STUDIES ON THE INHIBITION OF PLANT PATHOGENIC FUNGI BY CYCLOHEXYLAMINE AND SPERMIDINE ANALOGUES

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STUDIES ON THE INHIBITION OF PLANT PATHOGENIC FUNGI BY CYCLOHEXYLAMINE AND SPERMIDINE ANALOGUES

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for the degree of Doctor of Philosophy in respect of research carried out
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by

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CONTENTS

Acknowledgements i
Dedication v
Publications vi

List of Tables vii
List of Figures x
List of Plates xi

Summary xii

Chapter 1 INTRODUCTION

1.1 Review of literature 2
1.1.1 Polyamines: an introduction 2
1.1.2 Functions of polyamines 2
1.1.2.1 Cell proliferation 4
1.1.2.2 Cell differentiation and organism growth 5
1.1.2.3 Regulation of stress 6
1.1.2.4 Regulation of senescence 7
1.1.2.5 DNA methylation 8
1.1.2.6 Protein synthesis and phosphorylation 9
1.1.3 Biosynthesis and regulation of intracellular polyamines 10
1.1.3.1 Polyamine biosynthesis 10
1.1.3.2 Regulation of polyamine biosynthetic enzymes 12
1.1.3.3 Other means of controlling intracellular polyamine levels 14
1.1.4 Inhibition of polyamine biosynthesis 17
1.1.4.1 Inhibitors of ODC 17
1.1.4.2 Inhibitors of AdoMetDC 18
1.1.4.3 Inhibitors of spermidine synthase 19
1.1.4.4 Inhibitors of spermine synthase 20
1.1.4.5 Inhibitors of ADC 20
1.1.4.6 Polyamine analogues 20
1.1.5 Cancer research 23
1.1.6 Protozoal, bacteria, animal-infecting fungi and insecticide research 26
1.1.6.1 Protozoal infections 26
1.1.6.2 Bacteria 29
1.1.6.3 Animal-infecting fungi 30
1.1.6.4 Insecticides 32
1.1.7 Fungal research 33
1.1.7.1 Inhibitors of ODC 33
1.1.7.2 Inhibitors of AdoMetDC 39
1.1.7.3 Inhibitors of spermidine synthase 41
1.1.7.4 Inhibitors of spermine synthase 41
1.1.7.5 Inhibitors of ADC 41
1.1.7.6 Polyamine analogues 43
1.1.8 Fungal research - further considerations 48
1.1.8.1 Effects of polyamine biosynthetic inhibitors on plants 48
1.1.8.2 Effects of polyamine biosynthetic inhibitors on mycorrhizal fungi 48
1.1.8.3 Control of plant pathogenic fungi - an overview 49
1.2 Aim of study 51

Chapter 2 Effect of Cyclohexylamine and Norspermidine on Fungal Infection of Host Plants

2.1 Introduction and Objectives 53
2.1.1 Broad bean rust 53
2.1.2 Broad bean chocolate spot 53
2.1.3 Barley powdery mildew 55
2.1.4 Apple powdery mildew 55
2.1.5 Potato late blight 56

2.2 Materials and Methods 57
2.2.1 Effect of CHA and norspermidine against rust on broad bean 57
2.2.1.1 Growth of plant material 57
2.2.1.2 Maintenance of pathogen 57
2.2.1.3 Inoculation of plant material and treatment with CHA and norspermidine 57
2.2.2 Effect of CHA and norspermidine against chocolate spot on broad bean 58
2.2.2.1 Growth of plant material 58
2.2.2.2 Maintenance of pathogen 58
2.2.2.3 Inoculation of plant material and treatment with CHA and norspermidine 58
2.2.3 Effect of CHA and norspermidine against powdery mildew on barley 58
2.2.3.1 Growth of plant material 58
2.2.3.2 Maintenance of pathogen 58
2.2.3.3 Inoculation of plant material and treatment with CHA and norspermidine 59
2.2.4 Effect of CHA and norspermidine against powdery mildew on apple 59
2.2.4.1 Growth of plant material 59
2.2.4.2 Maintenance of pathogen 59
2.2.4.3 Inoculation of plant material and treatment with CHA and norspermidine 59
2.2.5 Effect of CHA and norspermidine against late blight on potato 60
2.2.5.1 Growth of plant material 60
2.2.5.2 Maintenance of pathogen 60
2.2.5.3 Inoculation of plant material and treatment with CHA and norspermidine 60

2.3 Results 62
2.3.1 Effect of CHA and norspermidine against rust on broad bean 62
2.3.2 Effect of CHA and norspermidine against chocolate spot on broad bean 62
2.3.3 Effect of CHA and norspermidine against powdery mildew on barley 62
2.3.4 Effect of CHA and norspermidine against powdery mildew on apple 63
2.3.5 Effect of CHA and norspermidine against late blight on potato 63

2.4 Effects of CHA and norspermidine on germination and development of rust uredospores on broad bean leaves 68
2.4.1 Introduction and Objectives 68
2.4.2 Materials and Methods 68
2.4.3 Results 68

2.5 Discussion 72

Chapter 3 EFFECT OF CYCLOHEXYLAMINE AND SPERMIDINE ANALOGUES ON THE GROWTH OF PLANT PATHOGENIC FUNGI *IN VITRO*

3.1 Effect of CHA and spermidine analogues on mycelial growth of fungi on solid media 76
3.1.1 Introduction and Objectives 76
3.1.2 Materials and Methods 76
3.1.2.1 Maintenance of pathogens 76
3.1.2.2 Growth of fungi on inhibitor amended solid media 76
3.1.3 Results 78
3.1.3.1 Effects of CHA and spermidine analogues on mycelial growth of *Pyrenophora avenae* 78
3.1.3.2 Effects of CHA and norspermidine on mycelial growth of *Pyricularia oryzae* 79

3.2 Effect of CHA and spermidine analogues on growth of *Pyrenophora avenae* in liquid culture 85
3.2.1 Introduction and Objectives 85
3.2.2 Materials and Methods 85
3.2.2.1 Maintenance of pathogen 85
3.2.2.2 Growth of *Pyrenophora avenae* in inhibitor amended liquid media 85
3.2.3 Results 86

3.3 Effect of CHA and spermidine analogues on cell lengths of *Pyrenophora avenae* grown on solid media 91
3.3.1 Introduction and Objectives 91
3.3.2 Materials and Methods 91
3.3.3 Results 91

3.4 Discussion 93

Chapter 4 EFFECT OF CYCLOHEXYLAMINE AND SPERMIDINE ANALOGUES ON BIOSYNTHESIS AND CATABOLISM OF POLYAMINES IN *PYRENOPHORA AVENAE*

4.1 Initial determination of the effects of CHA and spermidine analogues on polyamine biosynthesis in *Pyrenophora avenae* 96
4.1.1 Introduction and Objectives 96
4.1.2 Materials and Methods 96
4.1.2.1 Growth of fungal material 96
4.1.2.2 ODC assay 96
4.1.2.3 AdoMetDC assay 97
4.1.2.4 Incorporation of radio-labelled ornithine into polyamines 97
4.1.2.5 Analysis of free polymines in fungal tissue 98
4.1.3 Results 99
4.1.3.1 ODC and AdoMetDC activities 99
4.1.3.2 Incorporation of radio-labelled ornithine into polyamines 99
4.1.3.3 Analysis of free polyamines in fungal tissue 99

4.2 Further determination of the effects of CHA and norspermidine on biosynthesis and catabolism of polyamines in *Pyrenophora avenae* 103
4.2.1 Introduction and Objectives 103
4.2.2 Materials and Methods 104

4.2.2.1 Growth of fungal material 104
4.2.2.2 PAO assay 104
4.2.2.3 DAO assay 104
4.2.2.4 Analysis of acetylated polyamines in fungal tissue 105
4.2.3 Results 107
4.2.3.1 PAO and DAO activities 107
4.2.3.2 Analysis of acetylated polyamines in fungal tissue 107

4.3 Determination of the effects of higher concentrations of CHA and norspermidine on polyamine biosynthesis in *Pyrenophora avenae* 109
4.3.1 Introduction and Objectives 109
4.3.2 Materials and Methods 109
4.3.3 Results 110
4.3.3.1 Effect of 4 days exposure to higher concentrations of CHA and norspermidine on growth of *Pyrenophora avenae* 110
4.3.3.2 Effect of 1 day exposure to higher concentrations of CHA and norspermidine on growth of *Pyrenophora avenae* 110
4.3.3.3 Incorporation of radio-labelled ornithine into polyamines 110
4.3.3.4 Analysis of free polyamines in fungal tissue 110

4.4 Further determination of the effects of higher concentrations of CHA and norspermidine on biosynthesis and catabolism of polyamines in *Pyrenophora avenae* 114
4.4.1 Introduction and Objectives 114
4.4.2 Materials and Methods 115
4.4.2.1 PAO activity 115
4.4.2.2 Analysis of acetylated polyamines in fungal tissue 115
4.4.2.3 Formation of acetylated polyamines 115
4.4.2.4 Determination of amine excretion 115
4.4.3 Results 116
4.4.3.1 PAO activity 116
4.4.3.2 Analysis of acetylated polyamines in fungal tissue 116
4.4.3.3 Formation of acetylated polyamines 116
4.4.3.4 Determination of amine excretion 116

4.5 Discussion 118

Chapter 5 EFFECT OF NOVEL SPERMIDINE ANALOGUES ON *IN VITRO* GROWTH AND POLYAMINE BIOSYNTHESIS IN *PYRENOPHORA AVENAE*

5.1 Introduction and Objectives 124

5.2 Effect of novel spermidine analogues on mycelial growth of *Pyrenophora avenae* on solid media 124
5.2.1 Materials and Methods 124
5.2.2 Results 124

5.3 Effect of novel spermidine analogues on growth of *Pyrenophora avenae* in liquid culture 130
5.3.1 Materials and Methods 130
5.3.2 Results 130

5.4 Effect of novel spermidine analogues on the incorporation of radio-labelled ornithine into polyamines in *Pyrenophora avenae* grown in liquid culture 131
5.4.1 Materials and Methods 131
5.4.2 Results 131

5.5 Discussion 133

Chapter 6 FIELD STUDIES

6.1 Introduction and Objectives 136
6.2 Materials and Methods 136
6.2.1 1995 Trial 136
6.2.2 1996 Trial 137
6.2.3 1996 Mini plots 138
6.3 Results 139
6.3.1 1995 Trial 139
6.3.2 1996 Trial 142
6.3.3 1996 Mini plots 145
6.4 Discussion 147

Chapter 7 EFFECT OF CYCLOHEXYLAMINE AND NORSPERMIDINE ON UPTAKE OF AMINO ACIDS, POLYAMINES AND SUGARS BY *PYRENOPHORA AVENAE*

7.1 Introduction 151
7.1.1 Review of literature 151
7.1.1.1 Transport mechanisms: an introduction 151
7.1.1.2 Transport mechanisms in fungi 152
7.1.1.2.1 Transport of sugars 152
7.1.1.2.2 Transport of amino acids 154
7.1.1.3 Transport of polyamines 155
7.1.1.4 Transport of other substances 156
7.1.1.5 Polyamine analogues 157
7.1.1.6 Factors affecting transport processes 158
7.1.2 Aim of studies 158

7.2 Materials and Methods 160
7.2.1 Experiment 1: Effect of CHA and norspermidine on the uptake of compounds by *Pyrenophora avenae* during a four hour period 160
7.2.2 Experiment 2: Effect of prolonged exposure to CHA and norspermidine on the uptake of compounds by *Pyrenophora avenae* during a four hour period 160
7.2.3 Experiment 3: Effect of higher concentrations of CHA and norspermidine on the uptake of compounds by *Pyrenophora avenae* during a 15 minute period 161
7.2.4 Experiment 4: Determination of uptake kinetics 161

7.3 Results 162
7.3.1 Experiment 1: Effect of CHA and norspermidine on the uptake of compounds by *Pyrenophora avenae* during a four hour period 162
7.3.2 Experiment 2: Effect of prolonged exposure to CHA and norspermidine on the uptake of compounds by *Pyrenophora avenae* during a four hour period 162
7.3.3 Experiment 3: Effect of higher concentrations of CHA and norspermidine on the uptake of compounds by *Pyrenophora avenae* during a 15 minute period 162
7.3.4 Experiment 4: Determination of uptake kinetics 165

7.4 Discussion 173

Chapter 8 HYPUSINE BIOSYNTHESIS

8.1 Introduction 179
8.1.1 Review of literature 179
8.1.1.1 Hypusine: an introduction 179
8.1.1.2 Biosynthesis of hypusine 179
8.1.1.3 Biological significance of eIF-5A and hypusine 179
8.1.1.4 Polyamine biosynthesis and hypusine 181
8.1.1.5 Inhibition of hypusine biosynthetic enzymes 183
8.1.1.5.1 Deoxyhypusine synthase 183
8.1.1.5.2 Deoxyhypusine hydroxylase 185
8.1.2 Aim of studies 185

8.2 Effect of various deoxyhypusine synthase inhibitors on mycelial growth of *Pyrenophora avenae* on solid media 187
8.2.1 Introduction and Objectives 187
8.2.2 Materials and Methods 187
8.2.3 Results 188

8.3 Effect of various compounds on the growth of *Pyrenophora avenae* in liquid culture 193
8.3.1 Introduction and Objectives 193
8.3.2 Materials and Methods 193
8.3.3 Results 194

8.4 Effect of various compounds on fungal hypusine and deoxyhypusine content 196
8.4.1 Introduction and Objectives 196
8.4.2 Materials and Methods 197
8.4.3 Results 199

8.5 Discussion 203

Chapter 9 GENERAL DISCUSSION 207

Chapter 10 BIBLIOGRAPHY 214

Chapter 11 APPENDICES 257

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The 1996 World Food Summitt, organised by The Food and Agriculture Organisation of the United Nations reported the following facts:

800 million people in the world are undernourished

200 million of those are children under the age of five

By 2030, the world population will have risen by 3 billion

With rising populations and reductions in availability of agricultural land, increased productivity is paramount.

The problem of hunger is, however, both a problem of production and distribution.

This thesis is dedicated to those farmers and scientists who strive to provide food for all, and to the politicians who should.

PUBLICATIONS

Parts of the work presented in this thesis appear in the following publications:

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LIST OF TABLES

1. Effect of CHA and norspermidine against powdery mildew on apple 67

2. Effect of CHA and norspermidine against late blight on potato 67

3. Effects of 2.0 mM CHA and 2.0 mM norspermidine on bean rust germling development on leaf surfaces 70

4. Effect of N^1- and N^8-acetylspermidine on mycelial growth of *Pyrenophora avenae* 80

5. Effect of varying concentrations of CHA and norspermidine on growth of *Pyrenophora avenae* in liquid culture for 4 days 81

6. Effect of 2.0 mM N^1- and N^8-acetylspermidine on growth of *Pyrenophora avenae* in liquid culture for 4 days 81

7. Effect of 1.0 mM CHA and 0.01 mM norspermidine on growth of *Pyrenophora avenae* in liquid culture for 2, 3 or 4 days 88

8. Effect of 0.5 mM N^1- and N^8-acetylspermidine on growth of *Pyrenophora avenae* in liquid culture for 2, 3 or 4 days 88

9. Effect of varying concentrations of CHA and spermidine analogues on cell lengths in *Pyrenophora avenae* grown on solid media for 8 days 92

10. Effect of CHA and spermidine analogues on ODC activity in *Pyrenophora avenae* grown for 2, 3 or 4 days 100

11. Effect of CHA and spermidine analogues on AdoMetDC activity in *Pyrenophora avenae* grown for 2, 3 or 4 days 100

12. Effect of CHA and spermidine analogues on the incorporation of radio-labelled ornithine into polyamines in *Pyrenophora avenae* grown for 2, 3 or 4 days 101

13. Effect of CHA and spermidine analogues on free polyamine levels in *Pyrenophora avenae* grown for 2, 3 or 4 days 102

14. Rf values of various compounds, developed on tlc plates using chloroform : isopropyl alcohol (95:5) (v/v) 106

15. Effect of 1.0 mM CHA on PAO and DAO activities in *Pyrenophora avenae* grown for 2, 3 or 4 days 108

16. Effect of 1.0 mM CHA and 0.01 mM norspermidine on acetylated polyamine levels in *Pyrenophora avenae* grown for 2, 3 or 4 days 108

17. Effect of 1 day exposure to higher concentrations of CHA and norspermidine on growth of *Pyrenophora avenae* 112

18. Effect of 1 or 4 day exposure to higher concentrations of CHA and norspermidine on the incorporation of radio-labelled ornithine into polyamines in *Pyrenophora avenae* 112

19. Effect of 1 day exposure to higher concentrations of CHA and norspermidine on free polyamine levels in *Pyrenophora avenae* 113

20. Effect of 4 day exposure to higher concentrations of CHA and norspermidine on free polyamine levels in *Pyrenophora avenae* 113

21. Effect of 1 or 4 day exposure to 2.75 mM CHA on PAO activity in *Pyrenophora avenae* 117

22. Effect of 1 or 4 day exposure to higher concentrations of CHA and norspermidine on acetylated polyamine levels in *Pyrenophora avenae* 117

23. Effect of 1 or 4 day exposure to 2.75 mM CHA on polyamine excretion from *Pyrenophora avenae* 117

24. Structures of novel spermidine analogues 125

25. Effect of novel spermidine analogues on growth of *Pyrenophora avenae* in liquid culture for 4 days 132

26. Effect of novel spermidine analogues on the incorporation of radio-labelled ornithine into polyamines in *Pyrenophora avenae* grown for 4 days 132

27. Effect of CHA, norspermidine, E-TED and a flutriafol and carbendazim mixture on powdery mildew infection of spring barley: 1995 Trial 140

28. Effect of CHA, norspermidine, E-TED and a flutriafol and carbendazim mixture on plant height, plant dry weight and grain dry weight of spring barley: 1995 Trial 141

29. Effect of CHA, norspermidine, a flutriafol and carbendazim mixture, and tebuconazole on powdery mildew infection of spring barley: 1996 Trial 143

30. Effect of CHA, norspermidine, a flutriafol and carbendazim mixture, and tebuconazole on plant height, plant dry weight and grain dry weight of spring barley: 1996 Trial 144

31. Effect of CHA, norspermidine and DFMO on powdery mildew infection of spring barley: 1996 Mini plots 146

32. Uptake of methionine, ornithine, polyamines and sugars by *Pyrenophora avenae* exposed to 1.0 mM CHA or 0.01 mM norspermidine for 2 days 163

33. Uptake of methionine, ornithine, polyamines and sugars by *Pyrenophora avenae* exposed to 1.0 mM CHA or 0.01 mM norspermidine for 4 hours 163

34. Uptake of polyamines by *Pyrenophora avenae* exposed to 1.0 mM CHA or 0.01 mM norspermidine for 4 days 164

35. Uptake of methionine and spermidine by *Pyrenophora avenae* exposed to 2.75 mM CHA or 0.2 mM norspermidine for 4 days or 15 minutes 164

36. Uptake of methionine by *Pyrenophora avenae* exposed to 2.75 mM CHA or 0.2 mM norspermidine for 4 days 167

37. Structures of deoxyhypusine synthase inhibitors 189

38. Effect of deoxyhypusine synthase inhibitors on mycelial growth of *Pyrenophora avenae* 190

39. Effect of various compounds on the growth of *Pyrenophora avenae* in liquid culture for 4 days 195

LIST OF FIGURES

1. Structures of some major polyamines 3

2. Biosynthetic pathways of the major plant polyamines 11

3. Polyamine interconversion pathways 16

4. Structures of spermidine, CHA and norspermidine 54

5. Effect of cyclohexylamine and norspermidine against rust on broad bean 64

6. Effect of cyclohexylamine and norspermidine against chocolate spot on broad bean 65

7. Effect of cyclohexylamine and norspermidine against powdery mildew on barley 66

8. Structures of spermidine, N^1-acetylspermidine and N^8-acetylspermidine 77

9. Effect of cyclohexylamine and norspermidine on mycelial growth of *Pyrenophora avenae* 81

10. Effect of cyclohexylamine and norspermidine on mycelial growth of *Pyricularia oryzae* 82

11. Effect of C59 and C73 on mycelial growth of *Pyrenophora avenae* 126

12. Effect of LAS 2/11 and LAS 2/12 on mycelial growth of *Pyrenophora avenae* 127

13. Effect of LAS 2/26 and LAS 2/27 on mycelial growth of *Pyrenophora avenae* 128

14. Effect of LAS 2/28 and LAS 3/8 on mycelial growth of *Pyrenophora avenae* 129

15. Eadie-Hofstee plot: Vo against Vo/[S] (Control) 168

16. Eadie-Hofstee plot: Vo against Vo/[S] (CHA treatment - expanded scale) 169

17. Eadie-Hofstee plot: Vo against Vo/[S] (CHA treatment - original scale) 170

18. Eadie-Hofstee plot: Vo against Vo/[S] (norspermidine treatment - expanded scale) 171

19. Eadie-Hofstee plot: Vo against Vo/[S] (norspermidine treatment - original scale) 172

20. Biosynthesis of hypusine 180

21. HPLC Chromatograms of authentic hypusine and extract from *Pyrenophora avenae* 202

LIST OF PLATES

1. Effect of 2.0 mM CHA and 2.0 mM norspermidine on mycelial growth of *Pyrenophora avenae*, grown on solid media for eight days 83

2. Effect of 2.0 mM CHA and 2.0 mM norspermidine on mycelial growth of *Pyricularia oryzae*, grown on solid media for eight days 84

3. Effect of 1.0 mM CHA on growth of *Pyrenophora avenae* in liquid culture, grown for four days 89

4. Effect of 0.01 mM norspermidine on growth of *Pyrenophora avenae* in liquid culture, grown for four days 90

5. Effect of 1.0 mM concentrations of various deoxyhypusine synthase inhibitors on mycelial growth of *Pyrenophora avenae,* grown on solid media for six days. 191

6. Effect of varying concentrations of various deoxyhypusine synthase inhibitors on mycelial growth of *Pyrenophora avenae,* grown on solid media for six days. 192

SUMMARY

The potential inhibitory activity of several compounds against plant pathogenic fungal growth was studied. Prevention of growth was hypothesised to occur by the inhibition of polyamine biosynthesis and/or an effect on cellular functions associated with spermidine, the predominant polyamine in fungi.

The spermidine synthase inhibitor, cyclohexylamine (CHA), and the spermidine analogue, norspermidine, demonstrated protective, eradicant and systemic activity against a range of plant pathogenic infections of host plants: broad bean rust and chocolate spot, barley and apple powdery mildew, and potato late blight. For example, both 2.0 mM CHA and 2.0 mM norspermidine, applied pre inoculation, reduced powdery mildew infection of barley by 88 %. CHA and norspermidine were equally effective although apple powdery mildew and potato late blight infections were slightly more responsive towards norspermidine than CHA treatment. Against bean rust and barley powdery mildew, little increased efficacy was afforded by increasing inhibitor concentrations from 0.5 mM to 2.0 mM. Higher concentrations were, however, more effective against the other pathogenic infections examined. Pre inoculation treatments tended to be more effective than post inoculation applications of the inhibitors, although the reason for this is unclear as microscopic examination of bean rust spores and developing germlings on bean leaves found little difference between the treated leaves and the untreated control, suggesting the compounds acted on later stages of fungal development.

CHA and norspermidine were also effective against powdery mildew infection of spring barley in the field. The compounds were as effective as a commercially available fungicide in reducing disease. Neither the commercial fungicide nor the inhibitors caused any increase in yield over the untreated control, although this may have been the result of additional environmental pressures.

The compounds controlled mycelial growth of two plant pathogens, *Pyrenophora avenae* and *Pyricularia oryzae*, on artificial media and reduced fresh weight of *P. avenae* grown in liquid culture. Exposure of *P. avenae* for 4 days to 3.0 mM CHA reduced fungal growth by 93 % while 0.5 mM norspermidine completely inhibited growth. *In vitro* growth of the fungus was thus more responsive to norspermidine than CHA. Additional commercially available spermidine analogues, N^1- and N^8-acetylspermidine, had limited efficacy against mycelial growth of *P. avenae in vitro*, although antifungal activity was apparent when the fungus was grown in liquid culture in the presence of the analogues. Varying antifungal

activities were found for eight novel spermidine analogues although four compounds did indeed successfully inhibit growth of *P. avenae in vitro*.

Neither 1.0 mM CHA, 0.01 mM norspermidine nor 0.5 mM N^1- and N^8-acetylspermidine affected ODC, AdoMetDC or spermidine synthase activities in *P. avenae*. Free polyamine levels were unaffected, with the exception of treatment with CHA, which increased putrescine content. For example, exposure of fungus to 1.0 mM CHA for 4 days increased putrescine by 75 %. Higher concentrations of the inhibitors, 2.75 mM CHA and 0.2 mM norspermidine, caused similar effects on polyamine biosynthesis in *P. avenae*. Thus, even the most dramatic effects on growth were unlikely to have been caused by aberrant polyamine biosynthesis.

