen consumption by GGM only, and the effect of adding DMSO only were also undertaken. Results are expressed as mean % inhibition of oxygen consumption compared to the control rate (±SE) and are duplicates from 2 separate occasions.

6.2.4.2 Protoplasts

Freshly isolated protoplasts were resuspended in GGM stabilised by Phos-KCl to a concentration of 1×10^8 ml^{-1}, added to the electrode chamber and aerated by rapid pipetting. The rate of oxygen consumption was recorded for approx. 5min prior to the addition of the inhibitors and subsequent rate measurements as before. Results are expressed as mean % inhibition of oxygen consumption compared to the control rate (±SE) and are duplicates from 2 separate occasions.

6.2.5 Screening of test compounds against isolated *B. cinerea* mitochondria

6.2.5.1 Mitochondrial isolation

Two 1L flasks of *B. cinerea* liquid culture were grown as for protoplast isolation (section 2.2.3.1). The harvested mycelia were resuspended to a "soupy" consistency in extraction buffer (300mM sucrose, 1mM EDTA, 0.2% v/v defatted BSA, 25mM Tris/HCl, pH 7.5) containing 5 mg ml^{-1} lysing enzyme (as before) and incubated for 1h at 22°C with gentle shaking. The partially digested mycelia were then homogenised (Potter) until little resistance remained (approx. 25 strokes), transferred to cooled centrifuge tubes and centrifuged at 2500 x g for 20 min at 5°C to pellet cellular debris. The supernatant was centrifuged at 40 000 x g for 5 min at 5°C to pellet the mitochondria. Mitochondrial pellets were resuspended in 600µl resuspension buffer (1mM EDTA, 25mM Tris (free-base)/HCl, pH 8), dispensed into 1.5ml Eppendorf tubes as 60µl aliquots, and rapidly frozen in dry ice prior to storage at -80°C.

6.2.5.2 Assay of electron transport inhibition

The isolated mitochondria were defrosted and added to cooled solubilization buffer (0.25% CHAPS, 150mM KCl, 100ml standard buffer, containing 150mM K_2HPO_4, pH 7.2 with KH_2PO_4), at a ratio of 60µl mitochondria: 2ml buffer. The mitochondria were left on ice for 5 minutes to allow membrane solubilization to occur prior to use. The assay was carried out in 1.6ml semi-micro polystyrene cuvettes (Fisher Scientific). Contents of the cuvette were:

150µl solubilized mitochondria

300µl assay buffer (9ml standard buffer, 1ml 0.181M KCN)

450µl cytochrome C/NADH solution (39mg cytochrome C [from horse heart, Sigma], 2 mg NADH [reduced form disodium salt, Sigma], 20ml standard buffer).

Inhibitors were dissolved in DMSO and 1µl added to the cuvette (final concentration 1µM) prior to the mitochondrial enzyme preparation. Cuvette contents were mixed by rapid pipetting and absorbency determined spectrophotometrically at 550nm (Shimadzu UV-visible recording spectrophotometer, Shimadzu Europa, Milton Keynes, UK). The activity of each mitochondrial enzyme preparation used was determined prior to testing the inhibitors and the assay standardised with reference to this activity. After establishing a control activity rate, standard inhibitors of complex I and complex III were assayed and this inhibition was used to represent 100%. Inhibition of cytochrome c reduction caused by the tested compounds was calculated as a percentage of the control/standard inhibitor data. All compounds were tested in triplicate using mitochondria isolated on 3 separate occasions.

6.3 Results and discussion

6.3.1 Biological screening of test fungicides against *B. cinerea.*

Compounds 1, 2, 3, 4, 7, 8, 14 and 15 exhibited poor biological activity against *B. cinerea* on tomato (AgrEvo - unpublished data). When applied as protectant fungicides on tomato plants, compounds 5, 6, 9, 10, 11, 12 and 13 also offered little protection against *B. cinerea* infection (Figure 6.3), less than 30% disease control was achieved in all instances at application rates up to 100ppm. By contrast, application of 100ppm iprodione to the tomato plants provided an average of 84% disease control.

Figure 6.3 Control of *B. cinerea* infection on tomato plants, achieved by the application of experimental compounds at various rates prior to inoculation with the pathogen. (Results are mean % disease control obtained compared to control plants, n = 4)

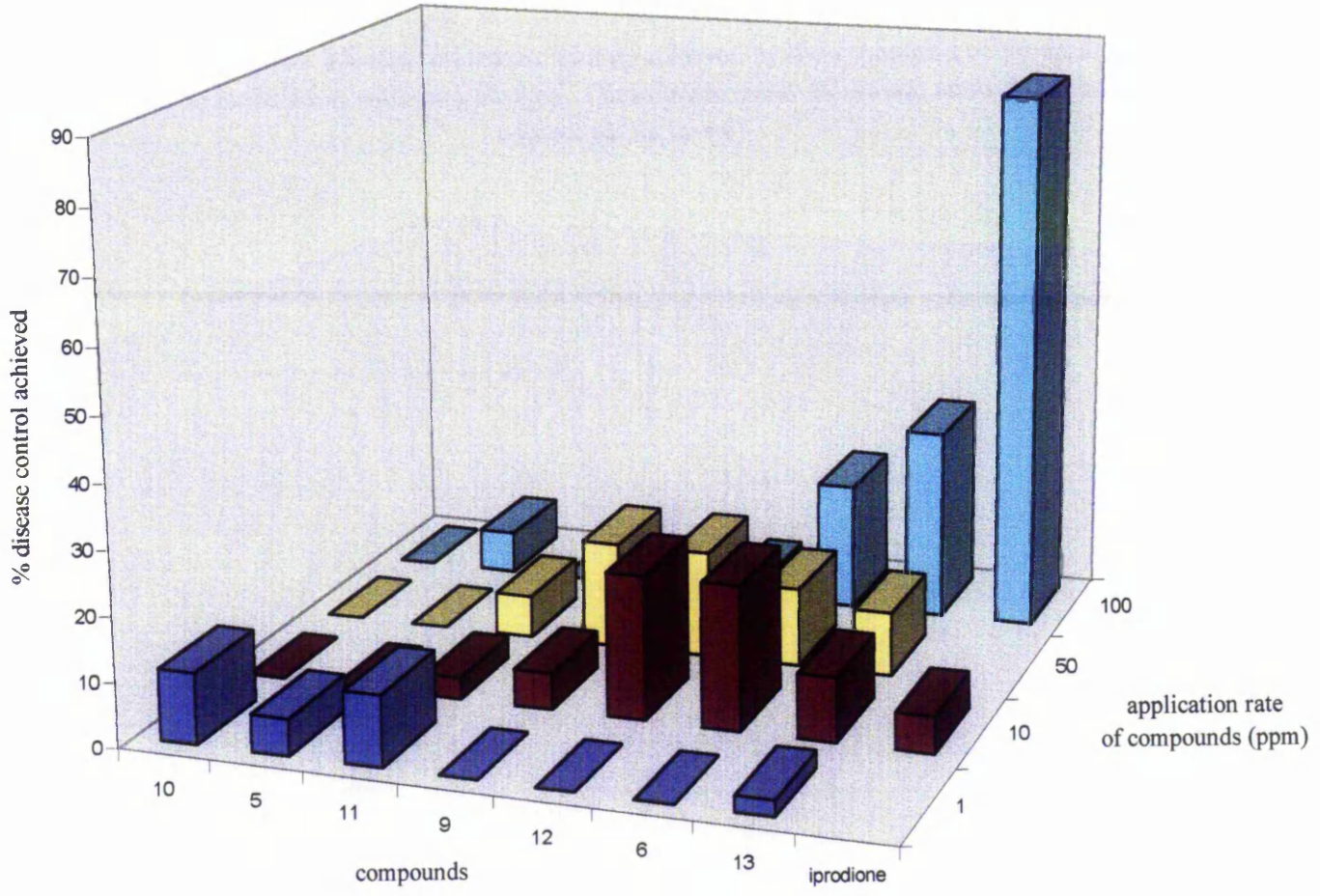


Figure 6.4 shows a plant protected from infection by application of 200ppm iprodione and a control plant, 5d after inoculation with the pathogen.

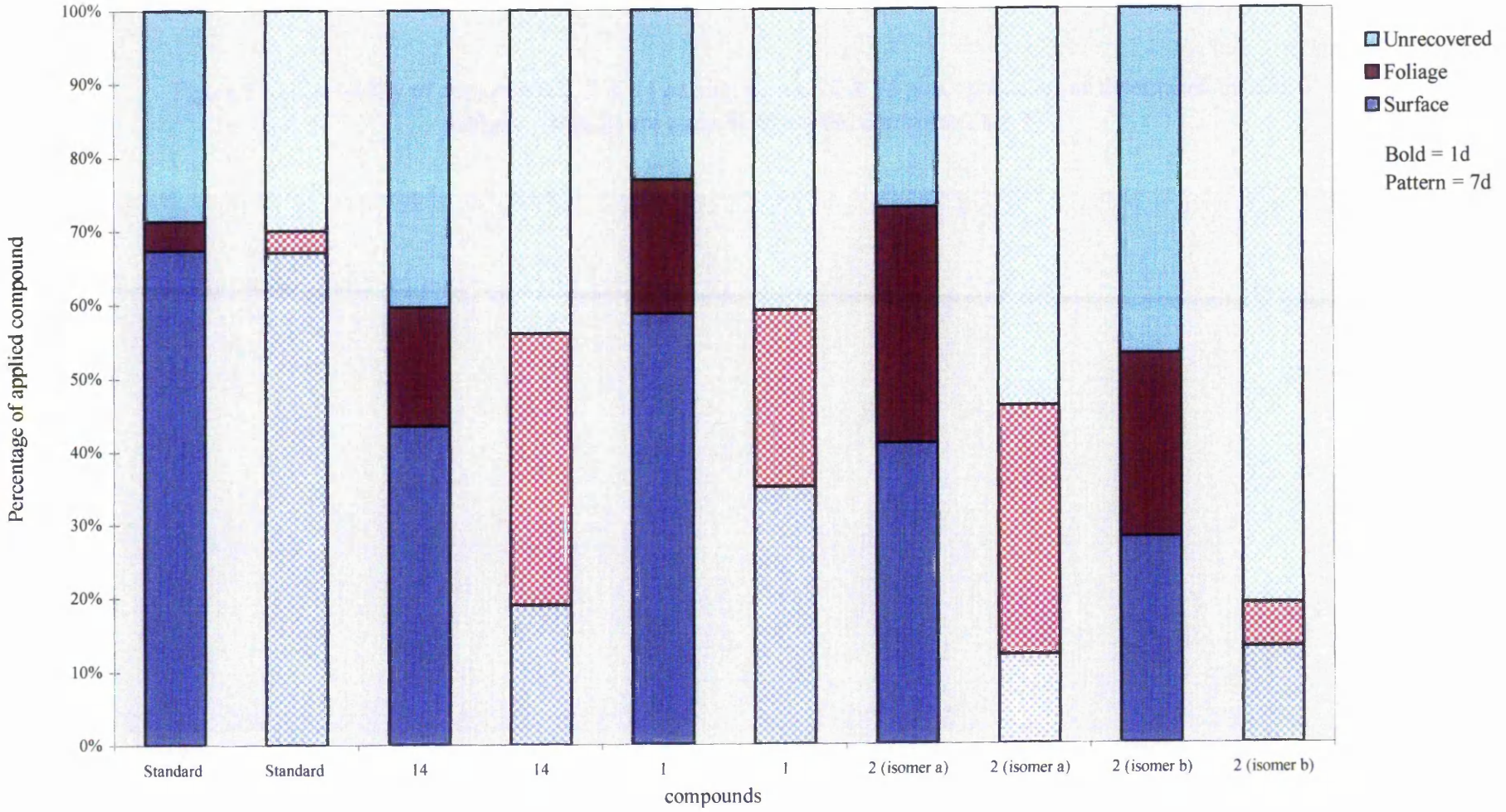
6.3.2 Biostability screening of test fungicides.

Compounds 1, 2 and 14 were tested for biostability with reference to an experimental standard. After 1d incubation on the vine plants, 68% of the standard compound was located on the surface of the leaf and 4% inside the foliage. The remaining 28% was unrecovered, lost either through chemical breakdown, plant metabolism or compound volatility. After 7d incubation, these figures were largely the same (Figure 6.5), indicating that the standard compound is stable under the test conditions. Compound 14 (azoxystrobin) also remained stable over the experimental period, 40% of the applied chemical was unrecovered at 1d, increasing slightly to 44% at 7d. It is interesting to note that after 1d, 43% of the compound was detected on the leaf surface dropping to just 19% after 7d. The difference was detected in the foliage at 7d demonstrating that between 1d and 7d the compound moves from the leaf surface into the foliage. This steady uptake into the leaf ensures that whilst the compound reaches the vascular tissues from where it can be redistributed to offer whole-plant protection, some chemical remains on the surface to combat spores (Zeneca Agrochemicals).

Only 23% of compound 1 was unrecovered at 1d, dropping to 41% at 7d. The percentage of applied compound detected on the leaf surface fell from 58% at 1d to 35% at 7d, representing an increased percentage of unrecovered compound at 7d. Compound levels in the foliage increased from 18% at 1d to 24% after 7d. Fifty nine percent of compound 1 was recovered 7d after application to the plants, indicating that this fungicide is quite stable under the test conditions.

Figure 6.4 A tomato plant protected from *B. cinerea* infection following application of 200ppm iprodione and an untreated plant, 5d after inoculation with the pathogen.

Figure 6.5 Biostability of compounds 1, 2 & 14 on vine plants, 1d & 7d post application as determined by HPLC analysis. (Results are mean % of applied compound, n = 4)

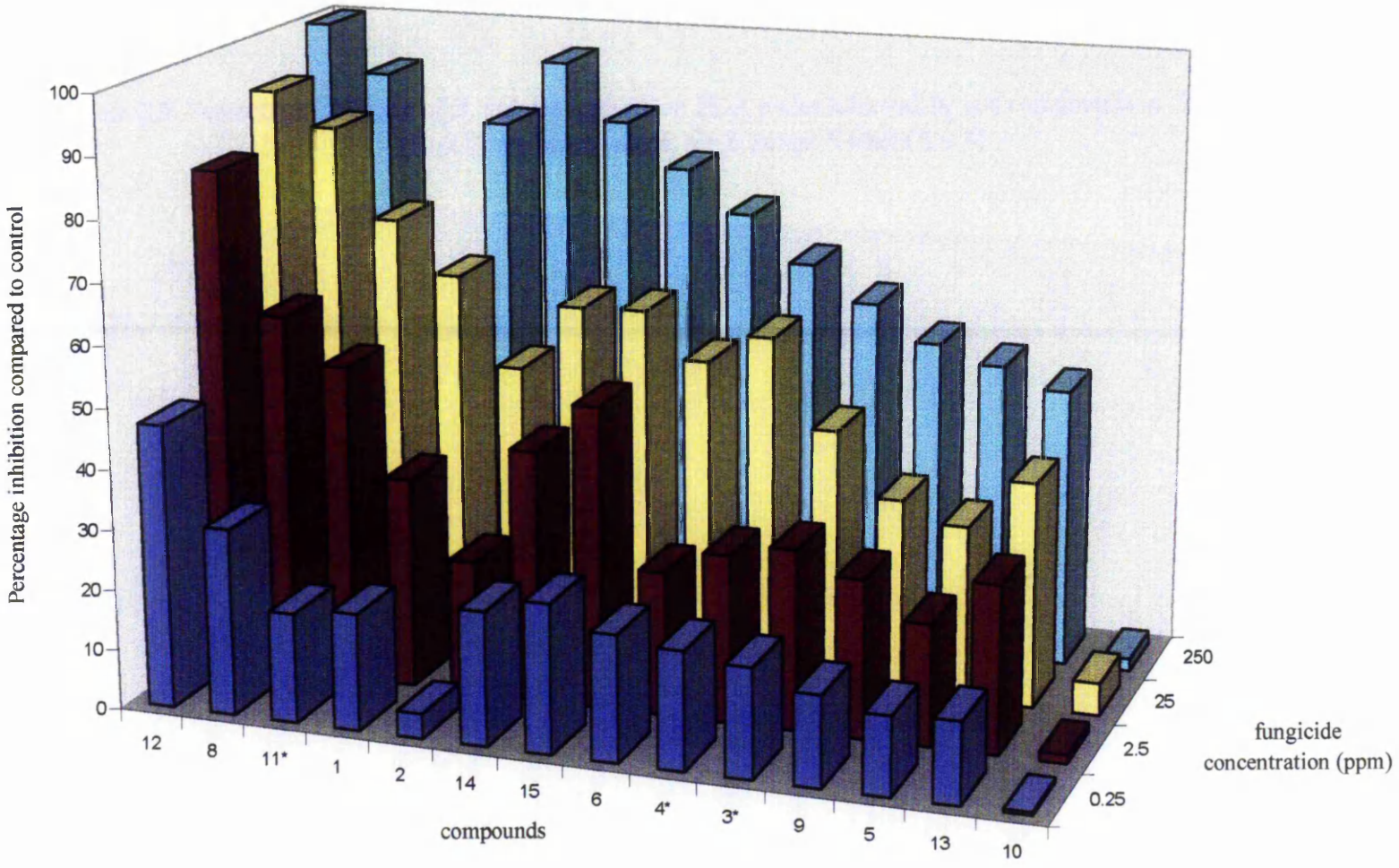


HPLC analysis of compound 2 revealed the existence of two isomeric forms in approximately equal quantities. Both exhibited similar patterns of compound distribution, although isomer a was more stable than isomer b. After 7d, 54% of isomer a and 81% of isomer b were unrecovered compared to 27% (a) and 47% (b) at 1d. In both cases this was reflected by a drop in amount of compound detected on the leaf surface. Isomer b also showed a decrease in foliar compound after 7d. The performance of compound 2 in these experiments reveals a biostability problem, which may account for the discrepancy between the good biochemical inhibition of the target site (section 6.3.5) and poor *in planta* activity (section 6.3.1) displayed by this fungicide. Similar biostability problems were not discovered for compound 1 or compound 14, eliminating poor biostability as an explanation for similar discrepancies in these fungicides. The results for compound 14 reflect the fact that azoxystrobin is a commercially successful, biostable fungicide. The reasons why this fungicide is ineffectual against *B. cinerea in planta* remain unclear.