The cause of the CHA induced increase in putrescine concentration remains unclear. Increased catabolism of higher polyamines was unlikely to be responsible since PAO activity was unaffected. Reduced putrescine catabolism was similarly not responsible, since DAO activity was unaltered. Breakdown of conjugated N-acetylputrescine was also not likely to be the cause of the increased putrescine concentration. However the increase was caused, it is unlikely to wholly account for the antifungal activity of CHA.

CHA and norspermidine did cause effects on uptake of amino acids, polyamines and sugars into *P. avenae*. Indeed, exposure of fungus to the inhibitors for 4 days completely destroyed the methionine uptake system. Disruption of membrane function and permeability may have been responsible for the dramatic effects on the kinetics of methionine uptake by *P. avenae* caused by this pre exposure of fungus to CHA or norspermidine.

Known inhibitors of deoxyhypusine synthase successfully inhibited mycelial growth of *P. avenae in vitro*. Attempts to determine fungal hypusine content were, however, unsuccessful and it remains to be seen whether CHA and spermidine analogues exert their antifungal and fungicidal modes of action via perturbation of hypusine synthesis.

The study therefore showed that CHA and spermidine analogues possessed both antifungal and fungicidal activity against a range of plant pathogens. Although differences in *in vivo* and *in vitro* studies exist, perturbation of polyamine biosynthesis was unlikely to account for such activity of the compounds. The compounds were most likely exerting their effects via some other cellular function related to polyamines.

Chapter 1

Introduction

1. INTRODUCTION

1.1 REVIEW OF LITERATURE

1.1.1 POLYAMINES: AN INTRODUCTION

Antoni van Leeuwenhoek is credited with the discovery of polyamines when, in 1678, he observed crystals whilst studying human semen. It was not until 1878, after many unsuccessful attempts, that these crystals were identified as the phosphate salt of an organic base and ten years later, named as spermine. An additional 38 years then passed before the chemical structure of spermine was finally elucidated, simultaneously in England and Germany (Smith 1982).

Since then, a number of polyamines have been described. The term 'polyamine' is, however, a misnomer as the compounds are in actual fact aliphatic diamines, triamines and tetraamines. Such organic compounds have low molecular weights, are soluble in water and are cationic at physiological pH, (around neutrality). Some, for example, putrescine (1, 4-diaminobutane), spermidine (4 azaoctane-1, 8-diamine) and spermine (4, 9 diazadodecane-1, 12-diamine) are widely distributed (Stevens & Winther 1979). Indeed, no eukaryotic or prokaryotic cell has been found in which polyamines are not present. Putrescine and spermidine are most common with spermine being less well distributed, some bacteria and filamentous fungi being devoid of the compound (Stevens & Winther 1979). The diamine cadaverine has been found in organisms such as plants (Smith 1985), mammalian cells (Pegg & McGill 1979) and *Neurospora crassa* (Paulus *et al.* 1982), but its occurrence in nature is generally restricted (Figure 1).

1.1.2 FUNCTIONS OF POLYAMINES

Polyamines have been implicated in many physiological functions, although their exact role in many of these is still not well understood (Tabor & Tabor 1985). Nevertheless, some of these functions with which polyamines are associated shall be reviewed including cellular proliferation and differentiation, and organism growth and development. Much work has concluded that polyamines are involved in regulating stress and senescence of plants and two additional specific cellular functions relating to polyamines, DNA methylation and protein phosphorylation, shall also be reviewed. Some of the work in this area has utilised polyamine biosynthesis inhibitors and such compounds will be discussed in later parts of this review. Other work used mutants of organisms, incapable of polyamine biosynthesis, to prove the essentiality of polyamines for growth (Tabor & Tabor 1985). Many studies have, therefore, concluded that polyamines are vital for a range of different functions associated with cellular growth and development.

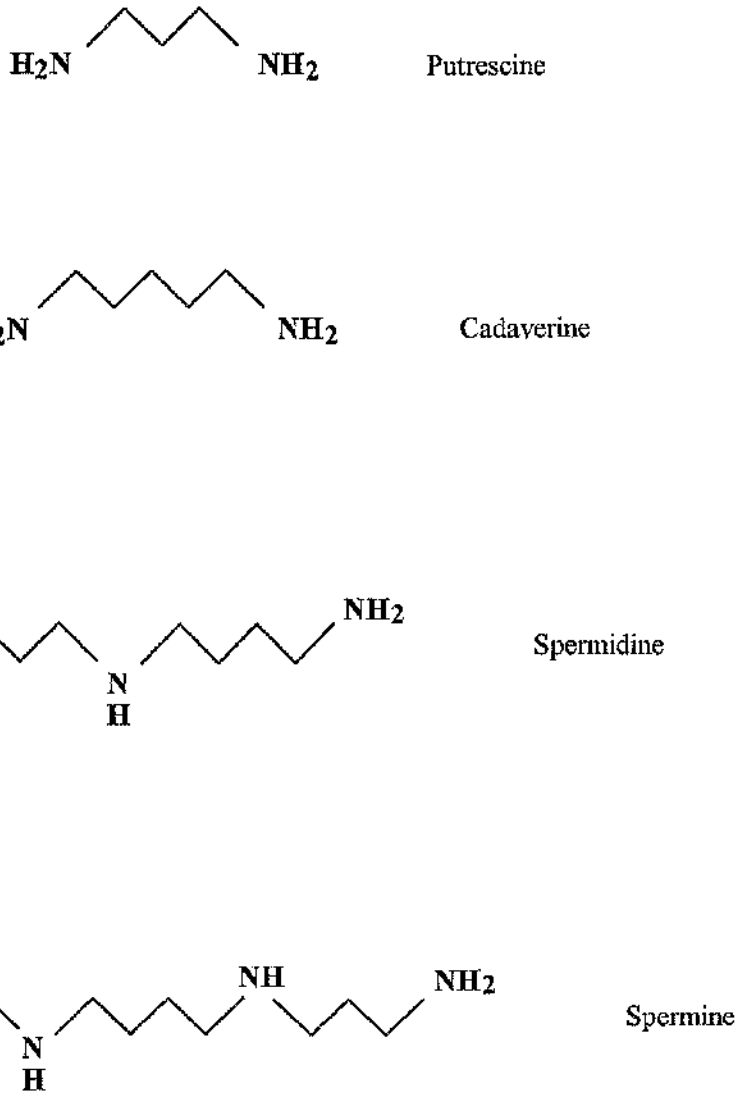


Figure 1. Structures of some major polyamines

1.1.2.1 Cell proliferation

Polyamines were initially associated with cellular proliferation in 1949 when Herbst and Snell observed that a factor in orange juice, later shown to be putrescine, was essential for growth of the bacterium *Hemophilus parainfluenzae* (Smith 1982). Since then, polyamines have been shown to stimulate cell division and growth in a number of bacteria, fungi, the beetle *Oryzaephilus*, tissue cultures of Chinese hamsters and tuber explants of Jerusalem artichoke (Tabor & Tabor 1964; Smith 1971). Also, an inhibitor of polyamine biosynthesis caused cessation of cell division in the tomato (Egea-Cortines *et al.* 1993), while Pfosser *et al.* (1992) demonstrated that polyamines decreased in cell suspension cultures of the alfalfa *Medicago varia* when cell division arrested.

The correlation between polyamines and cell division is thought to be largely due to their association with nucleic acids. Polyamines are the most cationic small molecules of the cell and therefore, bind readily to polyanionic macromolecules like DNA and RNA (Igarashi *et al.* 1982). Since the amines have a distributed charge, unlike multivalent ions, for example Mg^{2+}, they are able to interact more flexibly with the acidic phosphate groups of nucleic acids (Marton & Morris 1987). Thus, polyamines act by stabilising DNA and RNA molecules and indeed, several possible configurations for complexes formed between DNA and polyamines have been suggested through the use of X-ray crystallography (Smith 1982). The structural re-inforcement of DNA by polyamines is illustrated well by exposing the DNA double helix to increasing temperatures. High temperatures lead to an unwinding of the molecule to give a 'random coil form'. However, a considerably higher temperature is required to achieve the same degree of disorganisation when polyamines are present (Smith 1982).

Polyamines stimulate the DNA replicating enzyme although relatively little is known as to how this is achieved (Stevens 1970). Many reports provide evidence of the association between polyamines and DNA synthesis. Rapid accumulation of polyamines and in particular, putrescine, was a pre requisite for the enhancement of DNA synthesis in rat liver, induced by food intake (Kameji *et al.* 1979). Putrescine is known to increase in relation to the rate of DNA synthesis in the woody plant *Catharanthus* and other plant tissues (Serafini-Fracassini *et al.* 1980; Smith 1985; Minocha *et al.* 1991). Minocha *et al.* (1991) also observed that putrescine reversed inhibition of DNA synthesis caused by an inhibitor of polyamine biosynthesis in *C. roseus*, providing evidence of the absolute requirement for the polyamine in DNA synthesis.

Polyamines are also associated with RNA synthesis. Since the double stranded DNA separates for RNA synthesis, polyamines are suggested to enhance the rate of restoration of

the double helix, thus stimulating synthesis of RNA (Smith 1982). Polyamines may also stimulate RNA synthesis by facilitating the removal of transcripted RNA from the DNA single strand (Smith 1982). Gallardo *et al.* (1992) reported that the accumulation of polyamines during germination of chick-pea, *Cicer arietinum,* seeds coincided with DNA and RNA synthesis.

1.1.2.2 Cell differentiation and organism growth

Somatic embryogenesis in carrot cell cultures was accompanied by an increase in polyamines (Mengoli *et al.* 1989), while polyamine biosynthesis inhibitors reduced somatic embryo formation of the ligneous plant *Hevea brasiliensis* (El Hadrami & D'Auzac 1992). Polyamines were implicated in embryogenesis of celery where increased amine levels were necessary for embryo growth and plantlet organisation (Danin *et al.* 1993). Altamura *et al.* (1991) observed that rhizogenesis in tobacco explants was reduced by various inhibitors of polyamine biosynthesis. Polyamines were also associated with organogenesis in tissue culture systems derived from melon cotyledons (Leshem *et al.* 1991), while Cohen *et al.* (1982) demonstrated that the use of a polyamine biosynthesis inhibitor prevented tomato ovary development.

Polyamines are also implicated in higher developmental processes in plants. For example, pollen germination and pollen tube length in lily flowers were reduced following exposure to polyamine biosynthesis inhibitors (Rajam 1988). Harkess *et al.* (1992) observed that a correlation existed between polyamines and flowering in the long day plant *Rudbeckia hirta.* Polyamines were also reported to be involved in fruit growth during the initial stages of fruit development in apple trees (Biasi *et al.* 1991), while Matejko & Dahlhelm (1991) found that polyamine levels related to the physiological state of onion bulbs during dormancy.

Polyamines have been implicated in the development of fungi. Polyamine biosynthesis increased during germination of *Blastocladiella emersonii* (Mennucci *et al.* 1975), *Aspergillus nidulans* (Stevens *et al.* 1976) and morphogenesis of *Mucor racemosus* (Inderlied *et al.* 1980). In *Sclerotium rolfsii,* mycelial growth and sclerotium formation were associated with increasing levels of polyamines (Shapira *et al.* 1989). Polyamines were implicated in the dimorphic transition of *Mucor* species when biosynthesis increased during the yeast to mycelium conversion of *M. rouxii* and *M. bacilliformis* (Calvo-Mendez *et al.* 1987). Further evidence of the essentiality of polyamines in this process was provided when a mutant of *M. bacilliformis,* unable to grow myceliallly, possessed very low levels of a polyamine biosynthetic enzyme (Ruiz-Herrera *et al.* 1983).

Exactly how polyamines affect differentiation and growth of various organisms is unclear. They are known to be associated with cell division and Evans & Malmberg (1989) suggested that this influence may extend to affecting patterns of cell division and thus, organism form. Other workers, however, have suggested that polyamines act as types of growth regulator or hormonal secondary messenger (Galston 1983; Davies 1987; Galston & Kaur-Sawhney 1987). Ruiz-Herrera (1994) suggested that polyamine levels may affect fungal differentiation via DNA methylation and this subject will be discussed in more detail shortly.

1.1.2.3 Regulation of stress

A great deal of work has implicated polyamines in the regulation of plant stress. Richards & Coleman (1952) first reported elevated levels of putrescine in potassium deficient barley. Klein *et al.* (1979) and Adams *et al.* (1990, 1992) reported similar results for peas and grapes while increased putrescine was observed under conditions of magnesium deficiency in barley (Smith 1973). Likewise, low pH in barley (Smith & Sinclair 1967), excess ammonium in peas (Klein *et al.* 1979) and atmospheric pollutants such as sulfur dioxide in peas (Priebe *et al.* 1978) caused accumulation of putrescine. Mung bean plants under salt stress (Friedman *et al.* 1989) and barley plants under water stress (Turner & Stewart 1986) also demonstrated an accumulation of putrescine. Wheat plants exposed to osmotic stress accumulated polyamines (Foster & Walters 1991), while elevated levels of polyamines were found in barley leaves infected with powdery mildew (Walters *et al.* 1985) and in wheat leaves inoculated with black stem rust (Foster & Walters 1992b).

The exact mechanism by which polyamines regulate each stress is unknown. However, there are many protective functions that are associated with polyamines which may assist plants to withstand stress. Srivastava & Smith (1982) suggested that polyamines interact with anionic groups of cell membranes, preventing leakage and causing stabilisation under conditions of stress. Evidence of the stabilising effect of polyamines on membranes was obtained with apple cells grown in culture, storage tissue from swede and spinach leaf discs. Induced ion efflux from these tissues could be reversed by the addition of spermine (Srivastava & Smith 1982). Naik & Srivastava (1978) suggested that polyamines may partially replace calcium and maintain membrane integrity by binding to phospholipid components of the membrane. Marton & Morris (1987) similarly suggested that binding of polyamines to phospholipid groups of membranes would alter membrane stability and permeability. It should be noted that DiTomaso *et al.* (1989) suggested that increased levels of putrescine may have caused membrane damage in maize roots by producing hydrogen peroxide and free radicals via the action of diamine oxidase. However, Dumbroff (1991) suggested that polyamines possessed the potential to act as free radical scavengers.

Smith & Sinclair (1967) suggested that increased cytoplasmic putrescine could help maintain cytoplasmic pH at a constant value, and Altman & Levin (1993) did indeed find that polyamines acted as a metabolic buffer, maintaining cellular pH under conditions of ammonium accumulation in tobacco cell suspension cultures. The accumulation of putrescine has also been associated with enhanced protein synthesis (Kaur-Sawhney *et al.* 1980) and increased synthesis of protective alkaloids (Hartmann *et al.* 1988).

Such cases of polyamine association with plant stress have been observed in other species with Agrawal *et al.* (1992) suggesting that polyamines were involved in de-toxifying mercury from the green alga *Chlorogonium elongatum.*

1.1.2.4 Regulation of senescence

Polyamines are associated with the regulation of plant senescence, although the mechanisms by which this is achieved are not clear. Exogenous polyamines are known to retard plant senescence, as demonstrated by the application of putrescine or spermine to carnation buds (Upfold & van Staden 1991). Ethylene is known to induce senescence and since biosynthesis of polyamines and ethylene require a common pre cursor (S-adenosylmethionine), elevation of polyamines should reduce ethylene production and thus retard senescence (Evans & Malmberg 1989). Conversely, depletion of polyamines should elevate ethylene production and trigger senescence (Evans & Malmberg 1989).

Indeed, polyamines are known to inhibit ethylene formation in a number of plant tissues including apple fruits, bean and tobacco leaf explants (Apelbaum *et al.* 1981). However, elevated levels of polyamines are not always associated with reduced ethylene biosynthesis. Chen *et al.* (1991) showed that polyamines stimulated the production of ethylene in detached rice leaves, while Park & Lee (1994) reported that an accumulation of polyamines was ethylene induced in suspension cultures of tobacco cells. Lee & Chu (1992) showed that ethylene accumulation led to increased putrescine biosynthesis which was associated with the elongation of rice coleoptiles. Such findings suggest that reduction of ethylene synthesis may not be wholly responsible for polyamine associated retardation of plant senescence.

Botha & Whitehead (1992) and Chien *et al.* (1991) both dismissed ethylene biosynthesis inhibition as the main mechanism for regulation of senescence in *Petunia hybrida* and rice plants while putrescine application extended the effective pollination period in 'Comice' pears but was not associated with altered ethylene levels (Crisosto *et al.* 1992).

The prevention of softening of apples and tomatoes by polyamines was suggested to be caused by polyamines either rigidifying cell walls or altering the activity of degradation enzymes of such walls (Kramer *et al.* 1991; Law *et al.* 1991). Smith (1985) proposed that polyamines acted as antisenescence agents by binding to nucleic acids or membranes. Indeed, Cohen *et al.* (1979) implicated a polyamine induced stabilisation of chloroplast thylakoid membranes in the retardation of chlorophyll degradation in senescing barley tissue.

The accumulation of polyamines in diseased plants has been implicated in the formation of 'green islands' and a subsequent retardation of senescence in these regions of the infected leaf, although the exact mechanism for the cause of this effect is unknown (Walters & Wylie 1986; Coghlan & Walters 1990).

1.1.2.5 DNA methylation

Methylation of DNA bases, a reaction which occurs in both prokaryotes and eukaryotes, is known to be important in gene expression at different developmental stages of many organisms (Razin *et al.* 1984). Generally, methylation is indicative of gene inactivation (Jupe *et al.* 1986). Cytosine residues, the most common DNA base to be methylated in eukaryotes, are methylated in reactions involving methyl groups from S-adenosylmethionine, catalysed by methyl transferase enzymes (Magill & Magill 1989).

Cano *et al.* (1988) implicated an association between polyamines, DNA methylation and growth of dimorphic *Mucor rouxii* with different levels of methylation leading to either mycelial growth or yeast-like growth. Although polyamines were implicated in regulating the amount of methylation, the studies were unable to determine how exactly the polyamines acted (Cano *et al.* 1988).

Ruiz-Herrera (1994) also suggested that the association between polyamines and differentiation in Mucorales may be connected to DNA methylation and went on to suggest that increased polyamine levels may inhibit DNA methylation and thus allow expression of specific genes necessary for differentiation (Ruiz-Herrera 1994).

Demethylation has been suggested to occur not by direct removal of the methyl group from DNA, but by inhibition of cytosine methylation during DNA replication (Ruiz-Herrera 1994). Indeed, Ruiz-Herrera *et al.* (1995) showed how activities of cytosine DNA methyltransferases were sensitive to polyamine concentrations, in particular, high concentrations of spermidine reduced the number of methyl groups incorporated into *Escherichia coli* DNA. Interestingly, Cano and co-workers (1988) and Cano-Canchola *et*

al. (1992) found that an inhibitor of polyamine biosynthesis prevented DNA methylation although the exact mechanism by which the inhibitor operated is unknown.

1.1.2.6 Protein synthesis and phosphorylation

The cationic nature of polyamines allows them to interact with ribosomes, the site of protein synthesis. This association has been implicated in the ability of polyamines to speed up the transcription-translation process of protein manufacture (Stevens 1970). Spermidine has been reported to induce the initiation of protein synthesis in isolated chloroplasts of the unicellular green alga *Chlamydomonas reinhardtii* (Blättler *et al.* 1992).

As well as synthesis of proteins, polyamines have been associated with the regulation of protein activity. Protein phosphorylation is the most common covalent modification of proteins and is used as a means of activating or deactivating enzymes. For the various phosphorylase kinase enzymes to phosphorylate proteins, the kinases must also be phosphorylated and this is achieved by various protein kinases. Conversely, protein dephosphorylation is achieved through the action of phosphorylase phosphatases and such enzymes are phosphorylated by protein phosphatases (Palmer 1991).

The activities of several important phosphorylation enzymes are modulated by abberant polyamine levels including proteins from pea plumules (Datta *et al.* 1987), maize coleoptiles (Velutthambi & Poovaiah 1984) and wheat (Polya & Micucci 1985).

Varying effects are, however, reported. Enzymes from maize coleoptiles (Velutthambi & Poovaiah 1984) and pea nuclei (Datta *et al.* 1986) were stimulated by polyamines while polyamines inhibited activity of enzymes from wheat embryos (Polya & Micucci 1985), wheat anthers (Bothma & Dubery 1991) and soybean hypocotyls (Lin & Key 1980). Nevertheless, polyamines are clearly associated with protein phosphorylation although their exact role is unclear. Polyamines may exert their effects on organism growth and development through regulation of various enzyme activities.

1.1.3 BIOSYNTHESIS AND REGULATION OF INTRACELLULAR POLYAMINES

1.1.3.1 Polyamine biosynthesis

The initial step of polyamine biosynthesis in mammalian cells and protozoa is the decarboxylation of ornithine to form putrescine, catalysed by the enzyme ornithine decarboxylase (ODC: EC 4. 1. 1. 17) (Figure 2). In plants and bacteria, putrescine can also be formed from a series of intermediates following decarboxylation of arginine by arginine decarboxylase (ADC: EC 4. 1. 1. 19) (Walters 1995).

Although putrescine formation in most fungi occurs solely through the operation of ODC, there is some evidence for the operation of ADC in certain fungi. Examples include the phytopathogenic fungi, *Ophiostoma ulmi* [Khan & Minocha 1989a (as *Certatocystis ulmi*); Biondi *et al.* 1993], *Verticillium dahliae*, *Certatocystis minor* (Khan & Minocha 1989b) and the mycorrhizal fungus *Laccaria proxima* (Zarb & Walters 1994c).

The polyamines spermidine and spermine are synthesised from putrescine by subsequent additions of aminopropyl groups donated by decarboxylated S-adenosylmethionine, which is formed from S-adenosylmethionine (AdoMet) in a reaction catalysed by the enzyme S-adenosylmethionine decarboxylase (AdoMetDC: EC 4. 1. 1. 50). The aminopropyl group additions are catalysed by the aminopropyltransferases spermidine synthase (EC 2. 5. 1. 16) and spermine synthase (EC 2. 5. 1. 22) respectively (Walters 1995).

Cadaverine is formed by the decarboxylation of the amino acid lysine. In plants, cadaverine is formed via the enzyme lysine decarboxylase (LDC: EC 4. 1. 1. 18) (Smith 1985). However, in animal cells and *Neurospora crassa*, cadaverine formation is catalysed by ODC, albeit inefficiently (Pegg & McGill 1979; Paulus *et al.* 1982). In cultured animal cells, bacteria and *N. crassa*, starved of polyamines, cadaverine is synthesised along with its higher derivative aminopropylcadaverine (APC) (Dion & Cohen 1972; Alhonen-Hongisto & Jänne 1980; Paulus *et al.* 1982). Zarb & Walters (1994b) showed that APC and another higher derivative of cadaverine, N,N-bis(3-aminopropylcadaverine) (3APC) were routinely formed in a range of mycorrhizal fungi.