6.3.3 Agar plate screening of test fungicides against *B. cinerea.*

With the exception of compound 10 which proved ineffectual against *B. cinerea*, all other compounds inhibited growth of the pathogen (Figure 6.6). Inhibition of fungal growth decreased as the compounds were serially diluted from 250ppm through to 0.25ppm. As the mode of action of compound 10 is similar to that of the others tested, it is unlikely that the pathogen is exhibiting site of action resistance. This compound may be unstable and therefore inactive, may not be penetrating the fungus, or might be metabolised and detoxified by the pathogen.

Figure 6.6 Percentage inhibition of *B. cinerea* growth on PDA plates achieved by test compounds at different rates.
(Results are mean values, n = 6 except * where n = 4)



6.3.4 Screening of test compounds against *B. cinerea* mycelia and protoplasts in the oxygen electrode.

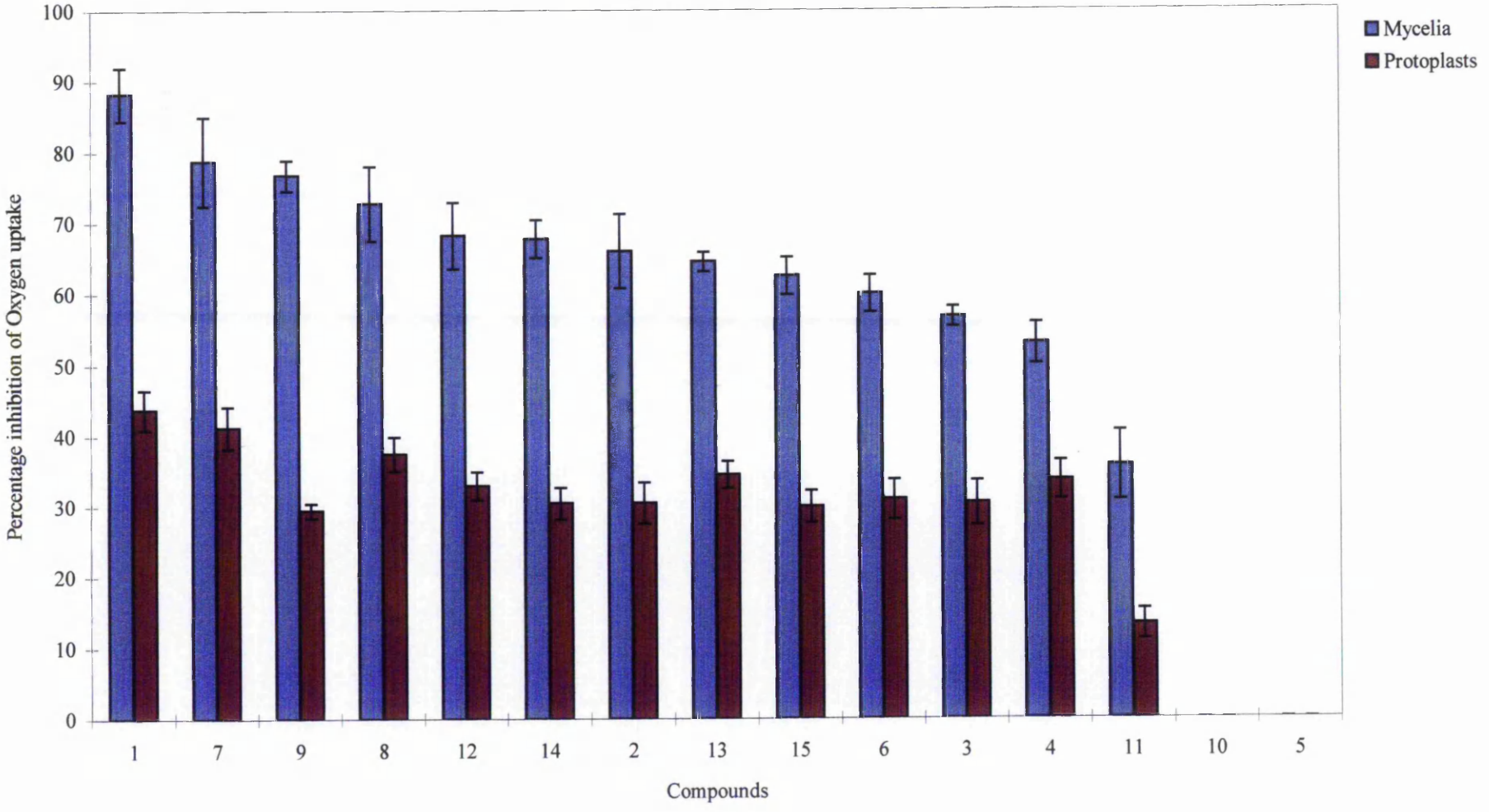
The inhibition of O_2 uptake by *B. cinerea* mycelia or protoplasts reflects the inhibition of respiration caused by the test compounds (Figure 6.7).

When protoplasts were used instead of mycelia, oxygen consumption was reduced to a lesser extent (Figure 6.7). In order to generate an initial rate of oxygen uptake similar to that achieved with mycelia, and therefore measure oxygen consumption and compound inhibition over the same time period, it was necessary to increase protoplast concentration beyond mycelial concentration *i.e.*, each mycelial assay used 0.1g mycelia and each protoplast assay used 1×10^8 protoplasts, which would have originated from approximately 1.0g mycelia.

This probably reflects both the reduced metabolic capacity of the isolated protoplasts compared to the actively growing mycelia, and an incomplete yield of protoplasts. The increased concentration of cells in the protoplast samples presumably resulted in a higher availability of active sites for the compound to bind to and inhibit, making the overall inhibition of oxygen uptake less than in the mycelial samples with a lower concentration of cells/active sites.

Compounds 10 and 5 did not alter oxygen uptake into either mycelia or protoplasts. Agar plate testing also found that compound 10 was ineffectual against *B. cinerea*. One explanation for this is that the compound is metabolised by the fungus. Investigating compound metabolism by the fungus would be a logical next step and could be achieved by extending the time period of the oxygen electrode assay, if the compound were metabolised, an increase in the oxygen uptake rate following initial inhibition would be seen. Metabolisable respiratory uncouplers such as dinitrophenol (DNP) are frequently used in studies involving oxygen electrodes, therefore it ought to be possible to study other respiratory inhibitors in this way.

Figure 6.7 Percentage inhibition of oxygen uptake into *B. cinerea* mycelia and protoplasts, achieved by the addition of 10μM compound. (Results are mean values ± SE, n = 4)