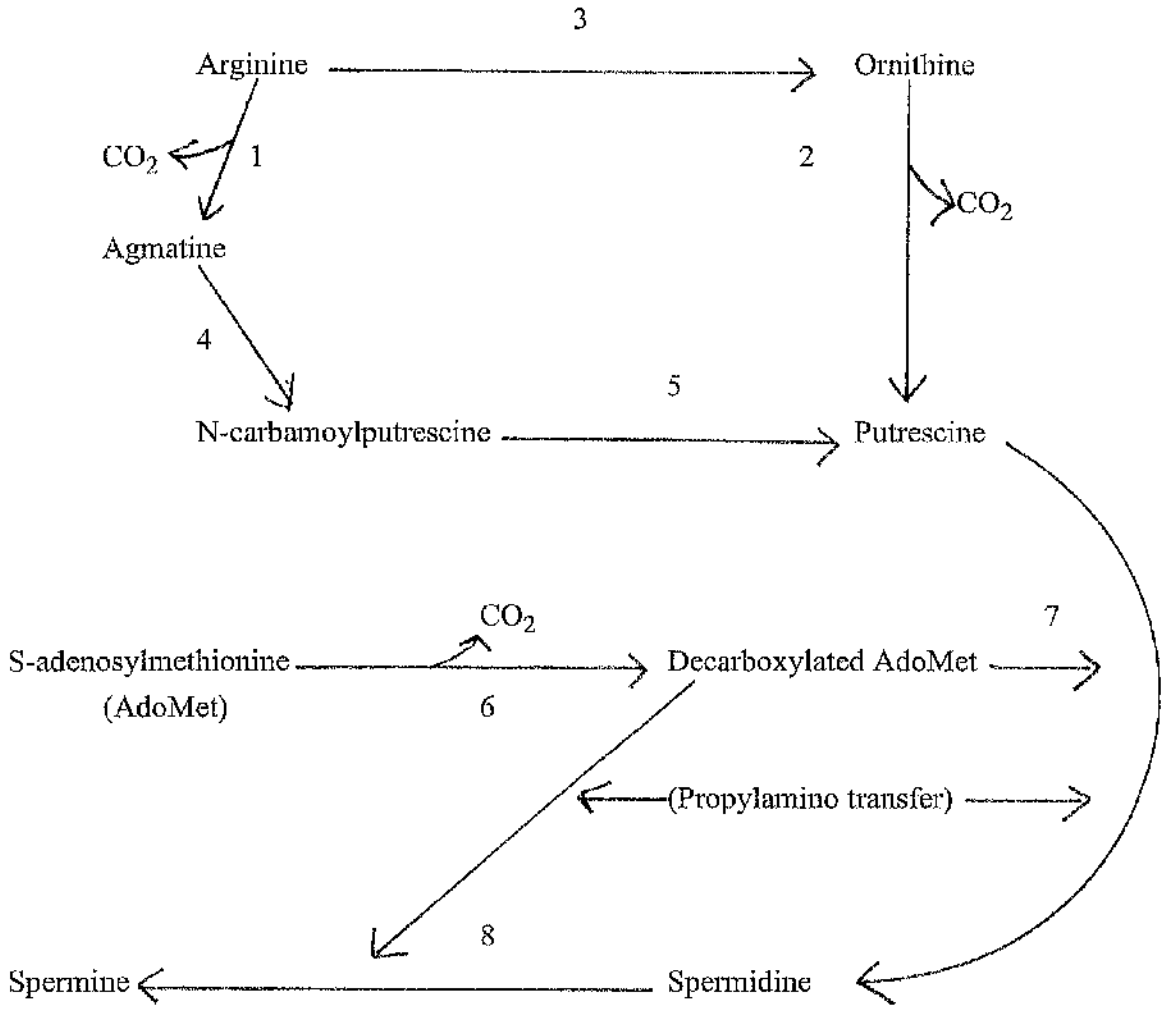


Figure 2. Biosynthetic pathways of the major plant polyamines
(Reproduced from Walters (1995))

1. arginine decarboxylase (ADC) 2. ornithine decarboxylase (ODC) 3. arginase 4. agmatine iminohydrolase 5. N-carbamoylputrescine amidohydrolase 6. S-adenosylmethionine decarboxylase (AdoMetDC) 7. spermidine synthase 8. spermine synthase

Note: Decarboxylation of arginine is common to plants and bacteria, however the intermediates preceeding putrescine differ; the plant biosynthetic route is shown.

1.1.3.2 Regulation of polyamine biosynthetic enzymes

The mammalian polyamine biosynthesis pathway consists of four irreversible steps, catalysed by the enzymes ODC, AdoMetDC, spermidine and spermine synthase. Putrescine synthesis appears to be regulated by the availability of ornithine and by the activity of ODC. The conversion of putrescine to spermidine and spermine is regulated by the activity of AdoMetDC, the supply of decarboxylated AdoMet determining the rate of synthesis of the higher polyamines. Whether decarboxylated AdoMet is utilised for the synthesis of spermidine or spermine depends on the amount of spermidine and spermine synthases available (Pegg 1988).

ODC activity is highly regulated and various mechanisms exist to facilitate enzyme response to fluctuating cellular polyamine levels. ODC also responds to a variety of growth stimulatory substances including hormones. In such responses, it has been shown that ODC activity is increased via an increased amount of ODC enzyme (Pegg 1986; Pegg 1987). This itself is mainly due to an elevated rate of protein synthesis although enzyme stabilisation may also occur (Pegg 1988).

In the presence of elevated levels of polyamines, ODC levels are reduced. Conversely, depletion of polyamines leads to an increase in cellular ODC content (Pegg 1988). Such changes in ODC concentration appear to be the result of changes in the rate of translation of the protein and in the rate of enzyme turnover (Pegg 1986; Pegg 1987). Indeed, all three key regulatory enzymes in the polyamine biosynthetic pathway, ODC, AdoMetDC and spermidine/spermine N^1-acetyltransferase (whose role will be discussed shortly), have very short half lives, enabling rapid turnover in response to cellular polyamine fluctuations (Pegg 1988).

Turnover of ODC protein may also be enhanced by its association with an inhibitory protein, 'antizyme'. This protein is induced by polyamines and inhibits ODC activity by forming a complex with the enzyme (Fong *et al.* 1976; Canellakis *et al.* 1979). Degradation of ODC in hepatoma tissue culture (HTC) cells and enzyme from Chinese hamster ovary (CHO) cells was indeed accelerated by 'antizyme' (Murakami *et al.* 1992a,b).

AdoMetDC activity is regulated by the availability of methionine which is the starting material for S-adenosylmethionine. Like ODC, AdoMetDC activity is highly regulated and is increased in response to various growth stimulatory substances (Pegg & McCann 1982; Pegg 1986). Such an increase has been reported to be the result of increased protein accumulation (Shirahata & Pegg 1985; Pegg *et al.* 1988b). This itself is probably due to

changes in rates of protein translation. Increased enzyme synthesis is less likely to be responsible (Pegg *et al.* 1988b).

Putrescine activates AdoMetDC directly, thus, a rise in putrescine levels will lead to an increase in decarboxylated AdoMet content so as to form spermidine and spermine (Pegg & McCann 1982; Pegg 1986). Elevated levels of spermidine and spermine act as products and repress AdoMetDC activity (Pegg 1984; Pegg 1986). Thus, as with ODC, depletion of polyamines results in increased AdoMetDC content while elevated polyamine levels lead to a reduction in AdoMetDC protein. These changes are mainly due to alterations in the rate of synthesis of the enzyme although degradation rates are also involved (Shirahata & Pegg 1985; Pegg *et al.* 1988b).

The third highly regulated enzyme involved in polyamine biosynthesis is the catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT). Enzyme activity can be induced by spermidine or spermine but is unaffected by putrescine. The increased activity of the enzyme results from both an increase in synthesis and a reduction in enzyme degradation (Pegg 1986).

The aminopropyltransferases are not as highly regulated as those enzymes discussed already. They do not turn over rapidly and fluctuations in intracellular polyamine levels do not generally lead to alterations in their activity (Pegg 1988).

Cells generally control their steady-state amount of biosynthetic end products through feedback inhibition (Davis 1990). Indeed, alterations in enzyme activity in Ehrlich ascites tumour cells treated with ODC inhibitors was attributed to the depleted polyamine concentrations exerting feedback control (Persson *et al.* 1989; Holm *et al.* 1989). However, control mechanisms involving the likes of enzyme turnover and protein translation rates are more elaborate and reflect the difficulty intracellular polyamines have in carrying out feedback inhibition (Davis 1990). Polyamines bind readily to cellular anions, thus Davis (1990) argued that feedback inhibition would be unworkable. Davis *et al.* (1985) suggested that only a small proportion of intracellular polyamines were involved in biosynthesis regulation while Paulus *et al.* (1983) demonstrated that the majority of polyamines in *Neurospora crassa* are bound to cell constituents. Bound polyamines can be released to overcome shortfalls in free polyamines, thus, the free cellular polyamine pool does not reflect the true amount of polyamines available to the cell (Davis *et al.* 1992). Adjustments to biosynthetic enzyme activity on the basis of the free polyamine pool, by feedback inhibition, would be largely inaccurate in terms of the whole cellular requirements (Davis

1990). Therefore, the elaborate mechanisms of enzyme regulation have evolved to respond to intracellular levels of polyamines (Davis *et al.* 1992).

1.1.3.3 Other means of controlling intracellular polyamine levels

Although polyamines are necessary for normal cell growth and development, polyamines are also toxic to organisms when present in excess (Davis 1990). Indeed, a mutant of *Neurospora crassa* which accumulated putrescine grew at a substantially reduced rate (Davis & Ristow 1991), while high levels of putrescine were reported to be toxic to the cyanobacterium *Anabaena* (Guarino & Cohen 1979).

As well as highly regulated biosynthetic enzymes, a number of mechanisms exist enabling cells to deal with excess polyamines. Mammalian cells have an elaborate polyamine catabolism system in which polyamines are interconverted and degraded back to putrescine (Pegg 1988). Spermine and spermidine are acetylated by the enzyme spermidine/spermine N^1-acetyltransferase (SSAT) to form N^1-acetyl derivatives of spermine and spermidine. Such derivatives are then substrates for polyamine oxidase (PAO: EC 1.5.3.3), and spermidine and putrescine are formed respectively (Pegg 1988). The spermidine formed from spermine can likewise form putrescine and the diamine either be excreted or degraded by diamine oxidase (DAO: EC 1.4.3.6) to form hydrogen peroxide (Pegg 1988). Thus, catabolism may prevent intracellular polyamines reaching toxic levels (Pegg 1986) (Figure 3).

Many plants and microbes also possess the ability to catabolise polyamines (Davis 1990). Indeed, catabolism in *Escherichia coli* is well characterised (Large 1992). In other microorganisms, however, the pathways taken are not well defined. Two major routes are available, one via N-acetyl derivatives, as described for mammalian cells, and the other via Δ^1-pyrroline (Large 1992). The N-acetyl route has been described for yeasts such as *Candida boidinii*, however, it is likely that other catabolic routes exist for such organisms (Large 1992). Polyamine conjugates such as glutathionyl-spermidine exist yet their function is unknown. Large (1992) suggests that they may be part of an as yet uncharacterised catabolic pathway. Little is known of polyamine catabolism in plant pathogenic fungi (Walters 1995).

Excess polyamines can be removed from cells by conjugation. Acetyl derivatives and glutathionyl-spermidine have already been discussed with respect to degradation. Such derivatisation may also act as a means of removing polyamines from the free pool (Large 1992) (Figure 3). Glutathionyl-spermidine conjugates have been reported for *E. coli* and trypanosomes (Tabor & Tabor 1985). Conjugation as hydroxycinnamic acid amide

derivatives has been observed in plants (Flores *et al.* 1989). However, Flores *et al.* (1989) suggested that conjugated forms of polyamines may have more significance as reserves than as detoxified forms of polyamines. Indeed, some evidence exists that conjugated forms of putrescine may be reversibly hydrolysed to maintain free putrescine levels in tissues treated with putrescine biosynthesis inhibitors (Slocum & Galston 1985).

Sequestration of polyamines from the cytosol into cellular organelles is known to control excess polyamines. Organisms such as *N. crassa*, other fungi and plants are capable of moving polyamines into vacuoles (Davis 1990).

Excretion of polyamines can also enable cells to control intracellular levels of amines. Cancer cells frequently release excessive polyamines into biological fluids, as do many normal mammalian tissues, probably by simple diffusion once intracellular levels of polyamines become too high (Davis 1990). *N. crassa* continues to synthesise spermidine whilst in the stationary phase and controls its intracellular polyamine levels by excretion (Davis & Ristow 1989). Control of extracellular uptake of polyamines can be used to regulate intracellular polyamine levels (Davis 1990), and this will be discussed later in the thesis.

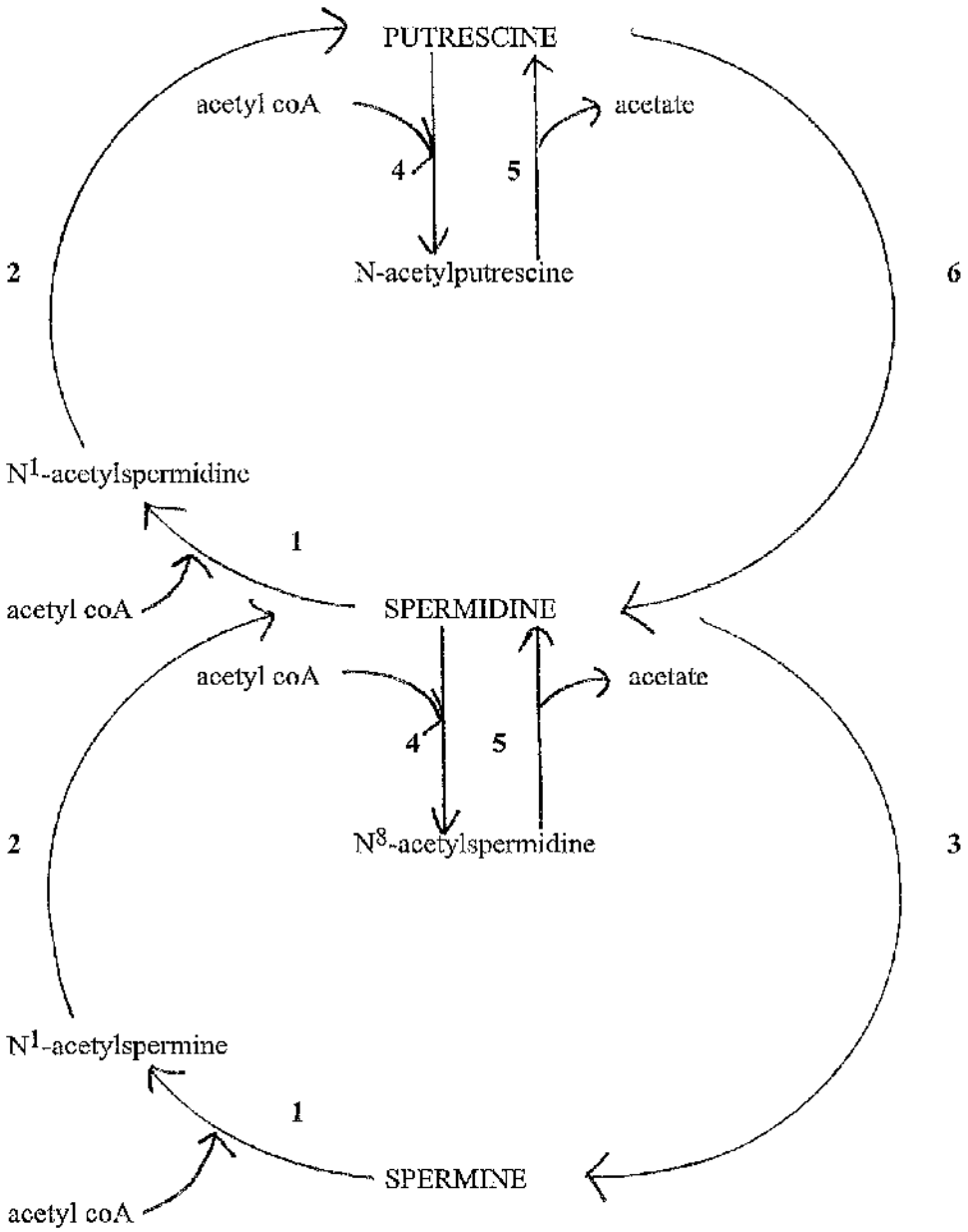


Figure 3. Polyamine interconversion pathways (Reproduced from Seiler (1988))

1. spermidine/spermine N^1-acetyltransferase (SSAT); 2. polyamine oxidase (PAO); 3. spermine synthase; 4. spermidine N^8-acetyltransferase; 5. N^8-acetylspermidine deacetylase; 6. spermine synthase

Note: N^8-acetylspermidine and N-acetylputrescine are conjugated forms of polyamines

1.1.4 INHIBITION OF POLYAMINE BIOSYNTHESIS

Although the physiological functions of polyamines are not fully understood, polyamines are undoubtedly vitally important to cellular processes such as proliferation. Indeed, elevated levels of polyamines and their biosynthetic enzymes are known to occur in rapidly proliferating tissues (Sunkara *et al.* 1987). This knowledge prompted workers, attempting to develop novel chemotherapeutic approaches for cancer treatment, to target the polyamine biosynthesis pathway since its inhibition was hypothesised to afford control of rapidly proliferating cells. Instigation of this work soon led to studies examining perturbation of polyamine biosynthesis as a means of controlling growth of many different types of organism (Porter & Sufrin 1986; Schechter *et al.* 1987; Pegg 1988). The following section is designed to provide a brief insight into the various inhibitors of polyamine biosynthetic enzymes although more details will be discussed in the subsequent sections relating to growth inhibition of specific organisms.

1.1.4.1 Inhibitors of ODC

Research into the inhibition of ODC has occurred via three different routes (Bey *et al.* 1987). The first of these involved the synthesis of substrate (ornithine) and product (putrescine) analogues as potential competitive reversible inhibitors of the enzyme. Compounds included the substrate analogues α-fluoromethylornithine (Bitonti *et al.* 1982), dehydroornithine (Relyea & Rando 1975) and the product analogue dehydroputrescine (Relyea & Rando 1975). The second route involved the synthesis of molecules capable of interacting or combining with the enzyme co-factor, pyridoxal phosphate. Such molecules were also competitive reversible inhibitors and examples included α-hydrazinoornithine (Inoue *et al.* 1975) and 1-aminooxy-3-aminopropane (APA) (Khomutov *et al.* 1985). Although α-hydrazinoornithine inhibited ODC from rat liver with an affinity constant of 0.5 μM (Inoue *et al.* 1975) and APA inhibited mouse kidney enzyme with a K_i of 0.0032 μM (Khomutov *et al.* 1985), these compounds lacked specificity, affecting other pyridoxal phosphate dependent enzymes (Khomutov *et al.* 1985).

The final route taken, in attempts to inhibit ODC, involved the design and synthesis of enzyme-activated or suicide irreversible inhibitors. Such compounds are chemically inert pseudosubstrates of the target enzyme. The inhibitors have a latent group incorporated into their structure which is transformed by catalytic turnover into a species that eventually inactivates the enzyme (Bey *et al.* 1987). Inhibitors belonging to this group include the ornithine analogues α-difluoromethylornithine (DFMO) and dehydromonofluoromethyl-ornithine (ΔMFMO) which have affinity constants of 39 μM and 2.7 μM respectively for enzyme from rat liver (Metcalf *et al.* 1978; Bey *et al.* 1983). Since ΔMFMO penetrates cell membranes poorly, *in vivo* use of the inhibitor utilises its methyl ester derivative,

AMFMO.CH_3 (Mamont *et al.* 1986). Putrescine analogues include (2R,5R)-6-heptyne-2,5-diamine (RR-MAP) which is a potent inhibitor of growth of rat hepatoma cells (Mamont *et al.* 1984).

Much exploration of ODC inhibition has been carried out with enzyme-activated irreversible inhibitors being particularly successful. It should be noted, however, that the relative potency of these inhibitors depends upon the source of the enzyme (Bey *et al.* 1987).

1.1.4.2 Inhibitors of AdoMetDC

Methylglyoxal bis(guanylhydrazone) (MGBG) is a powerful competitive inhibitor of mammalian AdoMetDC with an inhibition constant of less than 1 μM (Williams-Ashman & Pegg 1981). The inhibitor is less active towards AdoMetDC from other sources such as *Escherichia coli*, Chinese cabbage and the slime mold *Physarum polycephalum* (Pegg & Jacobs 1983; Pegg 1984; Yamanoha & Cohen 1985). A number of analogues of MGBG have been synthesised and ethylmethylglyoxal bis(guanylhydrazone) (EMGBG), was found to be a more potent inhibitor of AdoMetDC than MGBG (Pegg & Jacobs 1983).

Much work has been carried out using MGBG as a potential inhibitor of polyamine biosynthesis. Indeed, the compound has been reported to cause changes in the polyamine content of cells and spermidine has been shown to prevent the inhibitory action of MGBG (Pegg & Williams-Ashman 1987). In spite of this, it is unlikely that the compound exerts its effects wholly via inhibition of AdoMetDC activity (Pegg & Williams-Ashman 1987). Transport of the compound into cells utilises the same carrier system as polyamines. Prevention of growth by spermidine depletion may be due to competition between MGBG and spermidine for transport into cells (Pegg & Williams-Ashman 1987). MGBG inhibits cellular respiration and this has been implicated in severe mitochondrial damage observed in mammalian cells (Jänne *et al.* 1985). Nikula *et al.* (1984) reported that MGBG inhibited mitochondrial fatty acid oxidation and suggested this to be the cause of mitochondrial damage. EMGBG does not appear to affect cellular respiration, but although it is a more potent inhibitor of AdoMetDC than MGBG, it failed to demonstrate antiproliferative activity against mouse L1210 leukemia cells *in vitro* (Elo *et al.* 1986).

Many other inhibitors of AdoMetDC have been described including nucleoside analogues of AdoMet, such as the irreversible inhibitor S-(5'-deoxy-5'adenosyl)-methylthioethyl-hydroxylamine (AMA) (Artamonova *et al.* 1986). Secrist (1987) synthesised a number of irreversible inhibitors including 5'-deoxy-5'-{N-methyl-N-[2-(aminooxy)ethyl]}amino-adenosine (MAOEA) and 5'-deoxy-5'-[N-methyl-N-(3-hydrazinopropyl)]aminoadenosine

(MHZPA). These three compounds were reported to inhibit polyamine biosynthesis in L1210 cells (Pegg *et al.* 1988a; Kramer *et al.* 1989).

Recently, Regeness *et al.* (1994) described a cyclic analogue of MGBG, CGP 48 664 and reported how the compound inhibited AdoMetDC and exerted powerful antiproliferative effects against a variety of tumours (Regeness *et al.* 1994).

1.1.4.3 Inhibitors of spermidine synthase

Cyclohexylamine (CHA) has been sold as dicyclohexylammonium sulphate and confusion in the literature exists with many researchers incorrectly naming cyclohexylamine as dicyclohexylamine (Batchelor *et al.* 1986). The compound was initially reported as a strong inhibitor of mammalian spermidine synthase (Hibasami *et al.* 1980). Reports then followed of enzyme from trypanosomes, plants and various bacteria being inhibited by CHA (Pegg *et al.* 1983; Pösö *et al.* 1983; Paulin *et al.* 1983; Mattila *et al.* 1984; Sindhu & Cohen 1984). Enzyme from some bacteria were not, however, affected by the compound (Pösö *et al.* 1983; Mattila *et al.* 1984).

CHA is readily taken up into mammalian cells, inhibiting growth and leading to a depletion in spermidine levels (Ito *et al.* 1982; Mitchell *et al.* 1985; Feuerstein *et al.* 1985). Growth inhibitory effects on tumours in rodents were also observed (Ito *et al.* 1982). In extracts of *Helianthus tuberosus*, CHA decreased spermidine levels and increased putrescine concentration (Torrigiani *et al.* 1987). Such changes in polyamines were also observed in carrot embryos treated with cyclohexylammonium sulphate (Khan & Minocha 1991) and in pine cotyledons treated with CHA (Biondi *et al.* 1986). No effect was observed on the growth of pine cotyledons, however, an increase in spermine was suggested to be responsible for continued growth (Biondi *et al.* 1986). Such an increase in spermine and reduction in spermidine was also observed in protoplasts from Chinese cabbage leaves where spermidine synthase was inhibited by CHA *in vivo* (Greenberg & Cohen 1985). Feirer *et al.* (1985) also reported how CHA reduced growth and embryogenesis in carrots and decreased spermidine levels.

However, when studying *in vitro* propagation of potatoes on inhibitor amended medium, CHA reduced growth yet did not affect spermidine levels, suggesting that growth inhibiton was occurring by some other mechanism (Masse *et al.* 1988). Tiburcio *et al.* (1987), studying organogenesis in tobacco callus culture systems, reported how the effect of CHA was unclear and unlikely to be spermidine synthase inhibition. Indeed, Pegg & Williams-Ashman (1987) noted how information on the specificity of CHA was lacking and that the compound may affect other aspects of cellular physiology.

Various other inhibitors of spermidine synthase exist, including S-adenosyl-1,8-diamino-3-thiooctane (AdoDato) which successfully and specifically inhibited activity of spermidine synthase from various mammalian sources, bacteria, trypanosomes and plants (Pegg *et al.* 1983; Bitonti *et al.* 1984; Sindhu & Cohen 1984; Yamanoha *et al.* 1984). The compound did indeed deplete spermidine from mammalian cells and increased spermine levels (Pegg *et al.* 1982b). Hamaguchi and co-workers (1987) discovered an active compound juglorin in the culture filtrate of *Streptomyces malachiticus* which inhibited spermidine synthase but with weaker activity than that of CHA. Trans-4-methylcyclohexylamine was reported as inhibiting mammalian spermidine synthase with similar activity to CHA (Shirahata *et al.* 1988). Analogues of decarboxylated AdoMet had significant effects on polyamine levels in cultured L1210 cells, although they were suggested to affect both spermidine synthase and spermine synthase (Douglas *et al.* 1991).

1.1.4.4 Inhibitors of spermine synthase

Inhibitors of spermine synthase include S-methyl-5'-methylthioadenosine ($AdoS^{+}(CH_3)_2$) (Pegg *et al.* 1986) and S-adenosyl-1,12-diamino-3-thio-9-azadodecane (AdoDatad) (Coward & Pegg 1987). These compounds deplete cells of spermine yet have no effect on cell growth (Pegg *et al.* 1986). However, spermidine levels are increased following treatment of cells with the inhibitors and such elevated levels may account for the continuation of growth (Pegg *et al.* 1986). Combination of such inhibitors with one of AdoMetDC has therefore been suggested as an approach to effective depletion of spermidine and spermine (Pegg 1988).