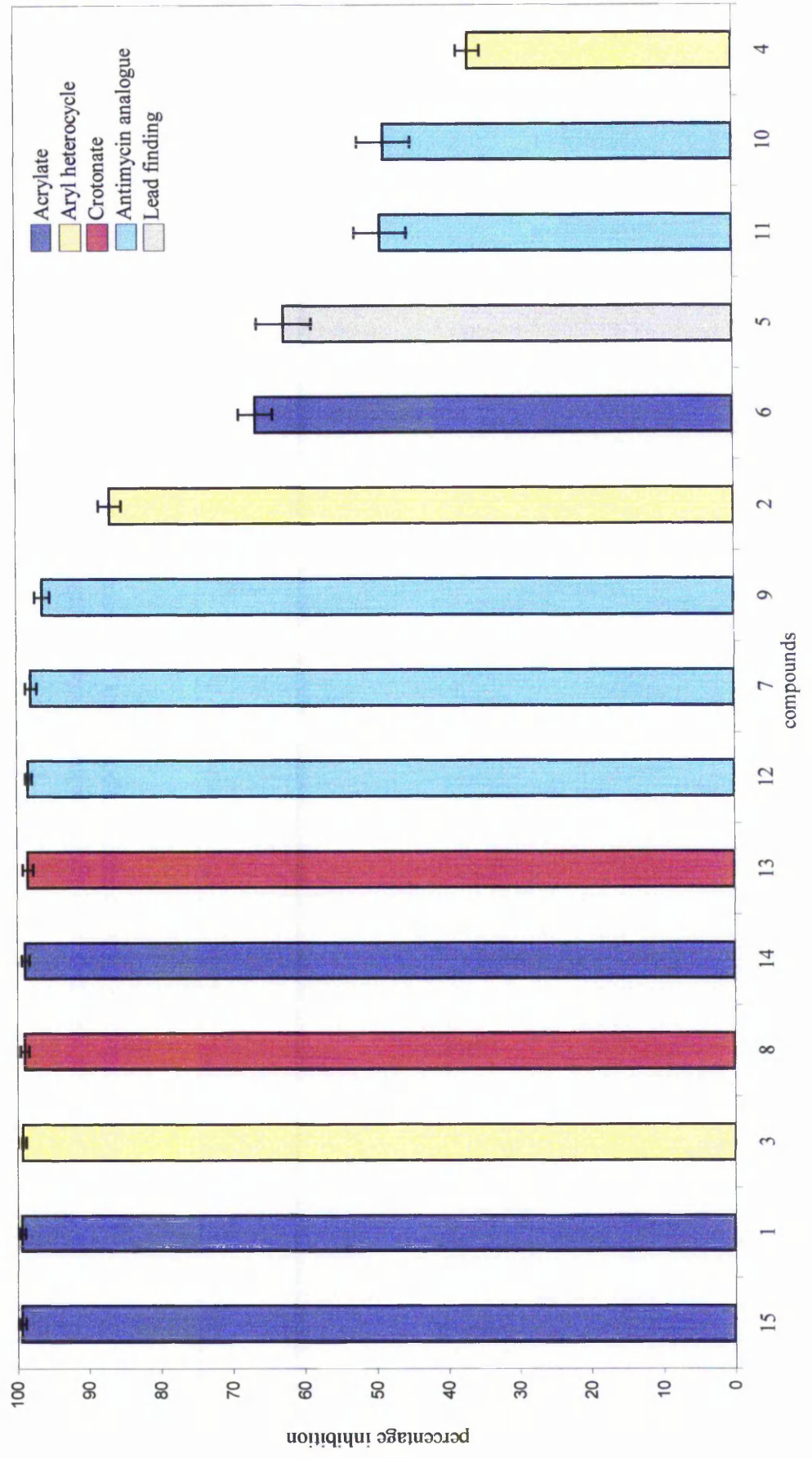
Clearly *B. cinerea* mycelia rather than protoplasts are a more effective way of testing compounds for activity in this assay, although if a compound were found to be inactive in a mycelial assay yet active in a mitochondrial screen, challenging protoplasts with the compound may be a useful way of ruling out problems with uptake into the pathogen which are often associated with mycelial surface mucilages. A protoplast screen based on detection of fluorescence (Chapter 4) is perhaps a more realistic option, discussed further in Chapter 7.

6.3.5 Screening of test compounds against isolated *B. cinerea* mitochondria.

Compounds 1, 3, 7, 8, 9, 12, 13, 14 & 15 all showed excellent activity against isolated *B. cinerea* mitochondria, inhibiting cytochrome c reduction by 95% or above (Figure 6.8). Compound 2 also displayed good activity, inhibiting cytochrome c reduction by an average of 87%. Compounds 4, 5, 6, 10 & 11 inhibited reduction by 66% or less. Although a 66% inhibition would be a good result in a biological screen, in this assay involving the enzyme and the inhibitor in solution, it is regarded as poor. Activity in excess of 90% is usually required at this stage for a compound to be considered for further development.

Compound 10 inhibited cytochrome c reduction by an average of 48.6% ± 3.7%. The poor performance of compound 10 in the mitochondrial enzyme assay suggests that instability, rather than poor penetration, could explain its ineffectiveness in the plate testing and oxygen electrode assay. If the compound is unstable and partially breaking down between synthesis and use, then using it at the same concentrations as the other compounds in the screen is, in effect, like using it at a lower concentration

Figure 6.8 Screening of test compounds against isolated *B. cinerea* mitochondria. (Results are mean % inhibition of cytochrome C reduction in the electron transport chain ,compared to control/standard data ± SE, n = 9)



Isolation of mitochondria from *B. cinerea* mycelia following degradation by protoplasting enzyme was successful. The isolated mitochondrial enzyme preparations were highly active and results of the assay were consistent across three separate isolations. Isolating mitochondria from fungi requires the cells to be disrupted. This can be achieved in a number of ways, for example grinding with sand, using a Potter-Elvehjem homogenizer, Waring blender or French press. However, these methods were not particularly successful when attempting to isolate mitochondria from *B. cinerea* mycelia grown under standard conditions (S. Dunn [AgrEvo] - Pers. Comm.), with the resulting mitochondrial enzyme preparations exhibiting low activity in the electron transport inhibition assay.

Subsequently, *B. cinerea* liquid cultures grown at higher than usual temperatures (approx. 30°C) were successfully used to isolate mitochondrial enzyme preparations with a higher activity. Whilst it is no more difficult to generate 'stressed' cultures by increasing the incubation temperature, the physiological and biochemical competence of such cultures may differ significantly from those grown under standard conditions, for example in these experiments the 'stressed' cultures were highly pigmented (dark grey/green) compared to those grown under standard conditions. This may have repercussions when relating data from different experiments. One potential advantage of using mitochondria isolated from *B. cinerea* using a protoplasting enzyme step, is that normal rather than stressed cultures can be used.

Isolating mitochondria from fungal or yeast protoplasts is a practised technique (Mizutani, Yukioka, Tamura, Miki, Masuko, & Takeda, 1995). Using enzymes to degrade the cell wall and releasing the cell contents directly into mitochondrial harvesting buffer (rather than protoplast buffer) as achieved here, allows a high mitochondrial yield to be obtained within a shorter isolation procedure. These advantages must however, be balanced against the relatively high cost of protoplasting enzymes that would be required to use this technique in a screening situation.

6.3.6 General discussion

These experiments were undertaken firstly to investigate the effectiveness of known and experimental fungicides against *B. cinerea* using a variety of assays; and secondly to establish the usefulness of protoplasts, or protoplast techniques in the context of such research. Large discrepancies were discovered between compound effectiveness *in planta*, where no compound offered good protection against *B. cinerea* infection (Figure 6.3), and *in vitro* where most compounds inhibited the pathogen to various extents depending on the assays employed (Figures 6.6, 6.7, 6.8).

Assimilating such data is potentially valuable in attempting to determine why *in planta/in vitro* discrepancies exist. For example, compounds which are good inhibitors of mitochondrial activity, mycelial growth on agar and mycelial respiration may have a biostability problem, as they are potent inhibitors of the target enzyme and are taken up into the pathogen yet still do not provide protection to the plant. Compounds 1 and 2 were two such candidates chosen for biostability investigation work, other compounds (e.g., 7, 8 and 12) would have also been tested but were not available at the time of testing.

Poor uptake of the compound into the pathogen could not however account for poor *in planta* activity for these compounds, as this would have been reflected in either the plate test or oxygen electrode results. Compound 2 was found to be unstable in the biostability screen, which could account for its poor performance as a fungicide. Compound 1 was found to remain quite stable over the experimental period, eliminating biostability as an explanation for poor activity. This compound may be metabolised by the fungus into inactive by-products, although this may arguably have been seen in the plate test experiment, which proceeded over a 7 day period. The oxygen electrode assay could provide a means of determining whether or not metabolism were occurring.

6.4 Conclusions

- Test compounds did not protect tomato plants from *B. cinerea* infection.

- When incorporated into agar plates, the same compounds (except #10) inhibited growth of *B. cinerea.* Mean inhibition ranged from 46.4% to 100% at 250ppm.

- In oxygen electrode tests, the compounds (except #10 and #5) inhibited oxygen uptake into mycelia from 35.8% to 88.3% and into protoplasts from 13.3% to 43.8% at 10μM.

- Active mitochondria were successfully isolated from *B. cinerea* mycelia digested with protoplasting enzyme. Reduction of cytochrome c in the electron transport chain was inhibited by addition of the test compounds at 1μM, from 36.8% to 99.4%.

- Compound 2 was found to be quite unstable in biostability experiments, which may account for the discrepancy between *in plant* and *in vitro* data for this chemical.

Chapter 7: General discussion

The first aim of this research was to develop reliable, reproducible procedures for the isolation of protoplasts from *B. cinerea, P. infestans* and *S. nodorum*. Based on techniques in the literature, isolation, purification and storage of protoplasts from *B. cinerea*, *P. infestans* and *S. nodorum* were successfully achieved, with yields comparable to published data (Shirane & Hatta, 1986; Braun & Heisler, 1990; Kinghorn *et al.*, 1991; Judelson & Michelmore, 1991; Cooley *et al.*, 1988). Protoplasts were isolated and purified from *B. cinerea* easier and more consistently than *P. infestans*, which proved difficult to purify and *S. nodorum*, where the sporulation of solid cultures was quite variable. For these reasons, *B. cinerea* was chosen as the pathogen to use in further experiments.

The purification of *P. infestans* protoplasts proved troublesome. Protoplasts of *P. infestans* have in the past been used mainly for genetic transformation purposes (Judelson & Michelmore, 1991; Judelson *et al.*, 1991; Judelson, 1993; Judelson *et al.*, 1993; Kinghorn *et al.*, 1991), which does not require high levels of purity as only protoplasts containing the transformed DNA, and hence the selectable marker, would regenerate when placed on selective media. Cellular debris, for example cell wall material would not be important in this context, and this may account for the purity of the protoplast samples obtained when following published isolation protocols. For the experiments presented here, the purity of the protoplast samples was considered to be important and it was felt that spending additional time attempting to purify *P. infestans* protoplasts was unnecessary, as isolation and purification of protoplasts from *B. cinerea* were so successful.

The second aim of this research was to characterise the isolated protoplasts with respect to their metabolic viability. Oxygen electrodes were successfully used to measure protoplast respiration, and through staining with fluorescein diacetate,

esterase activity and membrane integrity were also detectable.. Viability staining using FDA provided an avenue for further study, leading to the development of a quantifiable viability assay. Similar assays have been developed to quantify fluorescence in mammalian cells (Neytus *et al.*, 1991; Maeda *et al.*, 1993), plant cells and protoplasts (Aoyagi & Tanaka, 1994) and to determine microbial activity in wood (Bjurman, 1993). This is a new area of research for fungal protoplasts which may prove to be a useful tool for rapid screening of novel fungicides (discussed later).

The final aim of this research was to investigate the potential uses of protoplasts isolated from one of the pathogens in the context of fungicide research and development. Oxygen electrodes were utilised in an experiment to investigate the effects of a series of surfactants with varying carbon chain lengths upon respiration in protoplasts of *B. cinerea*. These experiments established the preferential carbon chain length for protoplast interaction (C8) and demonstrated that at levels which did not cause solubilization of the protoplast membrane, the surfactants did not significantly alter the respiration rates. It was hoped that this information would be important in future experiments, where fungicides able to inhibit electron transport were incubated with protoplasts in the oxygen electrodes (6.2.4.2). However, mycelia rather than protoplasts were discovered to be more useful in these experiments (Figure 6.7), consequently the effect of adding pre-rupture level C8 surfactant to the protoplasts in the presence of fungicides was not investigated.

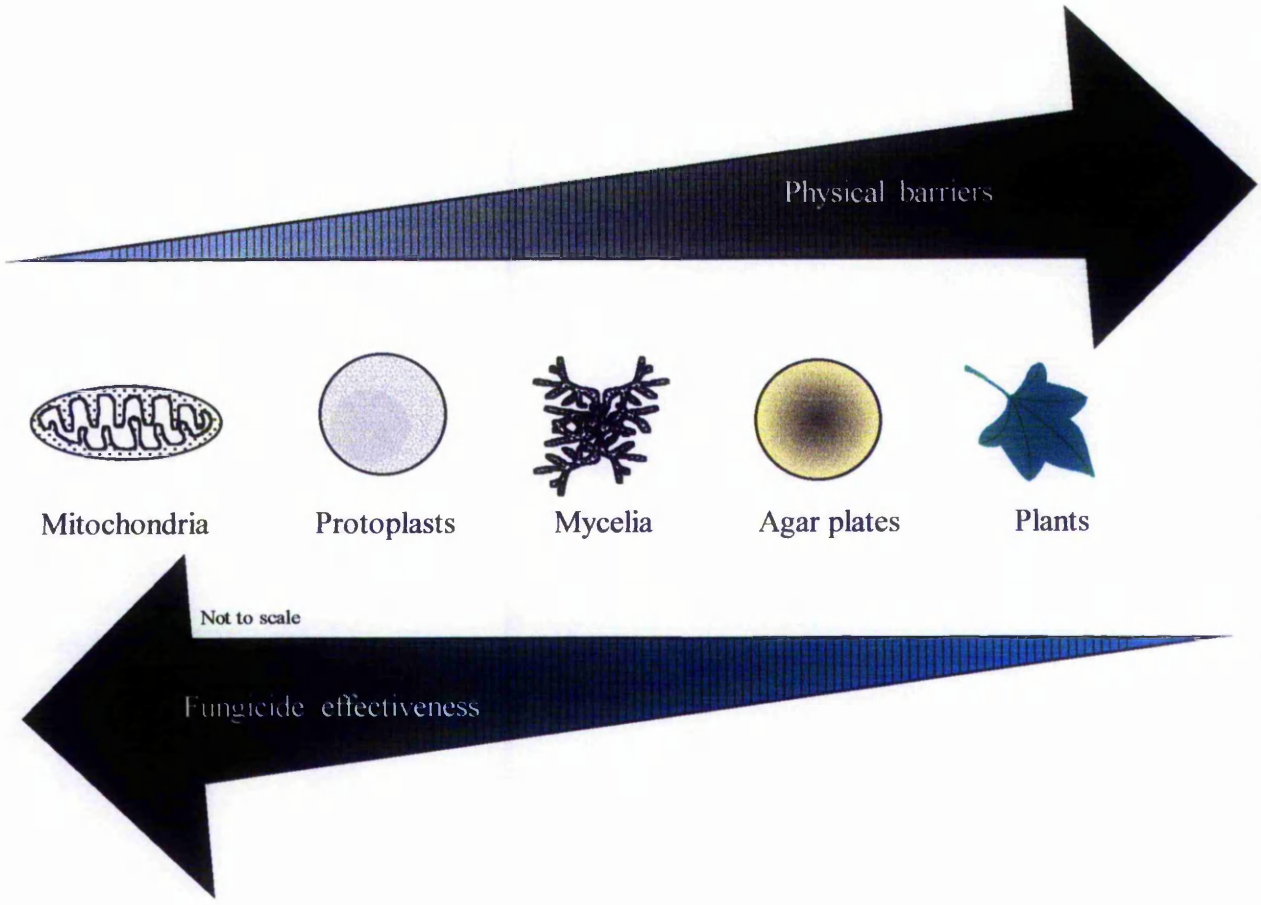
The problem of using protoplasts instead of mycelia to measure inhibition of oxygen uptake by different compounds, was that oxygen uptake by protoplasts was less than by the comparable quantity of mycelia, possibly reflecting reduced metabolic capacity of the isolated protoplasts compared to the actively growing mycelia, and/or an incomplete yield of protoplasts. Increasing the concentration of protoplasts to achieve an oxygen uptake rate to match that of the mycelia, presumably resulted in a higher availability of active sites for the compound to bind to and inhibit, making the

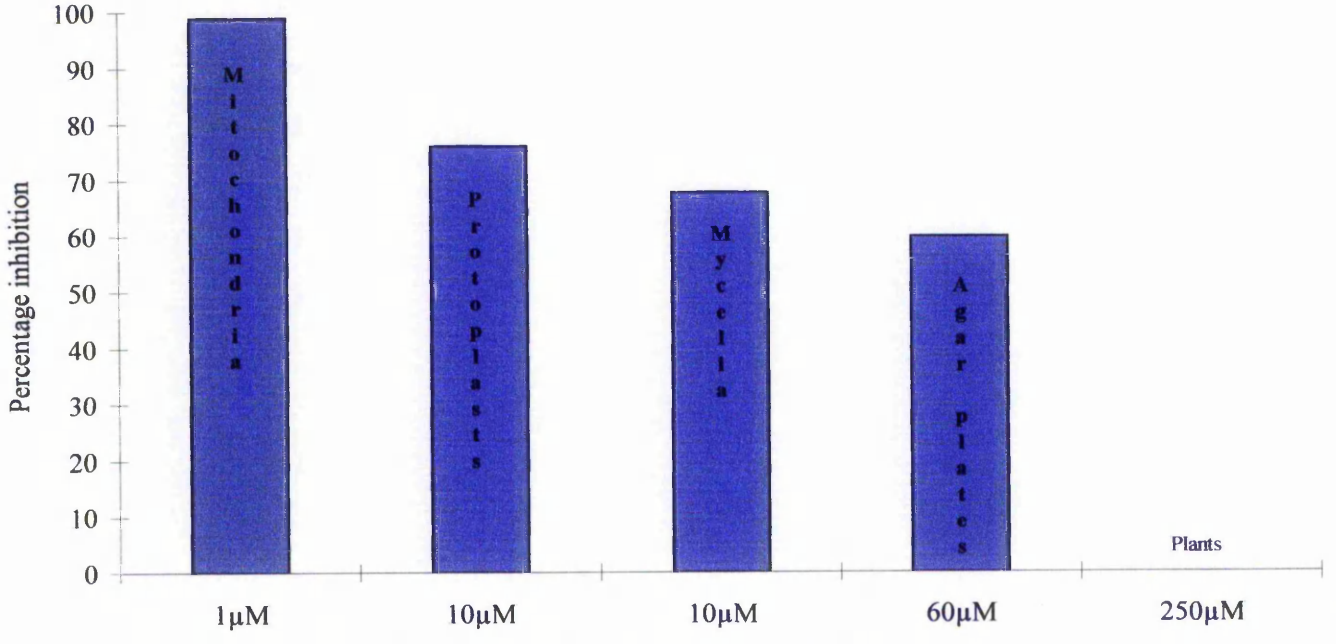
overall inhibition of oxygen uptake less than in the mycelial samples. Although mycelia were more useful than protoplasts in this assay, any compounds inactive against mycelia yet active against mitochondria, could be tested against protoplasts. Any observed activity against protoplast oxygen consumption, might indicate problems with uptake into the pathogen associated with mycelial surface mucilages not present in the protoplast samples.