1.1.4.5 Inhibitors of ADC

A range of α-fluoromethyl analogues of arginine were synthesised and found to be potent inhibitors of bacterial ADC, with the specific, enzyme-activated, irreversible α-difluoromethylarginine (DFMA) being one such inhibitor (Kallio *et al.* 1981). Analogues of arginine and agmatine, the enzymatic product, were also synthesised and found to inhibit ADC from *Escherichia coli* and oats with greater activity than DFMA (Bitonti *et al.* 1987a).

1.1.4.6 Polyamine analogues

The use of polyamine analogues as inhibitors of polyamine biosynthetic enzymes has already been discussed briefly in relation to each specific enzyme. Indeed, MGBG is an analogue of spermidine (Dave & Caballeri 1973). In work which will be discussed later, the irreversible enzyme-activated inhibitor DFMO was shown to have several short-comings with regards to inhibition of cell growth. Continuation of cell growth in the presence of DFMO was attributed to the drug being unable to totally deplete polyamines from cells (Pegg *et al.* 1982a; Mamont *et al.* 1982). Thus, Porter & Sufrin (1986) speculated that

polyamine analogues, differing only slightly in structure from naturally occurring polyamines, might be more capable of reducing cell growth than specific enzyme inhibitors.

Polyamines are taken up into most cells utilising a carrier system (Porter *et al.* 1982; Porter *et al.* 1984), which is specific for polyamine-like molecules (Seppänen 1981; Bartholeyns *et al.* 1984). Polyamine analogues may be able to utilise this system by binding to the carrier proteins at sites specific for polyamines. Analogues would be able not only to participate in binding but also to compete for sites with the polyamines. As an added advantage in cancer chemotherapeutic strategies, transport of polyamines into malignant tissues is elevated compared to normal tissues (Porter & Sufrin 1986). Once in the cell, analogues were hypothesised to exert antiproliferative effects by one or more of a number of mechanisms. Inhibition of polyamine biosynthetic enzymes could occur with analogues acting as either substrate or product analogues. Regulation of polyamine biosynthetic enzymes could also occur. Analogues could compete with polyamines for binding sites specific to cellular proliferation. Disruption of macromolecular structure and/or function could occur if analogues were to bind at associated polyamine sites and finally, analogues could act as vector molecules to deliver antiproliferative agents to cancerous tissues (Porter & Sufrin 1986).

Previous initial attempts using polyamine analogues as potential anticancer agents were limited since structural similarity to polyamines was minimal (Porter & Sufrin 1986). Analogues of spermidine and spermine were synthesised by various workers (Israel *et al.* 1964; Jorstad *et al.* 1980). However, only norspermidine inhibited cell growth *in vitro* (Porter & Bergeron 1983; Casero *et al.* 1984). Later work has shown this compound to possess antitumour activity against experimental animal tumours (Prakash *et al.* 1988). Norspermidine also inhibited HeLa cell growth in culture (Sunkara *et al.* 1988). Mitotic activity in such cells ceased while polyamines were depleted and ODC activity reduced. Interestingly, the growth inhibition could not be reversed by polyamines and norspermidine may thus have affected growth via an effect on mitosis of cells and/or perturbation of polyamine metabolism (Sunkara *et al.* 1988). Inhibition of spermidine biosynthesis was, however, associated with a retardation of growth of potatoes (Masse *et al.* 1988) and maize roots (Masse *et al.* 1985) on norspermidine amended media.

A number of spermidine analogues were designed, synthesised and evaluated (Porter *et al.* 1982; Porter *et al.* 1985). Spermidine analogues were chosen since studies with DFMO had indicated that it is the primary polyamine implicated in cell growth (Mamont *et al.* 1978). The analogues were evaluated against L1210 cell growth and three possessed significant inhibitory activity (Porter *et al.* 1985). Conclusions were made that two of the analogues,

N^1,N^8-bis(ethyl)spermidine (BES) and N^1,N^8-bis(propyl)spermidine (BPS), did indeed inhibit cell growth via polyamine depletion by regulating the biosynthetic enzymes as polyamines do (Porter *et al.* 1985). The analogues were incapable of promoting cellular growth (Porter *et al.* 1985). Thus, the potential of polyamine analogues as antiproliferative agents was indeed apparent although why some of the analogues do not inhibit growth and how the other exerts its mode(s) of action is unclear.

Many other polyamine analogues exist but their uses will be discussed in relation to the growth inhibition of various organisms.

1.1.5 CANCER RESEARCH

It is thus apparent that many inhibitors of polyamine biosynthetic enzymes exist. Their use in cancer research shall now be discussed before considering applications in the control of growth of other organisms and a detailed examination of their uses against growth of plant pathogenic fungi.

As discussed, perturbation of polyamine biosynthesis was suggested as a potential cancer chemotherapeutic strategy. Targetting of cellular proliferation is not wholly selective towards cancerous tissues, and as with most chemotherapeutic strategies, side effects were envisaged to occur. However, a number of cancers were known to be curable by chemotherapy and the development of a strategy involving inhibition of polyamine biosynthesis was worthy of consideration (Porter & Sufrin 1986). Over the past twenty years, much work has been carried out in this area and only a brief review of this work shall be given.

The initial polyamine biosynthetic enzyme to be targetted was ODC, the rate limiting enzyme (Sunkara *et al.* 1987). Relative success with various reversible inhibitors quickly instigated the design and synthesis of specific irreversible enzyme inhibitors by Merrell Dow scientists, the best known of these being DFMO (Metcalf *et al.* 1978). DFMO treatment typically reduced ODC activity and depleted mammalian cells of putrescine and spermidine (Mamont *et al.* 1978). Spermine levels were not reduced, probably because ODC activity would have continued at low levels, permitting small amounts of putrescine to be formed and converted through spermidine to spermine (Pegg *et al.* 1982a; Mamont *et al.* 1982). Alternatively, spermine pools may have been unaffected since growth is largely dependent upon spermidine. As ODC is inhibited, spermidine levels decrease by two means; by dilution since the cells continue to divide, and by conversion to spermine. Thus, when spermidine reaches a critical level and cell division ceases, the spermine pool is maintained (Porter & Sufrin 1986). It was this phenomenon which was to prove problematic with respect to use of DFMO. Cytostasis occurs when cells cease to divide in order to conserve their intracellular polyamine pools. However, upon removal of the drug, the residual polyamines allow resumption of cellular growth. Thus, DFMO inhibitory action was often temporary (Porter & Sufrin 1986).

Nevertheless, growth of various cultured cells including HTC and L1210 leukemia cells was inhibited by DFMO (Mamont *et al.* 1978). The inhibitory effects were reversed by the addition of putrescine and spermidine, indicating depletion of polyamines to be critical (Mamont *et al.* 1978). Inhibition of growth of many other types of cell was reported, including HeLa cells (Sunkara *et al.* 1980) and Ehrlich ascites carcinoma cells (Oredsson *et*

al. 1980). Effects of DFMO were generally cytostatic, however, cytotoxicity was apparent in some cell lines including mouse melanoma cells (Sunkara *et al.* 1983a). DFMO appeared to be cytostatic or cytotoxic, depending on the cell line employed (Sunkara *et al.* 1987).

In spite of the short-comings of the drug upon cells in culture, the compound was used in attempts to inhibit the growth of experimental tumours. Significant inhibitory effects were obtained against a variety of tumours including L1210 leukemia (Prakash *et al.* 1978), and melanoma in mice (Sunkara *et al.* 1983b). DFMO also inhibited growth of human small-cell lung carcinoma cells in experimental mice, with cytotoxicity effects reported. The cells did eventually die under sustained polyamine deprivation (Luk *et al.* 1983).

Given the antiproliferative effects of DFMO against cultured cells and tumours in rodent models, clinical studies of DFMO in patients with cancer commenced in 1979. Patients suffering from far-advanced disease for whom no other therapy could be offered were used in the trials. The advanced stage of their illnesses may have contributed to the relatively poor results observed. DFMO did not affect tumour growth or disease progression significantly. Occasionally, stabilisation of disease occurred but no general pattern of response to DFMO was observed from the patients (Schechter *et al.* 1987). In a later trial using patients suffering from malignant melanoma, more disease stabilisation was observed (Meyskens *et al.* 1986). Side effects such as gastrointestinal effects and hearing loss were reversible upon dose reduction or discontinuation of treatment (Schechter *et al.* 1987).

Although DFMO was not sufficiently effective alone, it was observed to have low toxicity and was thus hypothesised to be more useful in combination with other drugs, perhaps increasing the cytotoxicity of other antitumour agents (Porter & Sufrin 1986). Indeed, uptake of MGBG into Ehrlich ascites cells was increased after pre treatment with DFMO with an associated increase in cytotoxicity of MGBG (Alhonen-Hongisto *et al.* 1980; Seppänen *et al.* 1981). A similar phenomenon was observed with HeLa cells (Sunkara *et al.* 1983b). Marton *et al.* (1981) showed how DFMO increased the cytotoxicity of the DNA reactive drug, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in rat gliosarcoma cells in culture. However, Oredsson *et al.* (1982) failed to increase cytotoxicity of cis-platinum by pre treating cells with DFMO, demonstrating that results vary according to cell line and/or cytotoxic agent.

Such combinations of DFMO and other agents were observed to be effective against tumours in experimental model systems. The combination of DFMO with interferon suppressed growth of subcutaneous melanomas in mice (Sunkara *et al.* 1983a). Pre treatment with DFMO was hypothesised to deplete cellular polyamines, destabilising DNA

and thus making it more vulnerable to those drugs such as interferon which act upon DNA (Pegg 1988). Sequential therapy using DFMO followed by MGBG was also shown to be effective against L1210 cells in mice (Sunkara *et al.* 1993a). Similarly, DFMO treatment was envisaged to deplete cells of spermidine and since MGBG can utilise the spermidine uptake system, increased levels of MGBG could accumulate in the cell (Sunkara *et al.* 1987).

Initial clinical trials using combination therapy, for example utilising DFMO followed by MGBG against childhood leukemia were successful (Siimes *et al.* 1981). DFMO and interferon, together, were studied against malignant melanoma, however, limited responses were observed (Talpaz *et al.* 1986).

It was apparent that DFMO was not as effective as was originally hypothesised for the treatment of cancer. The cytostatic response of the drug has already been discussed. Other limitations include the rapid clearing of DFMO from the body (Grove *et al.* 1981) and its slow uptake into cells, via a passive diffusion mechanism (Erwin & Pegg 1982). However, the minimal host toxicity of DFMO was indeed promising and led to further work designing specific enzyme-activated irreversible inhibitors and studying other enzyme inhibitors and polyamine analogues, as discussed elsewhere in this review (Porter & Sufrin 1986).

Clearly, perturbation of polyamine biosynthesis and/or functions represents a promising approach to cancer treatment and much work is on-going in this area. Some of the most recent reported work includes an increase in cytotoxicity of cisplatin against L1210 leukemia cells by a polyamine analogue (Hawthorne & Austin 1996), lung tumour cell death caused by polyamine analogues (McCloskey *et al.* 1996) and an investigation into the use of a spermine conjugate as an inhibitor of polyamine transport into cells (Aziz *et al.* 1996).

1.1.6 PROTOZOAL, BACTERIA, ANIMAL-INFECTING FUNGI AND INSECTICIDE RESEARCH

1.1.6.1 Protozoal infections

Perturbation of polyamine biosynthesis was initially hypothesised by Cohen (1979) as a target for chemotherapeutic control of parasitic diseases. As a consequence of the relative success achieved with DFMO in inhibiting ODC and growth of mammalian cells, coupled with the low toxicity of the compound, work was instigated and showed DFMO to cure infection of mice with the protozoan cause of bovine trypanosomiasis, *Trypanosoma brucei brucei* (Bacchi *et al.* 1980). DFMO was also shown to be effective against mice infected with the human sleeping sickness pathogens *T. b. rhodesiense* and *T. b. gambiense* (McCann *et al.* 1986), while the compound reduced infection and prolonged survival time of mice infected with *T. evansi*, an important blood protozoan parasite of cattle in India (Damayanthi 1990).

Putrescine synthesis in *T. b. brucei* cultured *in vitro* was inhibited by DFMO, suggesting that the compound did indeed affect ODC (Bacchi *et al.* 1980). Inclusion of exogenous polyamines with the DFMO treatment reversed the curative action, confirming that DFMO caused an effect on polyamine levels (Nathan *et al.* 1981). Later work demonstrated that the compound inhibited cellular DNA and RNA synthesis (Bacchi *et al.* 1983). Interestingly, DFMO causes a morphological change in trypanosome populations from replicating parasitic bloodstream trypanosomes to non replicating short stumpy forms (Giffin *et al.* 1986)

Whatever the exact mechanism of action of DFMO against such trypanosomes, the parasites are much more sensitive to the compound than mammalian cells (Mamont *et al.* 1978). The reason for this is not clear as trypanosomal ODC from *T. b. brucei* has the same degree of sensitivity towards DFMO as ODC from mammalian cells (Bitonti *et al.* 1986a). DFMO is also known to enter the parasites by passive diffusion, as with mammalian cells (Bitonti *et al.* 1986a). Several suggestions have, however, been made to account for the difference in sensitivity observed between trypanosomes and mammalian cells with respect to DFMO. Enzyme from trypanosomes may turn over more slowly than that from mammalian cells (Phillips *et al.* 1987). ODC inhibition and subsequent depletion of spermidine could affect amounts of the trypanosome spermidine-containing enzyme co-factor, trypanothione, obligatory for glutathione reductase activity (Fairlamb *et al.* 1985). Also, the high multiplication rate of trypanosomes could place a greater dependance on spermidine for trypanosomes than mammalian cells (Byers *et al.* 1991).

In addition to DFMO, a number of ornithine and putrescine analogues, RR-MAP, ΔMFMO, ΔMFMO.CH_3 and ΔMFMO.C_2H_5 inhibited ODC from *T. b. brucei* as well as enzyme activity in trypanosomes (Bitonti *et al.* 1985). However, only ΔMFMO.CH_3 was as effective as DFMO against the infection in experimental mice (Bitonti *et al.* 1985). Indeed, Bacchi *et al.* (1987) found that ΔMFMO.CH_3 was effective at concentrations lower than those of DFMO in *T. b. brucei* infections of mice. Here, the compound was taken up by the host animals much more rapidly than DFMO (Bacchi *et al.* 1987). Evaluation of DFMO in combination with other drugs such as the antibiotic bleomycin (Clarkson *et al.* 1983) and the trypanocide suramin (Clarkson *et al.* 1984) against acute *T. b. brucei* infection in mice demonstrated synergistic action.

Interestingly, DFMO has no effect on *T. cruzi*, the cause of American trypanosomiasis (Kierszenbaum *et al.* 1987). However, Yakubu and co-workers (1992) demonstrated effective inhibition of *T. cruzi* growth by DFMA, the organism being unusual in synthesising putrescine via ADC.

Further studies using alternative polyamine biosynthesis inhibitors against trypanosomal parasites have been reported. MGBG inhibited AdoMetDC from *T. b. brucei* (Bitonti *et al.* 1986b). Another inhibitor of AdoMetDC, 5'-{[Z]-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine (AbeAdo) cured infections in mice caused by *T. b. brucei* (Bitonti *et al.* 1990). Indeed, the drug caused the parasites to be cleared from the bloodstream extremely rapidly, before significant depletion of spermidine had occurred, suggesting a mechanism of action other than polyamine depletion (Bitonti *et al.* 1990). Substantial increases in AdoMet levels were observed in trypanosomes exposed to AbeAdo (Byers *et al.* 1991). Since only smaller increases in AdoMet were observed in similarly treated mammalian cells, the phenomenon was suggested to be specific to parasites (Byers *et al.* 1991).

Similarly, reduced AdoMetDC activity and elevated AdoMet levels were observed in trypanosomes taken from rats which had been infected with *T. b. brucei* and treated with DFMO (Byers *et al.* 1991). Thus, inhibitors may act against trypanosomes via the induction of elevated AdoMet levels (Byers *et al.* 1991). Since AdoMet donates a methyl group for methylation, the mechanism by which elevated AdoMet may lead to control of growth of trypanosomes may be associated with aberrant methylation (Byers *et al.* 1991).

AdoMetDC activity of *T. b. brucei* was also inhibited by the trypanocidal agents Berenil (4,4'-diamidinodiazoaminobenzene) and pentamidine (*p, p'*-(pentamethylenedioxy)-dibenzamidine (Bitonti *et al.* 1986b). Two compounds, AdoDato and dicyclohexylamine,

shown to inhibit spermidine synthase from *T. b. brucei*, failed however, to prolong the life span of infected mice (Bitonti *et al.* 1984).

Given the promising results against trypanosomal infections in experimental models, clinical evaluations of DFMO against human sleeping sickness were initially reported by Sjoerdsma & Schechter (1984). Effective control of the disease was observed in twenty patients with parasites being eradicated from the blood, usually within days of commencing the treatment. One patient relapsed but most others remained symptom free for several months to two years. Taelman *et al.* (1987) reported the remarkable response of a comatose patient, suffering from late stage sleeping sickness, to DFMO administration. Rapid recovery was observed and thereafter, the patient remained parasite free. In spite of the efficacy demonstrated, DFMO did cause side effects although these were no more than those expected with chemotherapeutic strategies. Indeed, the side effects, such as nausea and diarrhoea were reversible (Schechter *et al.* 1987). Many other investigations have been reported for DFMO against human and animal trypanosomiasis and successful results led to DFMO now having been marketed for several years by Merrell-Dow Pharmaceuticals as Ornidyl, for treatment against human West African (Gambian) sleeping sickness. It should, however, be noted that drug resistant populations may emerge since resistance to DFMO was observed in a mutant strain of *T. b. brucei* (Phillips & Wang 1987).

Research into inhibition of polyamine biosynthesis as a chemotherapeutic strategy against other protozoan infections has been carried out. Malaria remains one of the most important infectious diseases in the world and since resistance to antimalarial drugs is common-place, the development of new drugs is paramount (Peters 1985). Initial studies demonstrated that DFMO inhibited proliferation of *Plasmodium falciparum in vitro* (McCann *et al.* 1981). The compound was later shown to inhibit proliferation of *P. berghei* in mice although it did not cure the infection (Bitonti *et al.* 1987b).

A number of bis(benzyl)polyamine analogues were found to be potent inhibitors of growth of chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum in vitro* (Bitonti *et al.* 1989). Analogues also inhibited growth of *P. berghei in vivo* and combination of the analogues with DFMO cured mice with established infections of *P. berghei* (Bitonti *et al.* 1989). Inhibition appeared to be cytotoxic, in contrast to effects of DFMO which tend to be cytostatic (Pegg & McCann 1982). Thus, prevention of parasitic growth by the analogues was suggested to be due to means other than the repression of polyamine biosynthesis (Bitonti *et al.* 1989). Indeed, binding of the analogues to DNA and subsequent disruption of macromolecular synthesis was concluded to be the major cytotoxic effect of the analogues against parasitic infections (Bitonti *et al.* 1989).

Clinical trials using DFMO against the protozoa *Pneumocystis carinii*, which causes pneumonia in immunocompromised patients, and is known as the most common lethal infection of patients with Aquired Immune Deficiency Syndrome (AIDS) have also been carried out (Schechter *et al.* 1987). While drugs are available for treatment of the infection, patients often suffer severe side effects or even fail to respond to these drugs (Golden *et al.* 1984). These patients were involved in the first clinical trials of DFMO against *P. carinii* with beneficial results obtained which warranted further study (Golden *et al.* 1984).

DFMO has also demonstrated a high degree of efficacy towards *Eimeria tenella*, the cause of coccidiosis in chickens (McCann *et al.* 1981) as well as other protozoan species; *Trichomonas* (Yarlett & Bacchi 1994) and *Leishmania* (Kaur *et al.* 1986). Clearly, perturbation of polyamine biosynthesis is useful against protzoan infections.

Much work is on-going with regards to the perturbation of polyamine biosynthesis and/or function in the sphere of protozoal research. Most recently, selective toxicity of DFMO for *P. carinii* has been reported (Merali & Clarkson 1996), MGBG derivatives have been shown to be effective against trypanosomes (Bacchi *et al.* 1996) and an inhibitor of AdoMetDC demonstrated inhibitory activity against growth of *Leishmania donovani* (Mukhopadhyay *et al.* 1996).

1.1.6.2 Bacteria

Putrescine biosynthesis in most bacteria occurs via both the ODC and ADC route, therefore, any desired inhibition of growth through polyamine biosynthesis inhibition must consider both pathways (Kallio & McCann 1981). Nevertheless, studies have been carried out to evaluate various inhibitors as potential bacterial growth retardants.

ODC activity in the bacteria *Escherichia coli* and *Pseudomonas aeruginosa* exposed to DFMO was reduced although, since ADC activity increased as a compensatory measure, no growth reduction was observed (Kallio & McCann 1981). Interestingly, ODC extracted from *E. coli* was unaffected by DFMO, yet the enzyme from *P. aeruginosa* was inhibited. Enzyme from another bacteria, *Klebsiella pneumoniae*, was unaffected by DFMO (Kallio & McCann 1981). Another ODC inhibitor, DL-α-monofluoromethylputrescine, inhibited enzyme from *E. coli* but failed to affect the enzyme from *P. aeruginosa* (Kallio *et al.* 1982). This is in direct contrast to those effects of DFMO (Kallio & McCann 1981). The studies showed differences in specificity for enzyme inhibitors by the bacterial enzymes as well as *in vitro* and *in vivo* differences.

Kallio and co-workers (1982) demonstrated that a combination of DL-α-monofluoromethylputrescine with DFMA reduced ODC and ADC activity in *E. coli*, agreeing with the suggestion that a two-pronged attack on bacterial putrescine synthesis was necessary to prevent compensatory increases in enzyme activity (Kallio & McCann 1981). However, putrescine levels were only reduced to one third of control values (Kallio *et al.* 1982).

Growth of *E. coli* and *P. aeruginosa* was inhibited using a combination of DL-α-monofluoromethylornithine (MFMO), DFMA and dicyclohexylammonium sulphate. Putrescine and spermidine levels were reduced and the growth inhibition reversed by the inclusion of exogenous polyamines in the growth medium, indicating that the growth reduction was indeed due to polyamine depletion (Bitonti *et al.* 1982).

In contrast, growth of *Agrobacterium tumefaciens* was inhibited through the use of two single inhibitors, DFMO and CHA. DFMO reduced putrescine and spermidine levels while CHA had no effect on intracellular polyamines. Growth inhibition by DFMO was reversed by putrescine but retardation of growth by CHA was not affected by exogenous spermidine, suggesting that CHA did not specifically inhibit polyamine biosynthesis (Ponappa *et al.* 1992). DFMA did not inhibit growth of the bacterium and indeed, ODC was observed to be the primary enzyme for the synthesis of putrescine in *A. tumefaciens* (Ponappa *et al.* 1992). However, the converse was observed by Speranza and Bagni (1977), with these authors identifying ADC as the primary enzyme. Such a difference may be attributed to the specific strain of bacterium (Speranza & Bagni 1977).

The ADC inhibitor DFMA reduced enzyme activity in *E. coli* and *P. aeruginosa* as well as enzyme extracted from these bacteria. DFMA also inhibited ADC from *K. pneumoniae* (Kallio *et al.* 1981). Arginine and agmatine analogues successfully inhibited ADC from *E. coli*, being more potent than DFMA (Bitonti *et al.* 1987a)

Inhibition of polyamine biosynthesis in *E. coli* has been further complicated by the reported finding of a probable new pathway for putrescine, not involving ODC or ADC (Cataldi & Algranati 1986). Nevertheless, perturbation of polyamine biosynthesis and/or function may prove useful in the control of bacterial growth.

1.1.6.3 Animal-infecting fungi

Inhibition of polyamine biosynthesis as a means of chemotherapeutic control of fungal infections of animals has also been studied. *In vitro* growth of *Candida albicans*, *C. tropicalis* and *C. parapsilosis* was inhibited by DFMO although *C. albicans* and

C. parapsilosis were less susceptible to the inhibitor than *C. tropicalis* (Pfaller *et al.* 1987). In later work, DFMO inhibited ODC activity in *C. albicans* and *C. tropicalis*, however, enzyme inhibition was greater for *C. albicans* compared to *C. tropicalis*. Such a result does not correlate with the given effects on yeast growth where *C. tropicalis* growth was more sensitive to DFMO than *C. albicans* (Pfaller *et al.* 1988). Growth of *Cryptococcus neoformans*, a pathogenic yeast associated with meningitis infections which occur in patients with AIDS (Patterson & Andriole 1989), was also inhibited by DFMO (Pfaller *et al.* 1990). In all of the fungal species, fluctuations in polyamine levels were consistent with inhibition of ODC and growth inhibition was reversed by exogenous polyamines, indicating that DFMO was indeed inhibiting ODC activity and not exerting non specific toxic effects (Pfaller *et al.* 1987, 1988, 1990).

Growth of *C. albicans*, *C. tropicalis* and *C. neoformans* was also inhibited by CHA. Fluctuations in polyamine levels, increases in putrescine and decreases in spermidine, and the ability of exogenous polyamines to reverse the growth effects indicated that CHA caused specific enzyme inhibition and not non specific toxic effects (Pfaller *et al.* 1988, 1990). A sequential treatment of DFMO followed by CHA in *C. albicans* proved to inhibit growth to a greater extent than CHA alone (Pfaller *et al.* 1988).

As well as pathogenic yeasts, inhibitors of polyamine biosynthesis have been studied against other animal-infecting fungal species. Growth of six species each of *Microsporum* and *Trichophyton* dermatophytic fungi were inhibited by DFMO and DFMA. *Trichophyton* species were generally more sensitive than *Microsporum* species and indeed, lower concentrations of DFMA compared to DFMO were required to inhibit growth (Boyle *et al.* 1988). The inhibitory activity of DFMA was thought to be the result of conversion of the compound to DFMO by arginase and the increased efficacy of DFMA over DFMO, due to increased uptake of DFMA (Boyle *et al.* 1988). Thus, ΔMFMO.CH_3, known to be taken up faster than DFMO by parasites (Bacchi *et al.* 1979) and mammalian cells (Mamont *et al.* 1986), was found to be more effective at inhibiting growth of the fungal species than DFMO (Boyle *et al.* 1988). The inhibition of putrescine synthesis in the fungi species was shown to be specific, being reversed by exogenous putrescine (Boyle *et al.* 1988).

Mycelial morphology was dramatically altered in the presence of DFMO or DFMA. Abnormal hyphae and swollen cells were characteristics of the fungal species observed after treatment with the inhibitors (Boyle *et al.* 1988).

In later work, DFMO and DFMA inhibited ten further dermatophytic fungal species and while DFMO-induced growth reductions were associated with decreased ODC activity and

reductions in polyamine levels, the mechanism of DFMA growth inhibition was unclear. ADC activity was not detected, nor arginase-mediated conversion of DFMA to DFMO (Gruhn & Boyle 1991).

1.1.6.4 Insecticides

Polyamine biosynthesis inhibitors have been suggested to act as novel insecticides. Insects synthesise putrescine solely via the ODC route while plants possess both the ODC and the ADC routes for polyamine formation, thus specific control of insects on crop plants should be possible (Rajam 1991). DFMO, MGBG and bis(cyclohexylammonium) sulphate all exhibited insecticidal activity against the tobacco caterpillar (*Spodoptera litura*) when larvae were allowed to feed on treated leaf discs. MGBG was the most effective compound in terms of mortality and reducing body weight of surviving larvae (Rajam 1991).

1.1.7 FUNGAL RESEARCH

As discussed in section 1.1.3.1, page 10, most fungi form putrescine from ornithine, in a reaction catalysed by ODC. Plants, however, can synthesise putrescine both via ODC and from arginine in a series of reaction steps, initially catalysed by ADC. Thus, specific control of fungal growth on plants should be possible by inhibiting ODC. Since plants have an alternative pathway to putrescine, they should not be adversely affected by ODC enzyme inhibition. It was this theory that instigated research into inhibition of polyamine biosynthesis in fungi. Such research will now be reviewed, begining with inhibition of ODC.

1.1.8.1 Inhibitors of ODC

Rajam & Galston (1985) initially studied the potential of polyamine biosynthesis inhibitors to control plant pathogenic fungi by examining the effects of DFMO on mycelial growth of *Botrytis sp*, *Botrytis cinerea*, *Rhizoctonia solani* and *Monilinia fructicola*. DFMO strongly inhibited growth of all the fungal species examined, with the highest dose of 1.0 mM almost completely depressing growth. *Botrytis sp* was the most sensitive species with regards to the effects of DFMO, differences being apparent between the responses of the different species to the inhibitor (Rajam & Galston 1985).

Later work by Birecka and co-workers (1986) not only demonstrated that DFMO inhibited mycelial growth of *Helminthosporium maydis*, but also noted that differences were apparent between sensitivity of different fungal species towards DFMO. These authors speculated that the differences may be due to variations in inhibitor uptake and distribution within the cell, enzyme sensitivity towards the inhibitor and fungal polyamine requirements (Birecka *et al.* 1986).

Fungal growth of *Botrytis sp* and *M. fructicola* on media amended with DFMO plus polyamines was greater than that growth on medium amended with only DFMO, suggesting that DFMO was indeed affecting growth through the depletion of polyamines (Rajam & Galston 1985). These findings were also observed for *H. maydis* when the inclusion of putrescine into the media prevented DFMO-induced inhibition of growth (Birecka *et al.* 1986).

Since then, there have been many reports of DFMO inhibiting mycelial growth of various phytopathogenic fungi on solid media. West & Walters (1989) demonstrated that 4.0 mM DFMO reduced growth of 'grey' *Pyrenophora teres* and *Gaeumannomyces graminis*. Culturing of 'grey' *P. teres* produced stable 'orange' variants and thus, both such variants were studied by these researchers. Smaller growth reductions of 'orange' *P. teres* and

Fusarium culmorum were obtained with DFMO while no inhibitory effect was observed against *Septoria nodorum*, indeed a 2.0 mM concentration of DFMO increased growth of that fungus (West & Walters 1989). Additional ODC inhibitors were examined; ΔMFMO, ΔMFMO.CH_3 and RR-MAP were less effective than DFMO in inhibiting growth of the various fungi. RR-MAP actually increased growth of 'grey' *P. teres* (West & Walters 1989). The addition of polyamines to DFMO amended media resulted in increased mycelial growth compared to DFMO treatment alone. Such a restoration of growth indicated that DFMO did indeed affect growth by reducing polyamine levels in the fungal species (West & Walters 1989).

Thus, the work of these authors agreed with the findings of Birecka *et al.* (1986) whereby differences in sensitivity of fungal species towards inhibitors was demonstrated. Indeed, West & Walters (1989) took these findings further with mutant variants of the same fungus, *P. teres*, displaying varying effects towards inhibitors. Interestingly, reduced efficacy of DFMO towards *F. culmorum* was later observed not to be a consequence of reduced inhibitor uptake (West & Walters 1991a).

Various growth increases observed by enzyme inhibitors may have been the result of compensatory increases in non-target enzyme activities (West & Walters 1989). As discussed in section 1.1.3.2, page 12, organisms possess a variety of means enabling them to overcome fluctuations in their intracellular polyamine concentrations, including enzyme activity increases (Jänne *et al.* 1985).

Mycelial growth of various other pathogens has been inhibited by DFMO. Mussell and co-workers (1987) reported the inhibition of *Verticillium dahliae* at concentrations as low as 5μM. The wheat pathogen *Neovossia indica* (Singh *et al.* 1989), *Botrytis cinerea* (Smith *et al.* 1990b), *Pyrenophora avenae* (Foster & Walters 1990) and *Ophiostoma ulmi* (Biondi *et al.* 1993) were all inhibited on solid media by DFMO. Khan & Minocha (1989a) observed DFMO-induced inhibition of *Helminthosporium carbonum* and *Fusarium oxysporum*. Inhibition was reversed in all these cases by the inclusion of polyamines into the medium.

More recently, Bharti & Rajam (1996) demonstrated inhibition by DFMO of mycelial growth of four plant pathogenic fungal species; *Helminthosporium oryzae* (causing leaf spot of rice), *Curvularia lunata* (black rot of chilli), *Pythium aphanidermatum* (damping off disease in chilli and pea) and *Colletotrichum capsici* (fruit rot of chilli). *H. oryzae* and *C. lunata* were more sensitive towards DFMO than the other fungi and all instances of growth inhibition were prevented by the addition of putrescine to inhibitor amended media (Bharti & Rajam 1996).

Other ODC inhibitors have also demonstrated inhibition of mycelial growth of fungi; growth of *B. cinerea* was reduced by ΔMFMO (Smith *et al.* 1990a) while ΔMFMO and RR-MAP reduced growth of *Phytophthora infestans* (Barker *et al.* 1993).

Mycelial morphology and cell size were dramatically altered by DFMO in *Botrytis sp* and *M. fructicola* where fewer hyphae resulted and fungal cell lengths were reduced (Rajam & Galston 1985). West & Walters (1989) observed varying effects on cell length caused by ODC inhibitors when exposed to fungi growing on solid media. DFMO reduced cell lengths of *P. teres* and *G. graminis* but increased those of *S. nodorum* and *F. culmorum*. The other ODC inhibitors examined, ΔMFMO, ΔMFMO.CH_3 and RR-MAP increased fungal cell lengths of *S. nodorum* and *F. culmorum* and reduced those of *P. teres*. Interestingly, mycelial growth of *G. graminis* was relatively sensitive towards the inhibitors yet only ΔMFMO affected its cell lengths, increasing them (West & Walters 1989). DFMO reduced cell lengths of *C. ulmi* (Garcia *et al.* 1991) while, in contrast, the inhibitor failed to alter cell lengths in *P. avenae* (Foster & Walters 1990).

It has already been noted that growth of some fungal species is unaffected by DFMO and other ODC inhibitors (for example, West & Walters (1989)). Other such instances of insensitivity towards inhibitors have been reported. DFMO failed to affect mycelial growth of *Pythium ultimum* at a concentration of 5.0 mM (Walters *et al.* 1995b). This result was interesting given that growth of the related fungus, *Phytophthora infestans,* was well controlled by the inhibitor (Walters *et al.* 1995b). DFMO reduced polyamine concentrations and ODC activity in *P. infestans* but failed to affect such enzyme activity and polyamine levels in *P. ultimum* when each fungus was exposed to the inhibitor in liquid culture. *In vitro* sensitivity of ODC from the two fungi to DFMO differed, with enzyme from *P. infestans* being more sensitive than that from *P. ultimum*. Inhibitor uptake was also greater in *P. infestans* compared to *P. ultimum* (Walters *et al.* 1995b). Barker *et al.* (1993) also reported differences in responses to DFMO by related fungi; growth of *P. infestans* was reduced while that of *Phytophthora cactorum* was unaffected. These authors speculated that the difference in growth inhibition was due to the lower amounts of polyamines found in *P. infestans*. Enzyme activity in the fungus was therefore thought to be lower and thus more sensitive towards inhibitors (Barker *et al.* 1993). Similarly, growth of both *Septoria tritici* and *Ustilago maydis* was insensitive towards DFMO and other ODC inhibitors. These findings, however, were unusual since exposure of the fungi to DFMO reduced ODC activity and polyamine levels (Smith *et al.* 1992). One possible explanation for continued growth in the presence of inhibition of polyamine biosynthesis would be the availability of an alternative pathway for putrescine synthesis (Smith *et al.* 1992).

Havis & Walters (1992b) reported how activity of ODC from *Pyrenophora avenae*, *Pyricularia oryzae* and *Uromyces viciae-fabae* was unaffected by 1.0 mM DFMO. Activity of ODC from *P. avenae* could be reduced by 5.0 mM DFMO and was also inhibited by MFMO. RR-MAP, however, did not affect activity of the fungal enzyme (Havis & Walters 1992b). Such work demonstrated that enzymes from different organisms vary in their sensitivity towards inhibitors (Havis & Walters 1992b).

Nevertheless, in addition to controlling plant pathogenic fungi grown using artificial media, ODC inhibitors have been shown to inhibit fungal infections on various plants. Rajam *et al.* (1985) initiated such studies by examining the effect of DFMO on rust infection (*Uromyces phaseoli*) of french beans (*Phaseolus vulgaris*). Complete protection was afforded by concentrations of DFMO of 0.5 mM and higher. At lower concentrations, post inoculation treatments were more effective than pre inoculation sprays (Rajam *et al.* 1985). DFMO also conferred protection to unsprayed parts of plants, demonstrating an ability to be translocated (Rajam *et al.* 1985). In further studies, complete inhibition of rust was achieved by 0.5 mM DFMO when it was applied up to three days post inoculation, while treatment 4 or 5 days after inoculation only resulted in partial pathogen control (Rajam *et al.* 1986).

Similarly, Walters (1986) found that 0.4 mM DFMO gave good control of rust (*Uromyces viciae-fabae*) infection on broad bean (*Vicia faba*) with post inoculation sprays being more effective than pre inoculation treatments. The translocational ability of DFMO was demonstrated when untreated leaf halves were protected from infection (Walters 1986).

Such translocational ability of DFMO was later observed against powdery mildew on barley seedlings when inhibitor applied to plant roots and other leaves reduced disease infection (Walters & Kingham 1990).

DFMO demonstrated good control of *Verticillium dahliae*, the cause of Verticillium wilt, a vascular disease of tomato, by post inoculation applications of the inhibitor (Mussell *et al.* 1987). Good control of *Botrytis cinerea* on various plants, for example, tomato and pepper, was afforded by concentrations of DFMO as low as 0.01 mM (Elad 1991).

Three biotrophic fungal pathogens, *Puccinia recondita* (leaf rust), *P. graminis* (stem rust) and *Erysiphe graminis* (powdery mildew) on wheat were controlled by DFMO at relatively low concentrations (Weinstein *et al.* 1987). In general, inhibitor application after inoculation with the pathogen was more effective than a pre inoculation treatment (Weinstein *et al.* 1987). Foster & Walters (1992a) also reported how DFMO reduced stem

rust infection of wheat. However, pre inoculation treatments were more effective than post inoculation sprays in this instance (Foster & Walters 1992a).

Powdery mildew infection on barley was reduced by a range of ODC inhibitors; DFMO, ΔMFMO, ΔMFMO.CH_3, RR-MAP and α-hydrazinoornithine in glasshouse experiments, with post inoculation treatments generally being more effective than pre inoculation sprays (West & Walters 1988). Control of powdery mildew on barley in the field by DFMO was demonstrated by Havis & Walters (1992a). Here, DFMO controlled early season powdery mildew infection as well as a commercial fungicide and although late season control was not as good, grain weights were increased compared to untreated controls (Havis & Walters 1992a).

While agreement exists as to the effectiveness of DFMO and ODC inhibitors against plant pathogens, disagreements are apparent with regards to the optimum timing of application. Foster & Walters (1992a) indicated that pre inoculation treatments were more effective while various other workers suggested a preference for post inoculation treatments (Rajam *et al.* 1985; Walters 1986; Weinstein *et al.* 1987). Weinstein *et al.* (1987) suggested that DFMO applied pre inoculation could enter the leaf and react with plant ODC enzyme, rendering the inhibitor unavailable to act upon fungal ODC. After observing DFMO to possess systemic activity, Walters (1986) suggested that the inhibitor may require to be applied prior to inoculation to allow time for translocation of the compound to untreated parts of the plant. Indeed, it may be translocation of DFMO, a metabolite of the inhibitor or an induced antifungal compound which is required for fungicidal activity (Rajam *et al.* 1985). Alternatively, post inoculation treatments may be more effective were DFMO to act upon early germling development of the fungus on the leaf surface, as suggested by Walters (1995).

Indeed, DFMO inhibited the germination of rust uredospores in a culture medium. In addition, spore germination was delayed and germ tube growth restricted (Rajam *et al.* 1989). Spore germination on plants was completely inhibited by 0.5 mM DFMO applied 1 day before inoculation, while application 1 day after inoculation caused greater damage to the uredosporelings (Rajam *et al.* 1989). DFMO inhibited the germination of *C. ulmi* spores *in vitro* (García *et al.* 1991) and inhibited sporidial formation of *Neovossia indica* in culture (Singh *et al.* 1989). Spore germination and hyphal growth of the mycorrhizal fungus *Glomus mosseae* were both reduced by DFMO (El Ghachtouli *et al.* 1996).
In contrast, DFMO had little effect on germination and germ tube growth of wheat stem rust fungal spores on plants although production of spores was reduced (Machatschke *et al.* 1990). These authors speculated that enough endogenous polyamines were present in

spores to support germination and growth for some time, thus accounting for the lack of inhibitor effects initially observed (Machatschke *et al.* 1990). Germination of wheat bunt fungal spores *in vitro* was unaffected by the inhibitor, however, DFMO did inhibit hyphal growth (Trione *et al.* 1988). Similarly, Reitz *et al.* (1995a) found that DFMO did not affect germination of rust uredospores on artificial membranes, however, the inhibitor did affect germling development by reducing appressorium formation almost completely when used at a concentration of 2.0 mM.

Anomalies exist between fungal growth on plants and that in culture; fungi on plants are exposed to inhibitors early on in their development while in culture, growing mycelium is used. Biotrophic fungi cannot be grown easily in culture and inhibitor effects on such fungi can only be suggested through studies using necrotrophic fungi grown on artificial media. Differences between *in vivo* and *in vitro* studies may exist and the exact mechanism(s) by which DFMO and other ODC inhibitors act against pathogenic fungi on plants may be unclear.

ODC inhibition has been demonstrated for some fungal species grown *in vitro*. DFMO reduced ODC and increased AdoMetDC activities of *P. avenae* whilst causing reductions in putrescine and spermidine concentrations (Foster & Walters 1990). The inhibitor reduced ODC activity in four phytopathogenic fungi, accompanied by decreases in putrescine and spermidine and an increase in spermine levels (Khan & Minocha 1989a). ODC activity in *P. infestans* was reduced by DFMO and accompanied by reductions in putrescine, spermidine and spermine (Walters *et al.* 1995b). Interestingly, cadaverine concentration decreased in *P. avenae* exposed to DFMO (Foster & Walters 1990). The exact mechanism for this reduction is unknown and it is in contrast to previous work where cadaverine actually increased in *Neurospora crassa* when polyamine depletion occurred (Paulus *et al.* 1982).

In vitro enzyme studies have also been carried out, although these may not always reflect *in vivo* effects on enzyme activities. Smith and co-workers (1990a) extracted ODC from *B. cinerea* and observed that DFMO did indeed inhibit the enzyme. Interestingly, RR-MAP and ΔMFMO also inhibited the enzyme well whilst they were less effective at inhibiting growth of *B. cinerea* on solid media (Smith *et al.* 1990a).

Nevertheless, given the many instances of inhibition reversal by polyamines, polyamine biosynthesis and/or function would appear to be involved in the antifungal and fungicidal effects of DFMO and other ODC inhibitors.

Only ODC inhibition can specifically inhibit polyamine biosynthesis in most species of plant pathogenic fungi since AdoMetDC, spermidine and spermine synthase are vital to both fungi and plants. However, research into inhibition of the other enzymes has been

carried out given the success of ODC inhibitors in controlling fungal growth and the possibility that such inhibitors may not wholly act through inhibition of polyamine biosynthesis but rather by disrupting function(s) of the amines. While ADC is primarily a plant enzyme, some fungi are known to possess both the ODC and ADC pathways to putrescine. Thus, research into inhibition of ADC activity has also been carried out.

1.1.8.2 Inhibitors of AdoMetDC

West & Walters (1989) observed that MGBG inhibited the growth of four necrotrophic fungi species, *P. teres, G. graminis, S. nodorum* and *F. culmorum* Indeed, MGBG was the most effective inhibitor examined, being more effective than DFMO and CHA, and causing substantial reductions in fungal growth when used at a concentration of 2.0 mM. Combination with DFMO was effective in reducing growth of all pathogens but no more so than MGBG alone (West & Walters 1989). Such combinations of inhibitors, targetting different enzymes, was proposed to be more effective in inhibiting polyamine biosynthesis than a single inhibitor, providing greater reductions of intracellular polyamines (Jänne *et al.* 1985). Indeed, mycelial growth of *B. cinerea* was reduced by MGBG and this reduction increased when the inhibitor was used along with DFMO (Smith *et al.* 1990b). Mycelial growth of *O. ulmi* was controlled successfully by a mixture of MGBG and CHA (Biondi *et al.* 1993).

Foster & Walters (1990) demonstrated that growth of *P. avenae* was inhibited by both MGBG and EMGBG. Indeed, mycelial growth was almost completely depressed by 2.0 mM MGBG. When combined with DFMO, MGBG successfully inhibited mycelial growth of *P. avenae*, although no more effectively than MGBG alone (Foster & Walters 1990). Interestingly, inhibitory effects of MGBG, EMGBG and MGBG/DFMO were not reversed by the inclusion of putrescine or spermidine in the media. However, the respiration rate of the fungus was reduced by MGBG and the mixture of MGBG and DFMO, thus, the inhibitory effects of these treatments may not have been solely due to effects on AdoMetDC (Foster & Walters 1990).

Recently, Bharti & Rajam (1996) observed that MGBG successfully inhibited mycelial growth of four pathogenic fungi. Indeed, MGBG was more effective than DFMO and CHA in controlling fungal growth of the species examined (Bharti & Rajam 1996).

Various effects on fungal cell lengths were caused by exposure to MGBG (West & Walters 1989). Cell lengths of *P. teres* and *G. graminis* were reduced while those of *F. culmorum* were increased (West & Walters 1989). Similar findings were reported for MGBG and DFMO combined (West & Walters 1989). Foster & Walters (1990) found little effect on

cell size in *P. avenae* following exposure to MGBG or EMGBG. A mixture of MGBG and DFMO did, however, reduce cell lengths (Foster & Walters 1990).

Powdery mildew on barley was reduced by MGBG applied both as pre and post inoculation treatments, although post inoculation sprays were generally more effective (West & Walters 1988). A concentration of 2 mM almost completely prevented mildew infection. Mixtures of MGBG and DFMO were also effective in reducing disease and were generally more effective than either compound alone (West & Walters 1988).

Foster & Walters (1992a) reported that MGBG and MGBG/DFMO controlled infection of wheat by the stem rust fungus, although they found that pre inoculation treatments were more effective than post inoculation sprays. Mixtures of MGBG and DFMO were observed to be more consistent in reducing infection on the plants (Foster & Walters 1992a).

Thus variation exists with regard to the efficacy of pre and post applications of MGBG. As Weinstein *et al.* (1987) proposed for DFMO and ODC, pre inoculation treatments may be less effective if MGBG were to bind to plant AdoMetDC and thus render it unavailable to act upon fungal AdoMetDC. However, inhibitors may be acting upon development of the fungus on the leaf surface as suggested by Walters (1995). Indeed, Reitz *et al.* (1995a) observed that MGBG reduced germination and prevented appressorium formation of rust urcdospores on artificial membranes when used at a concentration as low as 0.025 mM.

Various effects of MGBG and EMGBG on intracellular enzyme activities and polyamine concentrations in *P. avenae* grown in liquid culture were observed by Foster & Walters (1990). MGBG reduced ODC activity while EMGBG had no effect on this enzyme. AdoMetDC activity was substantially reduced by EMGBG but only slightly by MGBG. Both EMGBG and MGBG reduced spermidine concentrations while MGBG also reduced spermine. A mixture of MGBG/DFMO reduced ODC activity while no effect on AdoMetDC was observed. Putrescine and spermidine concentrations were reduced and spermine remained unaltered (Foster & Walters 1990). Interestingly, cadaverine was also reduced by MGBG, EMGBG and the mixture of MGBG/DFMO (Foster & Walters 1990).

Further work on inhibitors of AdoMetDC was carried out by Walters and co-workers (1995a). The trypanocidal agents Berenil and pentamidine, shown to irreversibly inhibit AdoMetDC from rat liver, yeast, *Escherichia coli* and *Trypanosoma brucei brucei* (Bitonti *et al.* 1986b), reduced mycelial growth of *Pyricularia oryzae* and infection of barley powdery mildew. However, activity of AdoMetDC from fungus was inhibited by the compounds yet the enzyme in fungus exposed to the compounds was unaffected (Walters *et*

al. 1995a). Thus, *in vivo* and *in vitro* enzyme studies may not be comparable. Putrescine levels increased while spermidine was unaltered and spermine levels reduced. The authors, however, suggested that such altered polyamine concentrations were unlikely to account for the antifungal and fungicidal activity of the compounds observed (Walters *et al.* 1995a). Interestingly, cadaverine concentration of *P. oryzae* was greatly increased upon exposure of the fungus to both Berenil and pentamidine, however, the reason for such an increase without considerable depletion of polyamines in unknown (Walters *et al.* 1995a). Instances of cadaverine and derivative accumulation in response to polyamine depletion have recently been reported by Zarb & Walters (1994b).