Botrytis cinerea protoplasts were successfully used to investigate protein synthesis and fungicide uptake using radiolabelled compounds. Initially, samples were incubated with stirring for 4h, but it was found that after 2h of continual stirring, protoplasts were rupturing. This is a criticism of the developed technique, which could not therefore be used to investigate uptake over long time periods. Incubating the samples without stirring was not investigated, as protoplasts were found to settle and clump together when not stirred for long periods (*i.e.*, in excess of 2 hours). This would be undesirable in these experiments, as protoplasts in the centre of a 'clump' would not have access to the radiolabelled compounds. Whilst resuspension is straightforward, protoplasts that had no association with the radiolabelled compounds due to their position during the incubation period, would effectively lower the protoplast activity ratio and skew the results.

To ascertain if protoplasts were a useful means of testing experimental compounds for activity, 13 experimental compounds plus 2 commercial products with a similar mode of action were used in a range of experiments. Protoplasts proved less useful than mycelia when oxygen electrodes were used to investigate compound inhibition of oxygen uptake. However, in the fluorescence-based viability assay, protoplasts proved a useful means of assessing azoxystrobin toxicity. Figure 7.1 places this assay in the context of *in planta* and *in vitro* testing. Inhibition of protoplast viability at 10μM was higher than inhibition of oxygen uptake of mycelia at the same concentration. These experiments demonstrated that as physical barriers

Figure7.1 As physical barriers between the fungicide and the active site increase, (from solubilised mitochondria through to intact plants), so fungicide effectiveness decreases. More compound is required at each stage to achieve similar levels of inhibition. This is exacerbated by problems with compound metabolism and biostability, resulting in large discrepancies between *in planta* and *in vitro* test data.





Molarity of Axozystrobin causing inhibition of *B. cinerea* growth (plants & agar plates), oxygen uptake (mycelia), viability (protoplasts) and cytochrome c reduction (mitochondria). Results are mean values.

between the active site and the fungicide increased, fungicide effectiveness decreased. More compound was required at each stage to achieve similar levels of inhibition. This, coupled with problems of compound metabolism and biostability resulted in large discrepancies between *in planta* and *in vitro* data. With the right assay, inhibition in protoplasts falls between that detectable in mycelia and that observed in mitochondrial enzyme preparations.

One of the potentially useful applications of protoplast technology within the agrochemical industry, must be within the development of high throughput biochemical screening. Current *in vitro* HTBS systems often involve dispensing fungal spores into microtitre plates and incubating these until mycelia grow to a sufficient concentration to allow testing to take place. There are several problems with this system. Firstly it is difficult to keep sterile during the growth stage, secondly growth is often uneven across the plate. Together these help to create a third problem, the time taken to reach the screening stage (G.Wayne [AgrEvo] - pers. comm).

The problems with sterility and uneven growth could be overcome were it possible to uniformly dispense flask-grown cultures into microtitre plates. However, this is virtually impossible to do as mycelia block up liquid dispensing systems. One solution has been to use yeast type pathogens, but there are few plant pathogens that exist in this form. *Saccharomyces cerevisiae* has been used as a model organism in HTBS, and whilst it has the advantage of having a fully sequenced genome and being well characterised at the molecular level (Corran *et al.*, 1998), *S. cerevisiae* is not a plant pathogen, nor a filamentous fungus. In tests, only 44% of compounds that showed *in vitro* activity against representative plant pathogenic fungi also inhibited *S. cerevisiae* at the same concentrations (Corran *et al*, 1998).

Further to these problems, *S. cerevisiae* is one of the few eukaryotic organisms in which the chytochrome bc1 complex is not an obligatory component of the mitochondrial respiratory chain (Schmitt & Trumpower, 1990). This has obvious

implications when using this organism to screen compounds that work by inhibiting the bc1 complex - this is particularly relevant at present as such compounds are proving an important area for research and development.

Protoplasts offer a representation of the adult mycelium in a dispensable form and for this reason, they could be important in HTBS. Problems with sterility and concentration uniformity would reduced, and real plant pathogenic filamentous fungi could be used instead of less valuable model organisms. One obvious important practical consideration would be the high cost of protoplasting enzymes. However, on site manufacture (or recovery/recycling) of such enzymes might prove possible, and this would help to keep costs down.

Another important aspect of using protoplasts in HTBS would be how to detect fungicide activity, and this may depend on the target site for inhibition. For example, the cell proliferation reagent WST-1 (Boehringer Mannheim Roche) is a colourimetric assay for the quantification of cell proliferation based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. This reagent effectively allows fungal respiration to be monitored over a period of several hours, where inhibition of respiration results in a 'flat-line' response, whilst inhibition by a different route can be seen as a gradual reduction in viability.

Measuring protoplast respiration in an oxygen electrode system was not as sensitive as measuring viability with a fluorescent probe (Chapters 4 & 6). WST-1 might be a useful and more sensitive alternative. Using fluorimetric or colourimetric dyes in a HTBS system, with protoplasts as the dispensable fungal material, could be an important new development. Protoplasts might be more suitable for this than intact mycelia, with dyes proving easier to load into wall-free cells (A. Daniels [AgrEvo] pers. comm.). A further potential application of protoplasts in HTBS would be the investigation of cell wall synthesis as a potential target. Given the correct conditions,

cell wall synthesis enzymes would be abundant in protoplast systems, and could therefore offer advantages over the use of intact mycelia.

The work presented in this thesis constitutes a step towards using fungal protoplasts in new compound screening procedures, structure optimisation processes and mode of action investigations within the agrochemical industry. As further advances in compound synthesis are made, and more genomic information becomes available, *in vitro* high throughput biochemical screening is likely to become more and more important as a lead finding tool. Within this field, protoplasts from real plant pathogens may offer distinct advantages over other forms of fungal material, and could prove to be an important tool in an integrated, multi-disciplinary approach to developing new, effective fungicides.

References

Agrios, G.N. (1988) *Plant Pathology*. 3rd Edition. Academic Press, New York.

Andres, I. and Peberdy, J.F. (1974) Release of invertase from *Aspergillus nidulans* by the action of lytic enzyme and invertase secretion by regenerating protoplasts. *Microbios* **11**, 15-20.

Aoyagi, H. and Tanaka, H. (1994) Measurement of viable plant-cell and protoplast concentrations with a specialized fluorimeter. *Journal of Fermentation and Bioengineering* **77** (5), 517-521.

Bailey A.M., Mena, G.L. and Herrera-Estrella, L. (1991) Genetic transformation of the plant pathogens *Phytophthora capsici* and *Phytophthora parasitica*. *Nucleic Acids Research* **19** (15), 4273-4278.

Barnard, C., Gilbert, F. and McGregor P. (1993) *Asking Questions in Biology*. Longman Scientific and Technical, UK.

Bartnicki-Garcia, S. (1968) Cell wall chemistry and taxonomy of fungi. *Annual Review of Microbiology* **22**, 87-108.

Becher, D., Wedler, H., Schulze, H., Bode, R., Kasuske, A. and Samsonova, I. (1991) Correlation of biochemical blocks and genetic lesions in leucine auxotrophic strains of the imperfect yeast *Candida maltosa*. *Molecular and General Gentics* **227**, 361-368.

Bennett, J.W. (1985) Protoplasts and secondary metabolism. In: *Fungal Protoplasts, Applications in Biochemistry and Genetics*. J.F. Peberdy and L. Ferenczy (Eds). Marcel Dekker, Inc. New York. pp 189-203.

Bianchi, V. and Fortunati, E. (1990) Cellular effects of an anionic surfactant detected in V79 fibroblasts by different cytotoxicity tests. *In Vitro Toxicology* **4** (1), 9-16.

Bjurman, J. (1993) Determination of microbial activity in molded wood by the use of fluorescein diacetate. *Material und Organismen* **28** (1), 1-16.

Braun, P.J. and Heisler, A. (1990) Isolation and cell wall regeneration of protoplasts from *Botrytis cinerea* pers. *Journal of Phytopathology* **128**, 293-298.

Brown, A.G.P. and Rosielle, A.A. (1980) Prospects for control of *Septoria*. *Journal of Agriculture* **21**, 8-11.

Butt, T.M., Hoch, H.C., Staples, R.C. and Leger, R.J. (1989) Use of fluorochromes in the study of fungal cytology and differentiation. *Experimental Mycology* **13**, 303-320.

Carlile, W.R. (1995) *Control of Crop Disease*. Second Edition, Cambridge University Press.

Caux, P.Y., Weinberger, P. and Szabo, A. (1993) Effects of pesticide adjuvants on membrane lipid composition and fluidity in *Lemna minor*. *Canadian Journal of Botany* **71**, 1291-1297.

Coley-Smith, J.R., Verhoeff, K. and Jarvis, W.R. [Eds.] (1980) *The Biology of Botrytis*. Academic Press.