1.1.8.3 Inhibitors of spermidine synthase

West & Walters (1989) reported how the spermidine synthase inhibitor, CHA, inhibited mycelial growth of *G. graminis,* with a concentration of 4.0 mM reducing growth by 80%. Little effect was however observed for *P. teres*, *S. nodorum* and *F. culmorum* (West & Walters 1989). Cell lengths of *P. teres* were reduced in the presence of CHA while those of *S. nodorum* were unaltered (West & Walters 1989). Recently, Bharti & Rajam (1996) reported inhibition of four pathogenic fungal species by bis-(cyclohexylammonium) sulphate (BCHA). CHA failed to affect mycelial growth of *B. cinerea* when used at a concentration of 1.0 mM (Smith *et al.* 1990b). Interestingly though, the compound acted as a synergist for DFMO mediated inhibition, increasing control of growth afforded by DFMO alone (Smith *et al.* 1990b).

Powdery mildew on barley was inhibited by CHA in pre and post inoculation treatments, although post inoculation treatments were generally more effective (West & Walters 1988). The mechanisms by which CHA affects fungal growth on plants has not been studied in any great detail, but Reitz *et al.* (1995a) found that rust uredospore germination was not affected although appressorium formation was reduced on artificial membranes by a concentration of 3.0 mM CHA.

1.1.8.4 Inhibitors of spermine synthase

No specific inhibitors of spermine synthase have been examined as potential inhibitors of plant pathogenic fungi. Some fungi are indeed capable of synthesising spermine, however, in other species, spermine is either absent, or present in only very low quantities (Stevens & Winther 1979).

1.1.8.5 Inhibitors of ADC

Rajam & Galston (1985) studied the effects of the ADC inhibitor DFMA on mycelial growth of four plant pathogenic fungal species. DFMA strongly inhibited growth of

Botrytis sp, *B. cinerea*, *Rhizoctonia solani* and *Monilinia fructicola* and was indeed, observed to be more effective than DFMO. Inclusion of polyamines with the inhibitor reversed the inhibition, suggesting that DFMA affected growth through an effect on polyamine levels.

Inhibition of mycelial growth of various other fungal species has been reported to have been caused by DFMA including *Helminthosporium carbonum*, *Fusarium oxysporum* and *Ceratocystis ulmi* (Khan & Minocha 1989a). In general, inhibition of growth of these fungal species was less pronounced with DFMA compared to DFMO. The inclusion of polyamines into media amended with DFMA reversed the growth inhibition and indicated that DFMA was acting via the depletion of polyamines (Khan & Minocha 1989a). Growth of *Ophiostoma ulmi* was inhibited by DFMA, DFMA being more effective than DFMO in this instance (Biondi *et al.* 1993). DFMA produced varying results on mycelial growth of four species of pathogenic fungi examined by Bharti & Rajam (1996). *Helminthosporium oryzae* and *Curvularia lunata* were inhibited while growth of *Pythium aphanidermatum* and *Colletotrichum capsici* was stimulated (Bharti & Rajam 1996). The inhibitor was ineffective against mycelial growth of *Neovossia indica* (Singh *et al.* 1989) and *Helminthosporium maydis* (Khan & Minocha 1989a).

Mycelial growth of *Botrytis cinerea* was reduced by DFMA, however, genuine ADC activity could not be detected in *B. cinerea* (Smith *et al.* 1990a,b). Since the inhibitor had no effect on activity of ODC extracted from the fungus, the growth inhibitory effect of DFMA was thought to be due to its conversion to DFMO by fungal arginase (Smith 1990a,b), as suggested by Rajam & Galston (1985). Nevertheless, genuine ADC activity has been reported to be predominant in *O. ulmi* (Biondi *et al.* 1993) and *C. ulmi* (Khan & Minocha 1989a,b). Generic differences between fungi may be responsible for the varying effects on growth observed for DFMA and DFMO, as suggested by Birecka *et al.* (1986). Indeed, instances of increased efficacy of DFMA compared to DFMO have been implicated with increased uptake of the compound (Boyle *et al.* 1988).

Mycelial morphology and cell size were dramatically altered by DFMA in *Botrytis sp* and *M. fructicola* with numbers of hyphae reduced and fungal cell lengths decreased (Rajam & Galston 1985).

DFMA did not offer protection against rust on french beans even at a concentration of 5.0 mM (Rajam *et al.* 1985). The inhibitor did, however, control stem rust and leaf rust on wheat plants albeit not as effectively as DFMO (Weinstein *et al.* 1987). Powdery mildew on barley was reduced by DFMA both pre and post inoculation, with pre inoculation

treatments being more effective (West & Walters 1988). The mechanisms by which DFMA affects fungi on leaf surfaces have not been studied in any detail. However, DFMA inhibited the germination of rust uredospores in a culture medium, delayed spore germination and restricted germ tube growth (Rajam *et al.* 1989). Also, DFMA had no effect on the germination of wheat bunt fungal spores *in vitro* but inhibited hyphal growth (Trione *et al.* 1988).

1.1.8.6 Polyamine analogues

Recent studies on the control of plant pathogenic fungi through the perturbation of polyamine biosynthesis have examined the potential of polyamine analogues as antifungal and fungicidal agents. As discussed previously (section 1.1.4.6, page 20), several reasons exist to suggest that polyamine analogues may be more effective in inhibiting polyamine biosynthetic enzymes than specific enzyme inhibitors.

Foster & Walters (1993) reported how the putrescine analogue, keto-putrescine, provided control of a number of plant pathogens. Powdery mildew on barley, rust and chocolate spot on broad bean, brown rust on barley, powdery mildew on apple and late blight on potato were all effectively controlled by the analogue. Pre and post inoculation sprays were both effective, however, post inoculation treatments tended to afford greater disease control. Keto-putrescine was most effective against chocolate spot infection of broad bean (Foster & Walters 1993).

The analogue reduced *in vitro* growth of *P. infestans, P. avenae* and *P. oryzae,* although it was less effective against *in vitro* growth than fungal infections on plants (Foster & Walters 1993). Indeed, keto-putrescine was relatively poor at inhibiting mycelial growth of *B. cinerea* (Smith *et al.* 1990b). Although such phenomena cannot be directly correlated, germination of *Aspergillus nidulans* was delayed by keto-putrescine in previous work (Stevens *et al.* 1977), suggesting that the analogue might indeed affect fungal development on the leaf surface, and be less effective against growing mycelium (Foster & Walters 1993). Alternatively, greater uptake and accumulation of the compound by a developing fungus could lead to greater effects *in vivo* as opposed to effects on mycelium (Foster & Walters 1993)

Interestingly, another putrescine analogue, N-acetylputrescine, did not affect barley powdery mildew infection of plants, nor *in vitro* growth of pathogens examined (Foster & Walters 1993). Similarly, no effect of the analogue on growth of *B. cinerea* was observed by Smith and co-workers (1990b). However, the lack of fungicidal and antifungal activity

reported may have been due to a deacetylase, found in some fungi, which can metabolise N-acetylputrescine (Haywood & Large 1986).

Both ODC and AdoMetDC activities in *P. avenae* were reduced when exposed to 1.0 mM keto-putrescine. Spermidine levels were decreased, spermine levels increased and fungal putrescine concentration remained unaffected (Foster & Walters 1993). Interestingly, Stevens and co-workers (1977) exposed *A. nidulans* to keto-putrescine and observed ODC activity to increase. This may have been due to enzyme stabilisation. An increase in putrescine concentration was also observed along with reductions in spermidine and spermine levels (Stevens *et al.* 1977). Clearly, the analogue has differing effects on polyamine biosynthesis in different fungal species. Keto-putrescine also caused a substantial increase in the concentration of cadaverine in *P. avenae* (Foster & Walters 1993). As discussed earlier, increased cadaverine concentrations have been observed to occur in response to polyamine depletion (Paulus *et al.* 1982; Zarb & Walters 1994b). Foster & Walters (1993) noted how the increased cadaverine levels could support fungal growth in the absence of spermidine.

Keto-putrescine was relatively successful as a fungicidal and antifungal agent. Varying effects on fungal polyamine biosynthesis have been reported and therefore, no conclusions with regards to the mode(s) of action of the analogue can be made. Nevertheless, novel putrescine analogues were synthesised in an attempt to control fungi via a perturbation of polyamine biosynthesis. Good control of a number of plant pathogen interactions; powdery mildew on barley, rust and chocolate spot on broad bean, powdery mildew on apple and late blight on potato, were afforded by (E)-1,4-diaminobut-2-ene (E-BED) and the cis isomer, Z-BED (Havis *et al.* 1994a). E-BED was generally more effective than Z-BED and while pre and post inoculation sprays both gave good control of infections, post inoculation treatments were more effective (Havis *et al.* 1994a). Best control of plant infections was observed against chocolate spot on broad bean with 1.0 mM E-BED (Havis *et al.* 1994a). E-BED also demonstrated good early season control of powdery mildew on barley in the field, being as effective as a commercial fungicide, and although later season control was not as effective, an equivalent increase in grain yield compared to untreated controls was observed (Havis *et al.* 1994b). (E)-N,N,N',N'-tetraethyl-1,4-diaminobut-2-ene (E-TED) and derivatives similarly controlled plant pathogenic fungal infections well (Havis *et al.* 1994c). As with the other analogues, post inoculation sprays were most effective and best control was achieved by E-TED against barley powdery mildew (Havis *et al.* 1994c).

Reitz *et al.* (1995b) observed that E-BED and E-TED reduced germination and appressorium formation of rust uredospores on artificial membranes. Such a result offers

evidence that, as suggested for keto-putrescine, the putrescine analogues affect fungal development on the leaf surface. Similarly, a range of polyamine biosynthesis inhibitors have been shown to inhibit conidial germination, germ tube growth and haustorial development of barley powdery mildew (S. Hannif & D. R. Walters unpublished results).

E-BED and Z-BED reduced mycelial growth of *B. cinerea* and *P. oryzae* and growth of *P. avenae* in liquid culture (Havis *et al.* 1994a). Both compounds displayed similar efficacy towards *P. oryzae*, however, E-BED was the more effective of the compounds against *B. cinerea* and *P. avenae* (Havis *et al.* 1994a). Indeed, *B. cinerea* and *P. avenae* were more sensitive towards the analogues than *P. oryzae* (Havis *et al.* 1994a).

E-TED completely inhibited growth of *P. avenae* in liquid culture, at a concentration of 1.0 mM. That same concentration, however, failed to affect mycelial growth of the fungus on solid media (Havis *et al.* 1994c).

ODC and AdoMetDC activities of *P. avenae* exposed to E-BED were substantially reduced. Putrescine and spermine concentrations were increased while spermidine levels decreased (Havis *et al.* 1994a). In contrast, Z-BED reduced ODC activity slightly while AdoMetDC activity was increased. Spermidine concentrations were reduced but putrescine and spermine levels remained unchanged (Havis *et al.* 1994a).

E-TED increased ODC activity in *P. avenae* while AdoMetDC activity was reduced. Putrescine and spermidine concentrations decreased while spermine levels were unaltered (Havis *et al.* 1994c).

A common effect in fungus exposed to all analogues described above was the reduction in spermidine levels. Since spermidine is the predominant polyamine in fungi (Stevens & Winther 1979), this may have been responsible for the observed antifungal effects. However, as substantial pools of spermidine generally remained, perturbation of polyamine biosynthesis was suggested by the authors not to be the major inhibitory mechanism of these putrescine analogues (Havis *et al.* 1994a,c). Often, an increase in putrescine was observed. Whatever the cause of this increase, it may have been responsible for the antifungal effects observed (Havis *et al.* 1994a).

More recent work has shown that E-BED inhibits DNA methylation (Walters 1997), and that it also causes a very substantial decrease in uptake of amino acids, polyamines and carbohydrates in *P. avenae* (Walters & McPherson 1997). Havis and co-workers (1996b) also reported how E-TED increased spermine concentration in *P. infestans* and suggested

the spermine was displaced from intracellular binding sites. Exactly how these effects of E-BED and E-TED relate to their antifungal activity is unknown at present.

Following studies on aliphatic diamines as analogues of putrescine, cyclic diamines were synthesised and tested for fungicidal and antifungal effects on plant pathogenic fungi. 1,2-bis(aminomethyl)-4,5-dimethylcyclohexa-1,4-diene (BAD) and various derivatives controlled powdery mildew on barley with post inoculation treatments providing greater control over pre inoculation treatments (Havis *et al.* 1996a). As with the aliphatic diamines, differences in efficacy were apparent between cis and trans isomers, trans-BAD being more effective against powdery mildew of barley than cis-BAD (Havis *et al.* 1996a). Unlike the aliphatic diamines, BAD and derivatives did not affect growth or polyamine biosynthesis in *P. avenae* to any great extent (Havis *et al.* 1996a).

Additional cyclic diamines were synthesised and their fungicidal activity tested against powdery mildew on barley. Trans-1,2-bis(diethylaminomethyl)cyclopentane (compound 1), trans-5,6-bis(aminomethyl)bicyclo[2.2.1]hept-2-ene (compound 2) and 1,2-bis(dimethyl-aminomethyl)-4,5-dimethylcyclohexa-1,4-diene (compound 3) afforded good control of powdery mildew infection with post inoculation treatments being more effective than pre inoculation treatments (Havis *et al.* 1997). Only limited activity was observed against chocolate spot and rust of broad bean (Havis *et al.* 1997). All three compounds reduced growth of *P. avenae in vitro*. However, differing effects were observed on polyamine levels in fungus exposed to the compounds. Compound 1 reduced levels of putrescine, spermidine and spermine, decreased ODC activity and slightly increased AdoMetDC activity. Compound 2 increased spermidine levels and reduced putrescine and spermine concentrations. ODC activity was increased while AdoMetDC activity was unaltered. Compound 3 reduced putrescine levels while spermidine and spermine concentrations were unaltered. ODC activity was reduced only slightly while AdoMetDC activity increased substantially (Havis *et al.* 1997). Such changes in polyamine levels could not be explained by the corresponding alterations in biosynthetic enzyme activities. Thus, as with the aliphatic diamines, the fungicidal and antifungal effects of these cyclic diamines is unlikely to have been wholly caused by an effect on polyamine metabolism (Havis *et al.* 1997).

Fungicidal activity of spermidine analogues has been briefly studied. Powdery mildew on barley was controlled in glasshouse experiments by 1.0 mM concentrations of N^1- and N^8-acetylspermidine. Mycelial growth of *P. avenae* and *P. oryzae*, however, was largely unaffected by 1.0 mM concentrations of the compounds (S. A. Foster unpublished results). Two novel spermidine analogues, C59 and C73, also gave good control of barley powdery

mildew, rust on broad bean and powdery mildew on apple when applied as post inoculation treatments in glasshouse experiments (N. D. Havis unpublished results).

1.1.8 FUNGAL RESEARCH - FURTHER CONSIDERATIONS

1.1.8.1 Effects of polyamine biosynthetic inhibitors on plants

Specific control of fungal growth via inhibition of polyamine biosynthesis should, in theory, not affect plants. Nevertheless, research into the effects of polyamine biosynthesis inhibitors on plants has been carried out, being necessary if inhibitors are to be used against fungi growing on plants.

In attempting to control rust on broad beans, Walters (1986), observed that no effect on plant growth nor polyamine levels of beans was caused by DFMO application. However, Rajam and co-workers (1986) demonstrated that putrescine and spermidine levels increased in french bean plants treated with DFMO. This increase was suggested to be the result of stimulated plant ADC activity in response to ODC inhibition (Rajam *et al.* 1986). Later, Rajam *et al.* (1991) examined the effects of DFMO and DFMA on endogenous polyamine levels in bean plants and although polyamine concentrations were slightly affected, growth continued unabated (Rajam *et al.* 1991). Application of DFMO, DFMA, MGBG and CHA, alone and in combinations did not significantly alter polyamine levels or affect growth of tomato seedlings (Felix & Harr 1989). Interestingly, the rate of photosynthesis of detached barley leaves decreased after only a 30 minute exposure to 1.0 mM DFMO. However, the return to near normal rates after 12 hours of exposure to the chemical indicates that the effect of DFMO on plant growth should be minimal (Coghlan & Walters 1992). Recently, MGBG and BCHA were shown to be non-phytotoxic towards wheat seedlings (Bharti & Rajam 1995). Thus, the potential of inhibitors to control plant pathogenic fungi is recognised as being viable.

1.1.8.2 Effects of polyamine biosynthetic inhibitors on mycorrhizal fungi

Mycorrhizal fungi are intimately associated with most plants and if polyamine biosynthesis inhibitors are to be used to control pathogenic infections, consideration must be given to their effects on such fungi.

Research has shown that different mycorrhizal fungi exhibit quite different responses when exposed to polyamine biosynthetic inhibitors *in vitro*. Zarb & Walters (1994c) found that mycelial growth of *Laccaria proxima* was only slightly reduced by DFMO yet slightly increased by DFMA. However, growth of *Paxillus involutus* was substantially reduced by DFMO (Zarb & Walters 1994a). Mycelial growth of *Crinipellis perniciosa* was reduced by both DFMO and DFMA as well as the ODC inhibitors MFMO.CH_3 and RR-MAP (Zarb & Walters 1993). DFMO also reduced mycelial growth of *Thelephora terrestris*, yet had no effect on *Hebeloma mesophaeum* (Zarb 1995). Varying effects on polyamine biosynthetic enzyme activities and polyamine concentrations were observed in the different fungal

species (Zarb & Walters 1993, 1994a,c; Zarb, 1995). Interestingly, in *P. involutus* and *L. proxima*, DFMO was suggested to affect cellular processes other than polyamine biosynthesis, for example, transmethylation reactions and amino acid synthesis (Zarb & Walters 1994a,c). Although different mycorrhizal fungi differ in their responses to polyamine biosynthetic inhibitors *in vitro*, the effect of such inhibitors on mycorrhizal infection and establishment on plant roots is not known and would have to be examined in detail should an effective polyamine biosynthetic inhibitor suggest itself for commercial use.

1.1.8.3 Control of plant pathogenic fungi - an overview

Even with effective disease control, around 12 % of crop yield potential is lost to diseases (Agrios 1988). Many methods are used to control plant disease including cultural measures and the use of resistant varieties. Fungicide use is, however, paramount.

In the UK, cereal diseases are the most important plant diseases which require consideration. These have been well controlled in the past by morpholines and triazoles, compounds which inhibit ergosterol biosynthesis. Ergosterol is a vital component of fungal membranes and its complicated biosynthetic pathway presents many potential enzymes for targetted inhibition. Morpholines inhibit biosynthesis at two particular sites, namely prevention of Δ^7-Δ^8 isomerisation and Δ^{14} reduction while triazoles inhibit the C14 demethylation step. Such specific inhibition has, however, allowed resistant pathogens to evolve and consequently, the fungicides tend to be used in mixtures (Hassall 1990).

Recent developments in fungicide research include the use of new compounds known as strobilurins which are synthetic analogues of naturally occuring antifungal compounds produced by the basidiomycete fungus *Strobilurus tenacellus*. Such compounds act by inhibiting the respiratory process of fungi. Two molecules are currently marketed, kresoxim-methyl is added to fenpropimorph (Ensign, BASF), and azoxystrobin (Amistar, Zeneca) is to be commercially launched fully in 1998. These new compounds offer a wide spectrum of disease control and as natural compounds, are environmentally acceptable (Anon 1997).

Other developments include cyprodinil (Unix, Novartis), as yet unavailable in the UK but offering control of eyespot, *Septoria nodorum* and mildew on wheat. Quinoxyfen (Fortress, DowElanco) is a quinolene and offers protectant action against mildew. Finally, Novartis are awaiting approval of their Plant Activator SAR which is not in actual fact a fungicide, but acts by activating the plants own resistance response (Anon 1997).

In spite of continuing developments, the number of available active ingredients for fungicide usage is decreasing. This is a result of increasing legislation with regards to toxicological and environmental considerations. Coupled with the threat of resistance, the need for novel fungicides with alternative modes of action is paramount (DeWaard *et al.* 1993).

1.2 AIM OF STUDY

The inhibition of polyamine biosynthesis has been recognised as a potential target for specific control of plant pathogenic fungi for over 10 years. During this time research has progressed and the most recent work has studied putrescine analogues in an attempt to inhibit ODC and/or affect cellular functions for which putrescine is vital.

Despite affording good control of a number of plant pathogens *in vitro* and *in vivo*, putrescine analogues failed to deplete spermidine pools completely. Since spermidine is known to be the predominant polyamine in fungi and in some species, is absolutely essential for growth, residual spermidine has been implicated in the limited efficacy of putrescine analogues. Thus, it was suggested that spermidine analogues might be more efficient at perturbing polyamine biosynthesis and/or cellular function(s) related to polyamines in fungi.

Although inhibition of spermidine synthase is not specific with regards to control of growth of pathogenic fungi on plants, putrescine analogues are now thought perhaps to afford control of fungal pathogens by mechanisms unconnected to polyamine biosynthesis. Thus, exploration of spermidine analogues as potential inhibitors of polyamine biosynthesis and/or functions in fungi on plants was warranted.

Analogues are proposed to be more effective than specific enzyme inhibitors since they may affect any function for which polyamines are essential. Spermidine synthase is not as highly regulated as ODC and AdoMetDC, thus perturbation of its activity is not likely to lead to elaborate compensatory measures within the cell.

This study was thus designed to explore fully the possibility of controlling growth of plant pathogenic fungi through the inhibition of fungal polyamine biosynthesis and/or an effect on cellular functions associated with spermidine by the use of spermidine analogues.

Chapter 2

Effect of cyclohexylamine and norspermidine on fungal infection of host plants

2. EFFECT OF CYCLOHEXYLAMINE AND NORSPERMIDINE ON FUNGAL INFECTION OF HOST PLANTS

2.1 INTRODUCTION AND OBJECTIVES

Preliminary experiments were designed to determine the fungicidal properties of two commercially available compounds: cyclohexylamine (CHA), an inhibitor of spermidine synthase and norspermidine, a spermidine analogue (Figure 4). *In vivo* effects of these compounds upon several plant pathogens were examined, in most cases investigating the protectant action of the compounds as well as their eradicant properties. Systemic activity of the compounds against one pathogen was also examined. Before detailing the experiments performed, the biology and control of each of the pathogens will be discussed briefly, along with an indication of their agricultural importance.

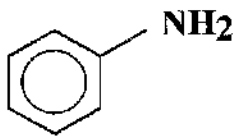
2.1.1 Broad bean rust

Broad beans are an important crop worldwide, providing protein for livestock feedstuffs. Production in the UK alone accounts for almost half of that in the European Union (Outsiders Guide 1995). The pathogen responsible for rust of broad bean (*Vicia faba* L.) is a basidiomycete, *Uromyces viciae-fabae* (Pers.) Schroet. In general, the family of rusts are among the most destructive of plant diseases (Agrios 1988). Rust on broad beans is usually seen late in the growing season with leaves becoming randomly covered in reddish-brown pustules, often surrounded by chlorotic tissue. Early infections can lead to defoliation of plants. The pathogen survives only on living plant material, such as volunteer beans or crop debris, primarily as uredospores. Infection is spread by air-borne spores. Disease control measures include cultural methods such as the removal of volunteer plants and crop debris. Chemical control is also available with, for example, a fungicide like fenpropimorph (Corbel, BASF) (Parry 1990).

2.1.2 Broad bean chocolate spot

Chocolate spot disease of broad bean is caused by two organisms: the deuteromycetes *Botrytis fabae* Sardina and *Botrytis cinerea* Pers. ex Fr. Together, these species are the most important pathogens of field beans. Symptoms of the disease are classified into two phases: the non-aggressive disease which can be caused by either pathogen, resulting in discrete brown lesions on leaves, stems and pods and the more aggressive phase, attributable mainly to *B. fabae* and leading to sizeable lesions and the destruction of large areas of tissue. Aggressive chocolate spot may also result in infected pods aborting, and if stems are affected, the occurrence of lodging. This phase of the disease is favoured by high humidity or prolonged rainfall. Both pathogens survive as sclerotia as well as on volunteers and green debris. Wind-borne conidia spread infection throughout crops. Chemical control

Spermidine



CHA

Norspermidine

Figure 4. Structures of spermidine, CHA and norspermidine

is widely practised with foliar-applied fungicides being used at regular intervals throughout the season. Fungicides used include vinclozolin (Ronilan FL, BASF) and carbendazim + chlorothalonil (Bravocarb, ISK Biosciences) (Parry 1990).