Cooke, B.M. and Jones, D.G. (1970) The effect of near ultraviolet irradiation and agar medium on the sporulation of *Septoria nodorum* and *S. tritici*. *Transactions of the British Mycological Society* **54**, 221-226.

Cooley, R.N., Shaw, R.K., Franklin, F.C.H. and Caten, C.E. (1988) Transformation of the phytopathogenic fungus *Septoria nodorum* to Hygromycin B resistance. *Current Genetics* **13**, 383-389.

Cooley, R.N., Franklin, F.C.H. and Caten, C.E. (1990) Cotransformation in the phytopathogenic fungus *Septoria nodorum*. *Mycological Research* **94** (2), 145-151.

Cooley, R.N., van Gorcom, R.F.M., van der Hondel, C.A.M.J.J. and Caten, C.E. (1991) Isolation of a benomyl-resistant allele of the β-tubulin gene from *Septoria nodorum* and its use as a dominant selectable marker. *Journal of General Microbiology* **137**, 2085-2091.

Corran, A.J., Renwick, A. and Dunbar, S.J. (1998) Approaches to *in-vitro* lead generation for fungicide invention. *Pesticide Science* **54**, 338-344.

Cunfer, B.M. and Johnson, J.W. (1981) Relationship of glume blotch symptoms on wheat heads to seed infection by *Septoria nodorum*. *Transactions of the British Mycological Society* **76**, 205-211.

Dancer, J., Schulz, Peine, T. and Wright, K. (1998) The value of biochemistry in pesticide discovery research. *Pesticide Outlook* (June), 36-41.

Davis, B. (1985) Factors influencing protoplast isolation. In: *Fungal Protoplasts, Applications in Biochemistry and Genetics*. J.F. Peberdy and L. Ferenczy (Eds). Marcel Dekker, Inc. New York. pp 171-187.

De Ruiter, H. and Meinen, E. (1996) Adjuvant increased glyphosate uptake by protoplasts isolated from quackgrass *Elytrigia repens* (L) nevski. *Weed Science* **44**, 38-45.

De Vries, O.M.H. and Wessels, J.G.H. (1972) Release of protoplasts from *Schizophyllum commune* by a lytic enzyme preparation from *Trichoderma viride*. *Journal of General Microbiology* **73**, 13-22.

De Vries, O.M.H. and Wessels, J.G.H. (1973) Release of protoplasts from *Schizophyllum commune* by combined action of purified α-1,3-glucanase and chitinase derived from *Trichoderma viride*. *Journal of General Microbiology* **76**, 319-330.

De Vries, O.M.H. and Wessels, J.G.H. (1975) Chemical analysis of cell wall regeneration and reversion of protoplasts from *Schizophyllum commune*. *Archives of Microbiology* **102**, 209-218.

Dowley, L.J. and O'Sullivan, E. (1991) Changes in the distribution of phenylamide-resistant strains of *Phytophthora infestans* (Mont.) de Bary in Ireland 1985-89. *Potato Research* **34**, 67-69.

Emerson, S. and Emerson, M.R. (1958) Production, reproduction and reversion of protoplast-like structures in the osmotic strain of *Neurospora crassa. Proceedings of the National Academy of Sciences USA* **44**, 668-671.

Elad, Y., Yunis, H. and Katan, T. (1992) Multiple fungicide resistance to benzimidazoles, dicarboximides and diethofencarb in field isolates of *Botrytis cinerea* in *Israel. Plant Pathology* **41**, 41-46.

Ersek, T., English, J.T. and Schoelz, J.E. (1995) Creation of species hybrids of *Phytophthora* with modified host ranges by zoospore fusion. *Phytopathology* **85**, 1343-1347.

Farkaš, V. (1985) The fungal cell wall. In: *Fungal Protoplasts, Applications in Biochemistry and Genetics.* J.F. Peberdy and L. Ferenczy (Eds). Marcel Dekker, Inc. New York. pp 171-187.

Faulkner, M.J. and Colhoun, J. (1976) Aerial dispersal of pycnidiospores of *Leptosphaeria nodorum. Phytopathologische Zeitschrift* **86**, 357-360.

Fritz, R., Lanen, C., Colas, V. and Leroux, P. (1997) Inhibition of methionine biosynthesis in *Botrytis cinerea* by the anilinopyrimidine fungicide pyrimethanil. *Pesticide Science* **49**, 40-46.

Gokhale, D.V. (1992) Fungal protoplast fusion - a tool for breeding industrial strains. *Journal of Scientific and Industrial Research* **51**, 497-506.

Hamlyn, P.F., Bradshaw, R.E., Mellon, F.M., Santiago, C.M., Wilson, J.M. and Peberdy, J.F. (1981) Efficient protoplast isolation from fungi using commercial enzymes. *Enzyme and Microbial Technology* **3**, 321-325.

Haughland, R.P. (1996) *Handbook of Fluorescent Probes and Research Chemicals*, 6th Edition. Molecular Probes Inc., Eugene, Oregon, USA.

Helenius, A. and Simons, K. (1975) Solubilization of membranes by detergents. *Biochimica et Biophysica Acta* **415**, 29-79.

Hollingworth, R.M. and Ahammadsahib, K.I. (1995) Inhibitors of respiratory complex I: mechanisms, pesticidal actions ans toxicology. *Review of Pesticide Toxicology* **3**, 277-302.

Holmes, S.J.I. and Colhoun, J. (1970) *Septoria nodorum* as a pathogen of barley. *Transactions of the British Mycological Society* **53**, 321-325.

Horsten, J. and Fehrmann, H. (1980) Fungicide resistance of *Septoria nodorum* and *Pseudocercosporella herpotrichoides*. 1. Effect of fungicide application on the frequency of resistant spores in the field. *Journal of Plant Diseases and Protection* **87**, 439-453.

Huang, C.N., Cornejo, M.J., Bush, D.S. and Jones, R.L. (1986) Estimating viability of plant protoplasts using double and single staining. *Protoplasma* **135**, 80-87.

Isaac, S. (1978) Biochemical properties of protoplasts from *Aspergillus nidulans.* Ph.D. thesis. University of Nottingham, Nottingham, England.

Isaac, S. (1985) Metabolic properties of protoplasts. In: *Fungal Protoplasts, Applications in Biochemistry and Genetics.* J.F. Peberdy and L. Ferenczy (Eds). Marcel Dekker, Inc. New York. pp 171-187.

James, C. (1998) Global Food Security. In: *Proceedings of the 7th International Congress of Plant Pathology.* Volume 1, 4.198.

Jayapal, V., Sharmila, K.M., Selvibal, G., Thyagaranjan, S.P., Shanmugasundaram, N. and Subramanian, S. (1991) Fluorescein diacetate and ethidium-bromide staining to determine the viability of *Mycobacterium-smegmatis* and *Escherichia-coli. Leprosy Review* **62** (3), 310-314.

Judelson, H.S. and Michelmore, R.W. (1991) Transient expression of genes in the Oomycete *Phytophthora infestans* using *Bremia lactucae* regulatory sequences. *Current Genetics* **19**, 453-459.

Judelson, H.S., Tyler, B.M. and Michelmore, R.W. (1991) Transformation of the Oomycete pathogen *Phytophthora infestans. Molecular Plant-Microbe Interactions* **4** (6), 602-607.

Judelson, H.S. (1993) Intermolecular ligation mediates efficient cotransformation in *Phytophthora infestans*. *Molecular and General Genetics* **239**, 241-250.

Judelson, H.S., Dudler, R., Pieterse, C.M.J., Unkles, S.E. and Michelmore, R.W. (1993) Expression and antisense inhibition of transgenes in *Phytophthora infestans* is modulated by choice of promoter and position effects. *Gene* **133**, 63-69.

Kapteyn, J.C., Milling, R.J., Simpson, D.J. and De Waard, M.A. (1994) Inhibition of sterol biosynthesis in cell-free extracts of *Botrytis cinerea* by Prochloraz and Prochloraz analogues. *Pesticide Science* **40**, 313-319.

King, J.E., Cook, R.J. and Melville, S.C. (1983) A review of *Septoria* diseases of wheat and barley. *Annals of Applied Biology* **103**, 345-373.

Kinghorn, J.R.., Moon, R.P., Unkles, S.E. and Duncan J.M. (1991) Gene structure and expression in *Phytophthora infestans* and the development of gene mediated transformation. In: *Phytophthora* J.A. Lucas., R.C. Shattock., D.S. Shaw. and L.R. Cooke (Eds.) British Mycological Society. Cambridge University Press pp. 295-311.

Knight, S.C., Anthony, V.M., Brady, A.M., Greenland, A.J., Heaney, S.P., Murray, D.C., Powell, K.A., Schulz, M.A., Spinks, C.A., Worthington, P.A. and Youle, D. (1997) Rationale and Perspectives on the development of Fungicides. *Annual Review of Phytopathology* **35**, 349-372.

Koeller, W. (1998) Mode of action of fungicides in real plant pathogens. In: *Proceedings of the 7th International Congress of Plant Pathology.* Volume 1, 5.5.1S.

Köhl, J., Molhoek, W.M.L., van der Plas, C.H. and Fokkema, N.J. (1995) Suppression of sporulation of *Botrytis* spp. as a valid biocontrol strategy. *European Journal of Plant Pathology* **101**, 251-259.

Lucas, J.A. (1998) *Plant Pathology and Plant Pathogens.* Third Edition, Blackwell Science.

Maeda, Y., Tanaka, K., Koga, Y., Zhang, X.Y., Sasaki, M., Kimura, G. and Nomoto, K. (1993) A simple quantitiative *in vitro* assay for thymocyte adhesion to thymic epithelial cells using fluorescein diacetate. *Journal of Immunological Methods* **157** (1-2), 117-123.

Milling, R.J. and Richardson, C.J. (1995) Mode of action of the anilino-pyrimidine fungicide pyrimethanil. 2. Effects on enzyme secretion in *Botrytis cinerea. Pesticide Science* **45**, 43-48.

Miura, I., Kamakura, T., Maeno, S., Nagata, T., Hayashi, S. and Yamaguchi, I. (1994) Effect of mepanipyrim on uptake of various substrates and macromolecular biosyntheses in *Botrytis cinerea. Journal of Pesticide Science* **19**, 103-109.

Mizutani, A., Yukioka, H., Tamura, H., Miki, N., Masuko, M. and Takeda, R. (1995) Respiratory characteristics in *Pyricularia oryzae* exposed to a novel alkoxyiminoacetamide fungicide. *Phytopathology* **85** (3), 306-311.

Morgan, A.J. (1983) Yeast strain improvement by protoplast fusion and transformation. *Experimentia Supplement* **46**, 155-166.

Necas, O. and Svoboda, A. (1985) Cell wall regeneration and protoplast reversion. In: *Fungal Protoplasts, Applications in Biochemistry and Genetics*. J.F. Peberdy and L. Ferenczy (Eds). Marcel Dekker, Inc. New York. pp 171-187.

Newbury, J. and Peberdy, J.F. (1996) Characterization of the heat shock response in protoplasts of *Aspergillus nidulans*. *Mycological Research* **100** (11), 1352-1332.

Newton, A.C. and Caten, C.E. (1988) Auxotrophic mutants of *Septoria nodorum* isolated by direct screening and by selection for resistance to chlorate. *Transcations of the British Mycological Society* **90** (2), 199-207.

Neyts, J., Snoeck, R., Schols, D., Himpens, B. and DeClercq, E. (1991) Sensitive, reproducible and convenient fluorometric assay for the in vitro evaluation of anticytomegalovirus agents. *Journal of Virological Methods* **35** (1), 27-38.

Parry, D.W. (1990) *Plant Pathology in Agriculture*. Cambridge University Press.

Peberdy, J.F. (1972) Protoplasts from fungi. *Sci. Prog. Oxford* **60**, 73-85.

Peberdy, J.F. (1976) Isolation and properties of protoplasts from filamentous fungi. In: *Microbial and Plant Protoplasts*. J.F. Peberdy; A.H. Rose; H.J. Rogers and E.C. Cocking (Eds.) Academic Press, New York, pp. 39-51.

Peberdy, J.F. (1978) Protoplasts and their development. In: *The Filamentous Fungi*. Vol 3. J.E. Smith and D.R. Berry (Eds.). Edward Arnold, London. pp 119-131.

Peberdy, J.F. (1979)a. Fungal protoplasts: Isolation, reversion and fusion. *Annual Review of Microbiology* **33**, 21-39.

Peberdy, J.F. (1979)b. Wall biogenesis in protoplasts. In: *Fungal Walls and Hyphal Growth*. J.H. Burnett and A.P.J. Trinci (Eds.) Cambridge University Press pp. 49-70.

Platt, H.W. (1994) Foliar application of fungicides affects occurrence of potato tuber rots caused by four foliar pathogens. *Canadian Journal of Plant Pathology* **16**, 341-346.

Ragsdale, N.N. and Sisler, H.D. (1970) Metabolic effects related to the fungitoxicity of carboxin. *Phytopathology* **60**, 1422.

Reglinski, T., Lyon, G.D. and Newton, A.C. (1995) The control of *Botrytis cinerea* and *Rhizoctonia solani* on lettuce using elicitors extracted from yeast cell walls. *Journal of Plant Diseases and Protection* **102** (3), 257-266.

Richards, W.C. (1994) An enzyme system to liberate spore and mycelial protoplasts from a dimorphic fungal plant pathogen *Ophiostoma ulmi* (buism.) nannf. *Physiological and Molecular Plant Pathology* **44**, 311-319.

Rosen, M.J. (1978) *Surfactants and interfacial phenomena.* John Wiley and Sons.

Royle, D.J., Shaw, M.W. and Cook, R.J. (1986) Patterns of development of *Septoria nodorum* and *S. tritici* in some winter wheat crops in Western Europe, 1981-1983. *Plant Pathology* **35**, 466-476.

Santos, M., Vallejo, I., Rebordinos, L., Gutiérrez, S., Collado, I.C. and Cantoral, J.M. (1996) An autonomously replicating plasmid transforms *Botrytis cinerea* to phleomycin resistance. *FEMS Microbiology Letters* **137**, 153-158.

Schmidt, K. (1995) Whatever happened to the gene revolution? *New Scientist* **145**, 21-25.

Schmitt, M.E. and Trumpower, B.L. (1990) Subunit 6 regulates half-of-the-sites reactivity of the dimeric cytochrome bc1 complex in *Saccharomyces cerevisiae*. *The Journal of Biological Chemistry* **265** (28), 17005-17011.

Scott, P.R., Benedikz, P.W., Jones, H.G. and Ford, M.A. (1985) Some effects of canopy structure and microclimate on infection of tall and short wheats by *Septoria nodorum*. *Plant Pathology* **34**, 578-53.

Shirane, N. and Hatta, T. (1986) Isolation of protoplasts from *Botrytis cinerea*. *Ann. Phytopath. Soc. Japan* **52**, 721-724.

Sietsma, J.H., Beth Din, A., Ziv, V., Sjollema, K.A. and Yarden, O. (1996) The localization of chitin synthase in membraneous vesicles (chitosomes) in *Neurospora crassa*. *Microbiology* **142**, 1591-1596.

Sinvan, A., Vanmoppes, D. and Arad, S.M. (1992) Protoplasts production from the unicellular red alga *Porphyridium* sp. (utex 637) by an extracellular bacterial lytic preparation. *Phycologia* **31** (3-4), 253-261.

Smith, D. and Onions, A.H.S. (1983) *The Maintenance and Preservation of Living Fungi*. Commonwealth Agricultural Bureaux, Slough, UK.

Sorensen, T.K, Wallis, G.L.F. and Peberdy, J.F. (1996) Fungal protoplasts as tools for studies on protein secretion. *Biotechnology Letters* **18** (12), 1375-1380.

Stock, D. (1996) Achieving optimal biological activity from crop protection formulations: design or chance? In: *Brighton Crop Protection Conference Proceedings* volume **3**, 791-800.

The Pesticide Manual. 11th Edition. (1997) C.D.S. Tomlin (Ed.) British Crop Protection Council, Surrey, UK.

Tilburn, J., Scazzocchio, C., Taylor, G.J., Zabicky-Zissman, J.H., Lockington, R.A. and Davies, W. (1983) Transformation by integration in *Aspergillus nidulans. Gene* **26**, 205-221.

United Nations. (1994) *World Population Prospects: the 1994 Revision Annex Tables.* New York, UN.

Vagvolgyi, C. and Ferenczy, L. (1991) Isolation of nuclei from *Aspergillus nidulans* protoplasts. *FEMS Microbiological Letters* **82**, 247-251.

Villanueva, J.R. and Garcia-Archa, I. (1971) Production and use of fungal protoplasts. In: *Methods in Microbiology*, Vol. 4. C. Booth (Ed.). Academic Press, New York, pp. 665-718.

Voet, D. and Voet, J.G. (1995) *Biochemistry.* 2nd Edition. John Wiley and Sons.

Walker, D.A. (1985) Measurement of oxygen and chlorophyll fluorescence. In: *Techniques in Bioproductivity and Photosynthesis*. Coombs, J., Hall, D.O., Long, S.P and Scurlock J.M.O. (Eds.) Pergamon Press, Oxford, UK, pp 95-106.

Walker, D.A. (1990) *The use of the oxygen electrode and fluorescent probes in simple measurements of photosynthesis*. 2nd impression. Oxygraphics Ltd., Sheffield, UK.

Wostemeyer, A. and Wostemeyer J. (1998) Fungal protoplasts: relics or modern objects of molecular research? *Microbiological Research* **153**, 97-104.

Zimmermann, K. (1995) An investigation into the efficacy and mode of action of fenpiclonil and fludioxonil using protoplasts, spores and mycelium of *Fusarium culmorum* and *Botrytis cinerea*. Nottingham Trent University, undergraduate degree thesis.