2.1.3 Barley powdery mildew

Powdery mildew (*Erysiphe graminis* DC f.sp. *hordei* Marchal) of barley (*Hordeum vulgare* L.) is the most important cereal disease in the UK (Carlile 1995). The ascomycete is an obligately biotrophic parasite, surviving only on living plant material. Volunteers are thus sources of infection. Another great source of infection arises from autumn sown crops, spreading disease to spring sown crops. Wind-borne conidia spread infection, a process which is favoured by warm, dry conditions. Leaves are most commonly affected although symptoms of white fluffy pustules can be found on all aerial parts of the plant. Control measures include cultural methods such as the removal of volunteers and the use of resistant varieties. Resistance, however, can be short lived and growers are recommended to make use of different varieties with varying forms of genetic resistance. Market demands, however, often make such choices impractical and fungicides remain the main method of disease control. Systemic seed treatments will protect against early attacks, for example, ethirimol + flutriafol + thiabendazole (Ferrax, Bayer), while foliar triazole and morpholine fungicides are used throughout the growing season, multiple applications often being required. Such fungicides have begun to encounter resistance problems and are therefore used together in mixtures to provide maximum disease control. Examples include fenpropimorph + propiconazole (Glint 500 EC, Novartis) and fenpropimorph + prochloraz (Sprint, AgrEvo) (Parry 1990). Recent additions to the mildewicide armoury include the quinolene, quinoxyfen (Fortress, DowElanco) and the strobilurin, azoxystrobin (Amistar, Zeneca) (Anon 1997).

2.1.4 Apple powdery mildew

Worldwide, apples are economically the most important fruit grown (de Rougemont 1989). Powdery mildew is an important disease of apples and is caused by the pathogen *Podosphaera leucotricha* (Ell. & Ev.) Salm. An ascomycete, the pathogen survives as mycelium in vegetative buds. Air-borne conidia spread infection to all types of tissue. Symptoms of fine white conidia and spreading mycelium are similar in appearance to barley powdery mildew. Severe infection may result in defoliation of plants, and fruits may fail to develop (Carlile 1995). Apple varieties vary in their susceptibility to the disease, but chemical control is available, with fungicides which provide control of apple scab also affording control of powdery mildew (Toms & Dahl 1976).

2.1.5 Potato late blight

Potatoes are the most important non-cereal crop in the world. The most historically important crop disease is still the most important disease of potatoes: potato late blight, caused by the oomycete pathogen *Phytophthora infestans* (Mont) De Bary. The disease epidemics of the 1840s led to one quarter of the 8 million population of Ireland dying and another one million emigrating, mostly to Northern America, thereby altering the course of world history (Parry 1990).

The pathogen survives as mycelium in tubers, such as seed stock, groundkeepers or dumped potatoes. Diseased tubers give rise to diseased haulms and the sporangiospores produced are dispersed by rain-splash or wind currents. Haulm symptoms of brown necrotic patches are often seen at the edges or tips of leaves. Infected tubers have inward progressing regions of brown rotted tissue. Zoospores are also produced and their contact with tubers can lead to infection. Such spores can be washed downwards through the soil or can contaminate tubers during harvesting. Disease progression is favoured by warm humid conditions, however, risk periods can be successfully predicted. Cultural control measures include the elimination of groundkeepers and infected haulms at dumps and the planting of certified seed. Resistant varieties are also available. Fungicide use is, however, routine and several sprays are used throughout the season. These include protectants such as fentin hydroxide (Du-Ter 50, AgrEvo) and mixtures of a protectant and a systemic fungicide, for example, mancozeb + metalaxyl (Fubol 58 WP, Novartis) (Parry 1990).

2.2 MATERIALS AND METHODS

2.2.1 Effect of CHA and norspermidine against rust on broad bean

2.2.1.1 Growth of plant material

Broad bean seeds (cv. Threefold White) were sown individually in 9 cm pots using Fison's Levington compost. Plants were grown in a ventilated glasshouse under natural daylight supplemented to a 16 hour photoperiod with 400W mercury vapour lamps. The average maximum temperature attained during the day was 24°C with an average minimum of 9°C at night.

2.2.1.2 Maintenance of pathogen

Bean seedlings were inoculated when they were approximately 20 days old by painting the leaves with a rust spore suspension (25 mg per 100 ml distilled water), using a small haired brush. The spore suspension was prepared with 0.01 % Tween 20 (v/v) to act as a surfactant. Plants were loosely covered with clear plastic bags for 48 hours to ensure the high relative humidity necessary for spore germination. New stock plants were inoculated at approximately 4 week intervals.

2.2.1.3 Inoculation of plant material and treatment with CHA and norspermidine

Previous work demonstrated that barley powdery mildew was reduced by post inoculation applications of 0.5 mM and 1.0 mM solutions of CHA (West & Walters 1988). Thus, to examine the effects of CHA and norspermidine on the various pathogens, 0.5, 1.0 and 2.0 mM solutions of the compounds were prepared with 0.01 % Tween 20 (v/v). Pre inoculation treatments were applied three hours before inoculation while post inoculation treatments were applied 3 days after inoculation. Timings were chosen on the basis of previous work in this laboratory which found them to be satisfactory for the study of fungicidal activity of putrescine analogues (Havis *et al.* 1994a, c).

Bean seedlings were used for experimentation when they were approximately 20 days old. Plants were sprayed to run off with either CHA or norspermidine solutions using hand held compressed air spray units, either pre or post inoculation. Seedlings were inoculated by painting a suspension of rust spores (25 mg per 100 ml distilled water) onto leaves using a small haired brush before covering the plants with clear plastic bags for 48 hours. Spore suspensions were prepared with 0.01 % Tween 20 (v/v). Disease infection was assessed by estimating the percentage area of each of the third pair of leaves affected, using a standard area diagram 19 days after inoculation. Controls consisted of plants which were inoculated only and eight replicates per treatment were used. As with other experiments performed to

examine the effect of CHA and norspermidine against plant pathogens, significance was assessed using Student's *t*-test. All experiments were repeated and similar results obtained.

2.2.2 Effect of CHA and norspermidine against chocolate spot on broad bean

2.2.2.1 Growth of plant material

Beans were grown as described in section 2.2.1.1.

2.2.2.2 Maintenance of pathogen

Stock cultures of *Botrytis fabae* were maintained on sterile medium X as described by Last and Hamley (1956)[1] . Culture plates were kept in a darkened incubator at a temperature of 24°C and fresh plates initiated approximately every ten days.

2.2.2.3 Inoculation of plant material and treatment with CHA and norspermidine

With a few exceptions, experiments were carried out as detailed in section 2.2.1.3. The suspension of *B. fabae* spores contained approximately 4 x 10^5 conidia per ml of distilled water. Infection intensity was assessed by estimating the percentage leaf area affected using a standard area diagram 7 days after inoculation.

2.2.3 Effect of CHA and norspermidine against powdery mildew on barley

2.2.3.1 Growth of plant material

Seeds of barley (cv. Delibes) were sown in 20 cm x 15 cm trays using Fison's Levington compost and grown under the glasshouse conditions described in section 2.2.1.1.

2.2.3.2 Maintenance of pathogen

Barley seedlings were inoculated when they reached GS 13 (Zadoks growth stage: three leaves unfolded), by simply dusting with powdery mildew conidia. Fresh stock plants were initiated approximately every 4 weeks.

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[1] Composition of media, made up in 1l of distilled water, was as follows: $KH_2.PO_4$ (1.52 g), $MgSO_4.7H_2O$ (0.52 g), $NaNO_3$ (6 g), KCl (0.52 g), Dextrose (10 g), Peptone (2 g), Casein hydrolysate (3 g), Yeast nucleic acids (0.5 g) and Agar (20 g).

2.2.3.3 Inoculation of plant material and treatment with CHA and norspermidine

Seedlings were used for experimentation when they reached GS 13 (Zadoks growth stage: three leaves unfolded). Plants were sprayed to run off with solutions of CHA or norspermidine using hand held compressed air sprayers either pre or post inoculation. Seedlings were inoculated using the method of Nair and Ellingboe (1962). Stock plants were shaken 6 hours prior to experimental inoculation, allowing the production of fresh conidia. Such fresh conidia were shaken onto microscope slides and cotton buds used to lift the conidia and place them onto the barley leaves, in a rolling fashion. This method ensured a more controlled inoculation rather than simply dusting the plants with the powdery mildew fungus. Infection intensity was assessed 14 days later by estimating the percentage area of the third leaf affected using a standard area diagram. Ten replicates were used per treatment with controls consisting of plants which were inoculated only.

2.2.4 Effect of CHA and norspermidine against powdery mildew on apple

2.2.4.1 Growth of plant material

Seeds of apple (*Malus communis* L.) were sown in 20 cm x 15 cm trays using a 3:1 mixture of Fison's Levington compost and horticultural sand. Seed trays were covered with a black plastic bag and seeds stratified by placing in warm storage (20-25°C) for two weeks before a 10-14 week period in cold storage (0-5°C). Seed trays were then uncovered and placed in the glasshouse to germinate under the conditions described in section 2.2.1.1. Before experimentation, seedlings were transplanted to individual 9 cm pots using the same compost/sand mixture.

2.2.4.2 Maintenance of pathogen

Apple seedlings were inoculated after they had been in the glasshouse for 4-5 weeks, having developed 3-4 leaves, by simply dusting with conidia of the powdery mildew fungus. Fresh stock plants were initiated approximately every 4 weeks.

2.2.4.3 Inoculation of plant material and treatment with CHA and norspermidine

Seedlings were used for experimentation when they had developed three unfolded leaves. Poor seed germination resulted in the production of lower numbers of seedlings and thus, for experiments using apples, plants were only treated post inoculation. Post inoculation was chosen as the treatment to be used, as opposed to pre inoculation, since previous work in this laboratory had used post inoculation treatment of apples to study the fungicidal activity of putrescine analogues against powdery mildew (Havis *et al.* 1994a, c). Seedlings

were sprayed to run off with solutions of CHA or norspermidine three days after inoculation using hand held compressed air sprayers. Inoculation was carried out by brushing conidia onto the leaves using a small haired brush. Infection intensity was assessed 21 days after inoculation using an infection key, assessing the third leaf. Six replicates were used for each treatment with controls consisting of plants which were inoculated only.

2.2.5 Effect of CHA and norspermidine against late blight on potato

2.2.5.1 Growth of plant material

Potato tubers (*Solanum tuberosum* L. cv. King Edward) were sown in 15 cm pots using Fison's Levington compost. Plants were grown in the glasshouse under the conditions described in section 2.2.1.1.

2.2.5.2 Maintenance of pathogen

Stock cultures of *Phytophthora infestans* were maintained on sterile potato dextrose agar (PDA). Plates were kept in a darkened incubator at a temperature of 24°C and fresh culture plates initiated approximately every 10 days. Prior to experimentation, the fungus had to be grown on whole potato leaves, since attempts to inoculate experimental leaf discs directly using spore suspensions prepared from artificially grown cultures were unsuccessful. This may have been due to a loss of vigour by the pathogen, having been grown on artificial media for some time. Leaves were removed from potato plants when they were approximately 4-5 weeks old and placed, upper surface downwards, on dampened filter paper in 90 mm plastic Petri dishes. A spore suspension (approximately 5 x 10^4 spores per ml of sterile distilled water) was prepared using fungus maintained on PDA. Leaves were inoculated by pipetting droplets of this suspension onto their surface. Petri dishes were sealed and placed in an incubator at 15°C with a photoperiod of 16 hours, for 10 days.

2.2.5.3 Inoculation of plant material and treament with CHA and norspermidine

Potato leaf discs (15 mm diameter) were cut from stock leaves when plants were approximately 4-5 weeks old. Discs were floated, upper surface downwards, on solutions of CHA or norspermidine in 90 mm plastic Petri dishes. Controls used distilled water only. Discs were used as opposed to whole plants as it is an established method of testing fungicidal activity against blight in the department. The method reduces the space required in incubators which are necessary for disease progression. Such a method examines the systemic activity of CHA and norspermidine against blight. Leaf discs were inoculated by pipetting spore suspension (20 µl) onto the centre point of each disc surface. The spore suspension (approximately 5 x 10^4 spores per ml of sterile distilled water), was prepared

using fungus that had been grown on whole plant leaves. Petri dishes were covered and placed in incubators as described in section 2.2.5.2. Infection intensity on each disc was assessed 8 days later using an infection key. Six replicates per treatment were used.

2.3 RESULTS

2.3.1 Effect of CHA and norspermidine against rust on broad bean

Disease effects aside, none of the plants in any of the experiments appeared to have suffered detrimentally as a result of CHA or norspermidine solution application. The compounds did not appear to have caused phytotoxic effects on any of the plants studied. Therefore, 'fungicidal' effects observed can indeed be attributed to such activity rather than non specific toxic effects of CHA and norspermidine.

All treatments reduced rust infection of broad bean although the reduction caused by the 1.0 mM CHA post inoculation treatment was not significant (Figure 5; Appendix 1). Little difference was observed between the treatments, with higher concentrations giving no better control than the lower concentrations of inhibitors. The efficacies of CHA and norspermidine at comparable concentrations were similar. Pre inoculation sprays appeared to be more effective than post inoculation treatments, although rarely significantly so. Best control was achieved using 1.0 mM CHA, applied pre inoculation, which reduced infection by 71.1 % ($p \leq 0.001$) (Figure 5; Appendix 1).

2.3.2 Effect of CHA and norspermidine against chocolate spot on broad bean

All treatments reduced chocolate spot infection of broad bean although several of the treatments did not do so significantly (Figure 6; Appendix 2). More effective control was achieved through the use of pre inoculation sprays with only the highest concentrations of CHA and norspermidine causing significant reductions in infection when applied post inoculation. Little difference was observed between the efficacies of CHA and norspermidine. Best disease control was achieved with the pre inoculation application of 1.0 mM CHA, which reduced infection by 40.0 % ($p \leq 0.001$) (Figure 6; Appendix 2).

2.3.3 Effect of CHA and norspermidine against powdery mildew on barley

All treatments reduced powdery mildew infection of barley, with 1.0 mM CHA providing best control; applied pre inoculation it reduced infection by 89.7 % ($p \leq 0.01$) (Figure 7; Appendix 3). Little differences were observed between the treatments, with higher concentrations providing no greater disease control. In addition, no differences in efficacy between CHA and norspermidine were detected. Pre inoculation treatments appeared to provide superior control to those treatments applied post inoculation, although this difference was rarely significant (Figure 7; Appendix 3).

2.3.4 Effect of CHA and norspermidine against powdery mildew on apple

All treatments afforded good control of powdery mildew infection on apple (Table 1). Higher concentrations of each of the compounds provided superior disease control compared to lower concentrations. Norspermidine appeared to provide better control than CHA at comparable concentrations (Table 1).

2.3.5 Effect of CHA and norspermidine against late blight on potato

All treatments reduced blight infection of potato leaf discs (Table 2). Increasing concentrations of each compound provided superior disease control when compared to the lower concentrations. Infection was completely prevented by 2.0 mM norspermidine and very substantially reduced by 2.0 mM CHA. Norspermidine thus appeared to provide superior disease control to CHA at comparable concentrations (Table 2).

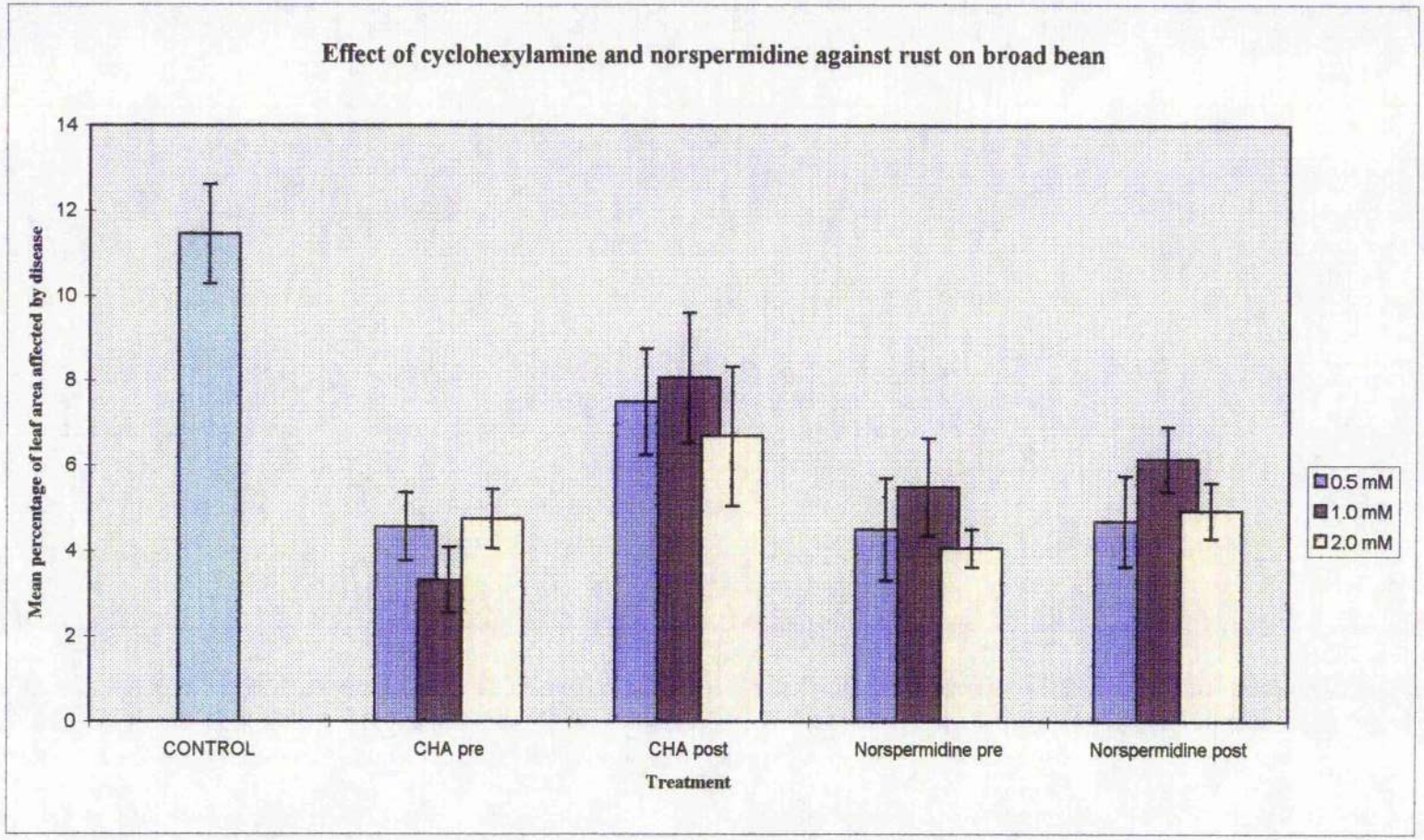

Figure 5. Effect of varying concentrations (0.5, 1.0 and 2.0 mM) of CHA and norspermidine on rust infection of broad bean seedlings. Treatments were applied either pre (3 hours) or post (3 days) inoculation. Values are the means of eight replicates with standard errors of means indicated. Significant differences from control are indicated in Appendix 1.

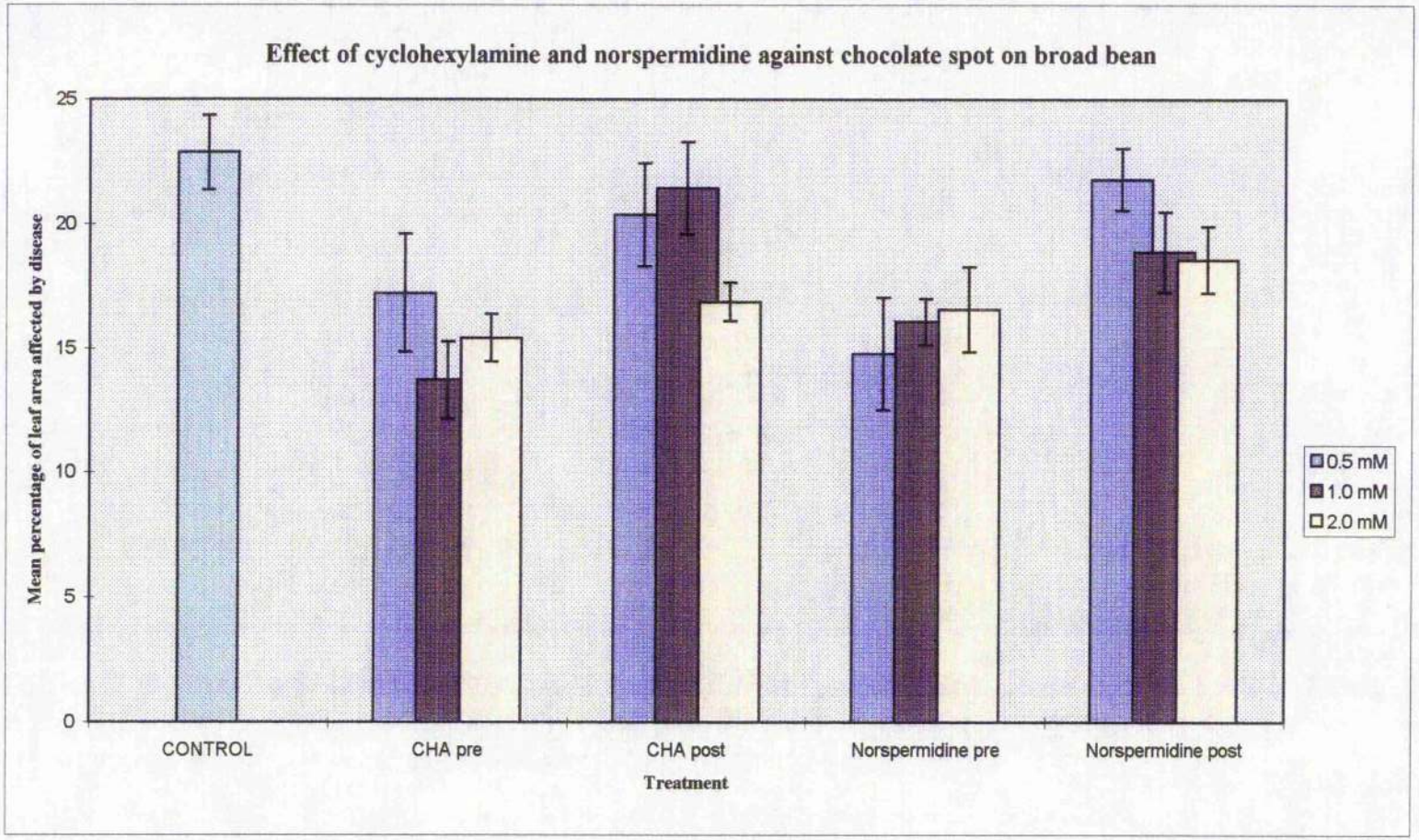

Figure 6. Effect of varying concentrations (0.5, 1.0 and 2.0 mM) of CHA and norspermidine on chocolate spot infection of broad bean seedlings. Treatments were applied either pre (3 hours) or post (3 days) inoculation. Values are the means of eight replicates with standard errors of means indicated. Significant differences from control are indicated in Appendix 2.

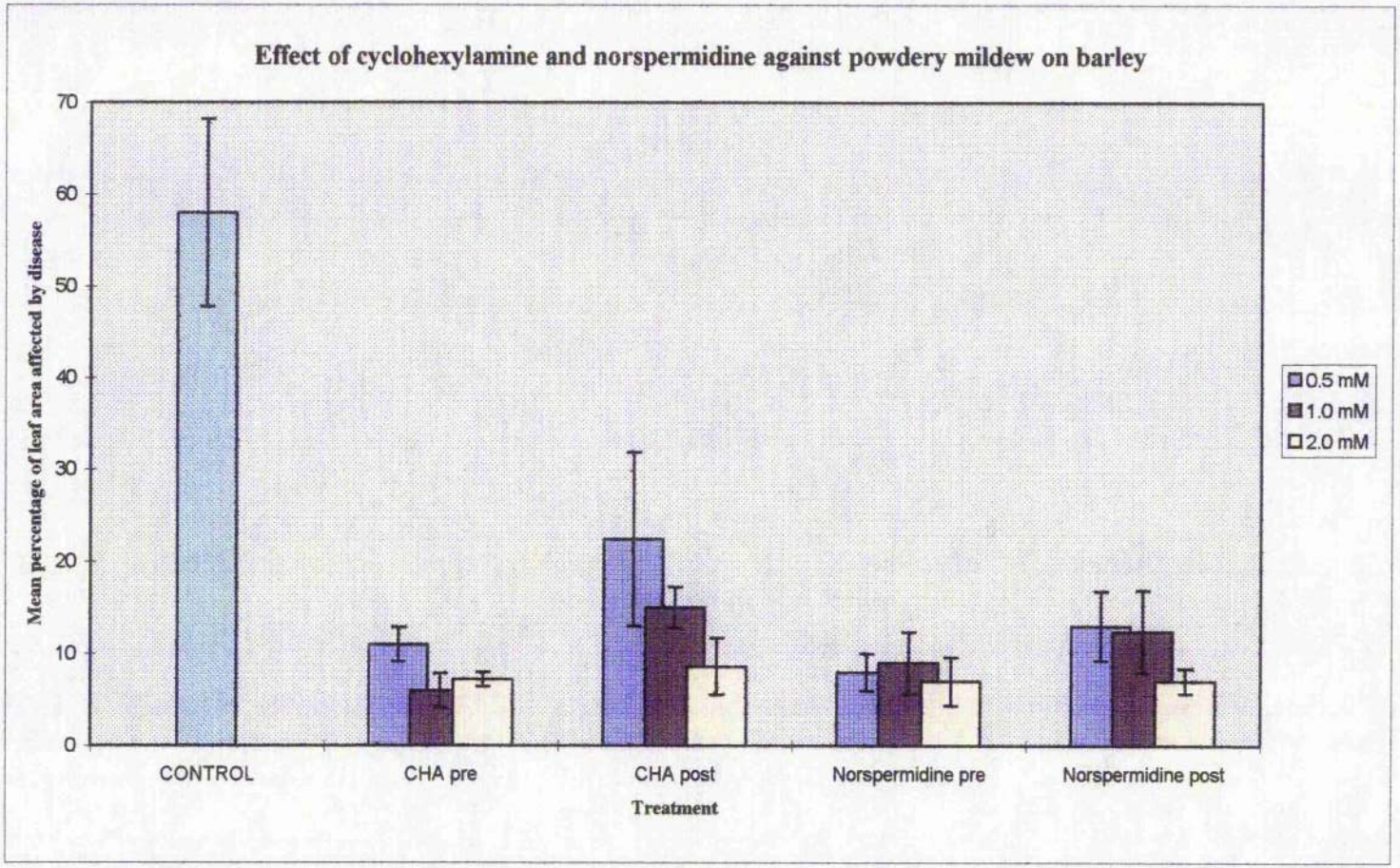

Figure 7. Effect of varying concentrations (0.5, 1.0 and 2.0 mM) of CHA and norspermidine on powdery mildew infection of barley seedlings. Treatments were applied either pre (3 hours) or post (3 days) inoculation. Values are the means of ten replicates with standard errors of means indicated. Significant differences from control are indicated in Appendix 3.

Table 1: Effect of CHA and norspermidine against powdery mildew on apple

Treatment	Mean disease score
Control	3
0.5 mM CHA	2
1.0 mM CHA	2
2.0 mM CHA	1.5
0.5 mM norspermidine	1.5
1.0 mM norspermidine	1
2.0 mM norspermidine	1

Note: Values are shown as the mean disease score of six replicates.

Infection key used for assessment:
3: ≥ 50 % infection
2: < 50 % infection
1: few isolated spores
0: no infection

Table 2: Effect of CHA and norspermidine against late blight on potato

Treatment	Mean disease score
Control	3
0.5 mM CHA	2.5
1.0 mM CHA	2.5
2.0 mM CHA	1
0.5 mM norspermidine	2.5
1.0 mM norspermidine	2.5
2.0 mM norspermidine	0

Note: Values are shown as the mean disease score of six replicates.

Infection key used for assessment:
3: ≥ 50 % infection
2: < 50 % infection
1: few isolated spores
0: no infection

2.4 Effects of CHA and norspermidine on germination and development of rust uredospores on broad bean leaves

2.4.1 Introduction and Objectives

The effects of CHA and norspermidine against rust and chocolate spot on broad bean and powdery mildew on barley indicated that pre inoculation treatments were more effective than post inoculation sprays. Although such differences were not always significant, further work was carried out in an attempt to extend these findings, examining the effects of the compounds on the development of rust germlings on the leaf surface. Bean rust was chosen since it was used previously in this laboratory to examine the effects of the putrescine analogues E-BED and E-TED, DFMO, MGBG and indeed, CHA, on germling development on artificial membranes (Reitz *et al.* 1995a, b). Only the highest concentrations of CHA and norspermidine (2.0 mM) were studied, using the specific treatment timings of either 3 hours before or 3 days after inoculaton.

2.4.2 Materials and Methods

Broad beans were grown as described in section 2.2.1.1 and rust maintained as detailed in section 2.2.1.2. When the beans were approximately 20 days old, they were treated with 2.0 mM solutions of either CHA or norspermidine and inoculated as described in section 2.2.1.3. At 12 hourly intervals, leaves were harvested from plants and placed into 100 % absolute alcohol, killing the growing fungus as well as removing chlorophyll from the leaves. Harvesting continued up to 120 hours after inoculation. Six replicates per treatment were used with controls consisting of plants that were inoculated only.

Upon completion of harvesting, leaves were re-hydrated by placing them in 80 % absolute alcohol for 3-4 hours, followed by similar periods in 50 % absolute alcohol and finally 20 % glycerol. Random sections of leaf were then cut, placed onto microscope slides and stained using a dilute solution of lacto-fuchsin. Leaves were examined using an Olympus CH2 microscope at a magnification of 10 x (eye lens) and 10 x (objective lens). Thirty spores were counted from each slide. The number of germinated spores was recorded, germ tube lengths measured using a calibrated micrometer, and the number of germinated spores forming appressoria noted. Significance was assessed using Student's *t*-test.

2.4.3 Results

Great variability within each replicate of each treatment was observed, a phenomenon reflected in the standard errors of the means. Little differences were apparent between any of the treatments at any of the timings examined (Table 3). CHA and norspermidine, applied pre inoculation, did appear to reduce germination at the earliest timings studied.

However, the effect was never significant. Likewise, germ tubes of those germinated spores appeared to be reduced in length and the percentage of them forming appressoria also reduced when beans were treated with CHA and norspermidine pre inoculation. However, again, these differences were rarely significant. Post inoculation treatments of CHA and norspermidine appeared to have little effect on the developing germling. Indeed, rust development had already considerably progressed when the post inoculation treatment of CHA and norspermidine was applied.

Table 3: Effects of 2.0 mM CHA and 2.0 mM norspermidine on bean rust germling development on leaf surfaces

Time after inoculation (hrs)	Treatment	% spores germinated	average length of germ tubes (μm)	% of germ tubes forming appressoria
12	Control	6.1 ± 1.02	73 ± 15.9	0
	pre CHA	3.3 ± 1.71	21 ± 10.1 *	0
	pre norspermidine	4.4 ± 2.68	32 ± 18.4	0
24	Control	11.1 ± 5.07	61 ± 17.1	0
	pre CHA	5.0 ± 2.39	48 ± 23.6	0
	pre norspermidine	5.6 ± 2.67	47 ± 23.7	0
36	Control	30.6 ± 10.90	60 ± 22.5	5.2 ± 3.28
	pre CHA	17.8 ± 5.35	31 ± 9.2	4.0 ± 2.62
	pre norspermidine	21.1 ± 6.97	23 ± 11.1	8.3 ± 8.33
48	Control	34.5 ± 10.70	61 ± 17.3	15.5 ± 8.68
	pre CHA	25.6 ± 6.75	46 ± 21.2	13.8 ± 10.30
	pre norspermidine	33.9 ± 5.74	40 ± 8.9	6.9 ± 4.52
60	Control	43.9 ± 11.20	76 ± 14.4	20.4 ± 8.03
	pre CHA	23.9 ± 4.08	44 ± 18.9	15.2 ± 8.24
	pre norspermidine	26.7 ± 7.10	56 ± 11.8	6.7 ± 4.94
72	Control	46.6 ± 9.51	76 ± 16.1	20.4 ± 8.03
	pre CHA	35.1 ± 3.86	51 ± 16.7	14.9 ± 8.17
	pre norspermidine	36.2 ± 5.87	56 ± 11.8	8.9 ± 4.30

... Table 3 continued overleaf.

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84	Control	41.7 ± 5.95	64 ± 11.9	24.2 ± 15.50
	pre CHA	33.9 ± 5.05	43 ± 6.6	16.6 ± 7.72
	pre norspermidine	32.8 ± 5.27	54 ± 4.8	18.8 ± 7.74
	post CHA	43.9 ± 4.75	66 ± 11.1	14.2 ± 8.21
	post norspermidine	43.3 ± 6.44	34 ± 5.6	10.4 ± 8.18
96	Control	42.2 ± 6.93	82 ± 8.0	17.3 ± 7.63
	pre CHA	37.8 ± 4.92	56 ± 17.0	14.9 ± 8.17
	pre norspermidine	32.8 ± 4.66	52 ± 8.2	6.4 ± 2.95
	post CHA	45.0 ± 8.55	66 ± 15.4	16.6 ± 7.72
	post norspermidine	41.7 ± 7.59	72 ± 16.2	19.9 ± 6.97
108	Control	40.6 ± 7.76	72 ± 14.5	19.3 ± 6.99
	pre CHA	35.0 ± 2.81	45 ± 9.1	15.7 ± 7.12
	pre norspermidine	75.6 ± 6.62	53 ± 9.0	14.0 ± 7.57
	post CHA	53.3 ± 6.62	44 ± 9.0	14.9 ± 8.17
	post norspermidine	46.1 ± 7.47	47 ± 7.6	16.0 ± 7.57
120	Control	43.3 ± 7.40	70 ± 12.2	16.6 ± 7.72
	pre CHA	33.3 ± 4.63	70 ± 16.6	9.9 ± 3.84
	pre norspermidine	33.3 ± 5.08	72 ± 20.0	14.6 ± 7.27
	post CHA	42.8 ± 5.27	57 ± 8.7	10.3 ± 3.87
	post norspermidine	45.0 ± 5.07	47 ± 7.7	9.6 ± 3.79

Note: Values are shown as the means ± SE of thirty measurements from each of six replicates.

Significant difference from control is shown as: * $p \leq 0.05$

2.5 Discussion

These studies have shown that CHA and norspermidine possess fungicidal activity against a number of plant pathogens. Such findings are in agreement with those of West and Walters (1988) who found that CHA reduced powdery mildew infection of barley in glasshouse studies. Little information exists as to the effect of norspermidine upon plant pathogenic fungi, although antiproliferative activity was observed in experimental animal tumours (Prakash *et al.* 1988). The present findings are in agreement with these studies and demonstrate the growth inhibitory properties of norspermidine.

The compounds demonstrated protective, eradicant and systemic activity against a broad range of plant pathogenic fungi. Such findings are in agreement with the broad fungicidal activity found for putrescine analogues (Havis *et al.* 1994a, c). As suggested by Rajam *et al.* (1985), the systemic action observed against late blight on potato leaf discs could be due to the compounds themselves, metabolites of the compounds or induced fungicidal compounds. Further work would be necessary to determine which of those possibilities is responsible for this systemic activity.

Various differences were apparent between the responses of the different pathogens towards the compounds. Against rust on broad bean and powdery mildew on barley, no differences were apparent between the higher and lower concentrations of inhibitors. The other pathogens responded more effectively to increasing concentrations of CHA and norspermidine. Bean rust and barley powdery mildew may be more sensitive to the compounds and thus, lower concentrations may be optimal for control of such plant infections.

Such differences in responses of fungi to inhibitors is well documented. Birecka *et al.* (1986) suggested that differences may be due to variations in uptake of inhibitor, enzyme sensitivity towards the inhibitor and fungal polyamine requirements. Similar fungal species can possess markedly different sensitivities towards a single inhibitor, for example, Walters *et al.* (1995b) found that *Phytophthora infestans* was well controlled by DFMO while *Pythium ultimum* was unaffected by the inhibitor. These authors found that differences in inhibitor uptake and enzyme sensitivity towards the inhibitor accounted for the differences observed against fungal growth. Further work would be necessary to elucidate the mechanism(s) by which bean rust and barley powdery mildew afforded increased sensitivity towards CHA and norspermidine.

Additional response differences were observed since bean rust and chocolate spot, and barley powdery mildew did not appear to be affected differently by CHA or norspermidine.

Apple mildew and potato blight, however, were more responsive to norspermidine than to CHA. Similarly, this effect could be due to differences in fungal uptake of inhibitor or enzyme sensitivity towards the inhibitor. Further work would be required to elucidate these findings.

The study indicated that pre inoculation sprays of the compounds were more effective than post inoculation treatments in all the plant/pathogen interactions examined, i.e. broad bean rust, chocolate spot on beans and powdery mildew of barley. This is in agreement with Foster and Walters (1992a) who concluded that a pre inoculation application of DFMO was more effective than a post inoculation treatment against the stem rust fungus on wheat. However, the findings are in contrast to much of the previous work carried out to examine the effects of various inhibitors on plant pathogenic fungi, where post inoculation treatments were invariably superior to pre inoculation sprays (Rajam *et al.* 1985; Walters 1986; Weinstein *et al.* 1987; Havis *et al.* 1994a, c). Walters (1995) suggested that inhibitors may effect fungal germination and/or development on the leaf surface. In recent work, Reitz *et al.* (1995a) found that CHA did not affect germination but reduced appressoria formation of rust uredospores on artificial membranes. While this would suggest that CHA should be more effective when applied post inoculation, the chosen timings of inhibitor application were 3 hours pre inoculation and 3 days post inoculation. Thus, the post inoculation treatments of CHA and norspermidine may have been too late to critically affect early fungal development on the leaf surface.

Thus, microscopy studies were carried out but little differences were found between pre inoculation applied CHA, norspermidine and the untreated control. A slight tendancy towards reduced germination, length of germ tube and appressoria formation was indicated on treated leaves, although this was rarely significant. No differences were apparent between the inhibitors applied post inoculation and the control. However, while microscopic examination of rust development on leaves found little differences between the treated leaves and the untreated control, it should be noted that the compounds had indeed effectively demonstrated an ability to reduce infection on plants. The inhibitors perhaps exert a much greater effect upon the growing fungus at a later stage of development than on spore germination or early germling development.

Great variability was observed even between the replicates of each treatment and perhaps a less variable fungus would have proved a more suitable subject for such microscopy studies. Powdery mildew development is well documented (Jenkyn & Bainbridge 1979), and future work in this area may be better utilising such a fungus.

However the compounds act, CHA and norspermidine undoubtedly possess fungicidal activity against a variety of plant pathogens and no phytotoxicity was observed. Previous work has indicated that inhibitors of polyamine biosynthesis do not adversely affect polyamine levels in plants (Walters 1986). However, future work would be necessary to confirm this for plants treated with CHA and norspermidine.

Chapter 3

Effect of cyclohexylamine and spermidine analogues on the growth of plant pathogenic fungi *in vitro*

3. EFFECT OF CYCLOHEXYLAMINE AND SPERMIDINE ANALOGUES ON THE GROWTH OF PLANT PATHOGENIC FUNGI *IN VITRO*

3.1 EFFECT OF CHA AND SPERMIDINE ANALOGUES ON MYCELIAL GROWTH OF FUNGI ON SOLID MEDIA

3.1.1 Introduction and Objectives

Preliminary laboratory experiments were carried out to determine the antifungal activity of CHA, norspermidine and two additional commercially available spermidine analogues: N^1- and N^8-acetylspermidine (Figure 8). *In vitro* effects of these compounds on mycelial growth on solid media were studied in two plant pathogenic fungi: the ascomycete *Pyrenophora avenae* Ito & Kuribay, causing leaf-stripe in oats and the deuteromycete *Pyricularia oryzae* Br. & Cav., responsible for rice blast.

As a plant pathogen *P. avenae* has declined in importance through the advent of routine effective seed treatments (Jones & Clifford 1978). Rice blast remains an important disease in those parts of the world, for example, the Far East, which rely heavily on rice production (Stapley & Gayner 1969). Such pathogens were, however, primarily chosen for experimentation as representative of those fungi able to grow on artificial media, and which had been shown in previous work to be moderately affected by polyamine biosynthesis inhibitors (Foster & Walters 1990; Havis *et al.* 1994a).

3.1.2 Materials and Methods

3.1.2.1 Maintenance of pathogens

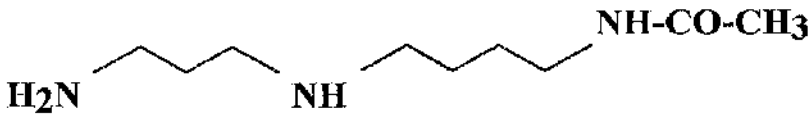
Stock cultures of *P. avenae* and *P. oryzae* were maintained on sterile PDA in a darkened incubator at a temperature of 24°C. Fresh culture plates were initiated approximately every ten days for *P. avenae* and every 20 days for *P. oryzae*.

3.1.2.2 Growth of fungi on inhibitor amended solid media

Previous work by West and Walters (1989) demonstrated that CHA reduced mycelial growth of *Pyrenophora teres* and *Gaeumannomyces graminis* at concentrations ranging from 0.5 mM to 4.0 mM. However, when Foster and Walters (1990) examined the effect of ODC and AdoMetDC inhibitors on the mycelial growth of *P. avenae*, they found concentrations ranging from 0.1 mM to 2.0 mM inhibited growth of the fungus. Havis and co-workers (1994a) demonstrated that E-BED and its derivatives, used at 1.0 mM, successfully inhibited mycelial growth of *P. oryzae* and *Botrytis cinerea*. For experiments

Spermidine

N1-acetylspermidine



N8-acetylspermidine

Figure 8 . Structures of spermidine, N ^{1}-acetylspermidine and N ^{8}-acetylspermidine

with *P. avenae*, the concentrations of CHA and norspermidine thus chosen for study ranged from 0.05 mM to 2.0 mM. N^1- and N^8-acetylspermidine were only examined at a concentration of 2.0 mM due to the limited amount of those chemicals available. The higher concentration was chosen since work had already shown mycelial growth of *P. avenae* to be unaffected by 1.0 mM concentrations of these compounds (S. A. Foster unpublished results).

Higher inhibitor concentrations (1.0 mM - 5.0 mM) were chosen for experiments with *P. oryzae* since an initial investigation had shown that concentrations ranging from 0.05 mM - 2.0 mM had little effect on mycelial growth of the fungus (data not shown). Effects of N^1- and N^8-acetylspermidine upon growth of *P. oryzae* were not examined due to the limited amounts of chemicals available. Higher concentrations of these compounds, compared to CHA and norspermidine, were required to affect growth of *P. avenae*. Since CHA and norspermidine concentrations had to be increased to affect *P. oryzae*, excessively high concentrations of N^1- and N^8-acetylspermidine would have been required to affect growth of *P. oryzae*.

Filter-sterilised inhibitor solutions (10 ml) were added to 70 ml of sterile PDA at 45-47°C to obtain the final desired concentration of each inhibitor. Sterile medium (20 ml) was added aseptically to a 90 mm sterile plastic Petri dish. An 8 mm cork borer was used to remove plugs of stock mycelium which were inverted and placed in the centre of each Petri dish. Inoculated plates were incubated in the dark at 24°C. Radial mycelial growth, excluding the mycelial plug, was measured 3, 6 and 8 days after inoculation with *P. avenae* and 7, 14 and 21 days after inoculation with the slower growing *P. oryzae*. Three measurements were taken from each plate. Control plates contained culture medium only. Four replicates were used for each treatment with significance assessed using Student's *t*-test. All experiments were repeated with similar results obtained.

3.1.3 Results

3.1.3.1 Effects of CHA and spermidine analogues on mycelial growth of *Pyrenophora avenae*

Mycelial growth of *P. avenae* on solid media was reduced in the presence of 2.0 mM CHA by 55.1 %, eight days after inoculation ($p \leq 0.001$) (Figure 9; Appendix 4) (Plate 1). Lower concentrations of CHA had little effect on fungal growth. Norspermidine produced a slightly greater effect on fungal growth, with 1.0 mM reducing growth by 41.0 % and 2.0 mM reducing growth by 63.1 % eight days after inoculation ($p \leq 0.001$) (Figure 9; Appendix 4) (Plate 1). N^1- and N^8-acetylspermidine had little effect on fungal growth at a concentration of 2.0 mM (Table 4).

3.1.3.2 Effects of CHA and norspermidine on mycelial growth of *Pyricularia oryzae*

Mycelial growth of *P. oryzae* on solid media was reduced by all of the concentrations of CHA examined although the reduction caused by 1.0 mM CHA after 21 days growth was not significant (Figure 10; Appendix 5). Fungal growth was reduced by 5.0 mM CHA, by 35.9 %, 21 days after inoculation ($p \leq 0.001$) (Plate 2). Norspermidine had a greater effect on fungal growth with a concentration of 5.0 mM reducing growth by 50.6 % after 21 days growth ($p \leq 0.001$) (Figure 10; Appendix 5) (Plate 2).

Table 4. Effect of N^1- and N^8-acetylspermidine on mycelial growth of *Pyrenophora avenae*

	Mycelial growth, measured as mean colony radius (mm)		
Treatment	3 days	6 days	8 days
Control	12.0 ± 0.26	28.7 ± 0.42	36.2 ± 0.79
N^1-acetylspermidine	12.1 ± 0.42	26.7 ± 0.80 *	36.0 ± 0.33
N^8-acetylspermidine	12.2 ± 0.48	29.2 ± 0.48	36.8 ± 0.31

Note: Values are shown as the means ± SE of three measurements from each of four replicates.

Significant difference from control is shown as: * $p \leq 0.5$

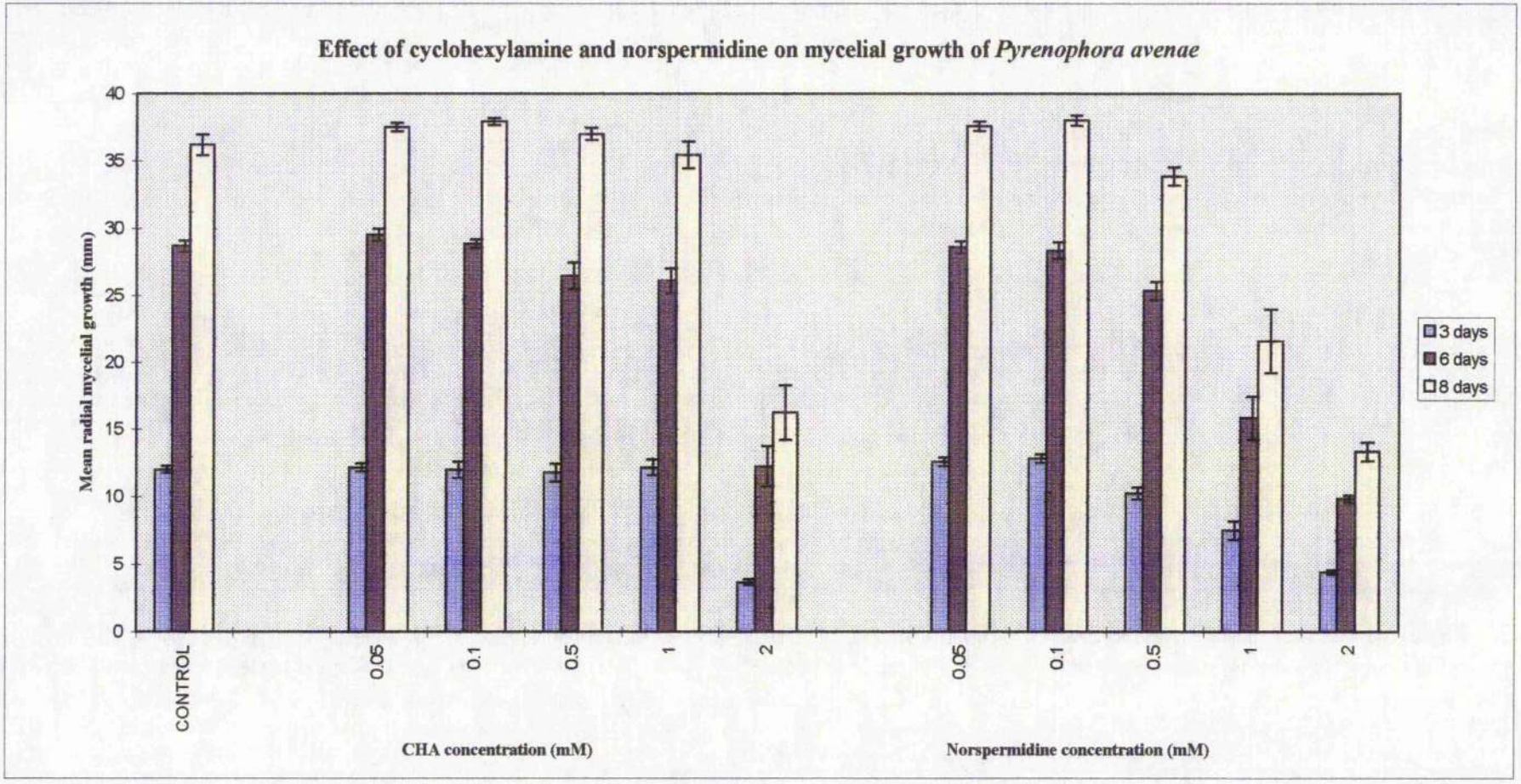


Figure 9. Effect of varying concentrations (0.05 - 2 mM) of CHA and norspermidine on mycelial growth of *Pyrenophora avenae* on solid media. Radial mycelial growth was measured 3, 6 and 8 days after inoculation of plates. Values are the means of 3 measurements from each of 4 replicates with standard errors of means indicated. Significant differences from control are indicated in Appendix 4.

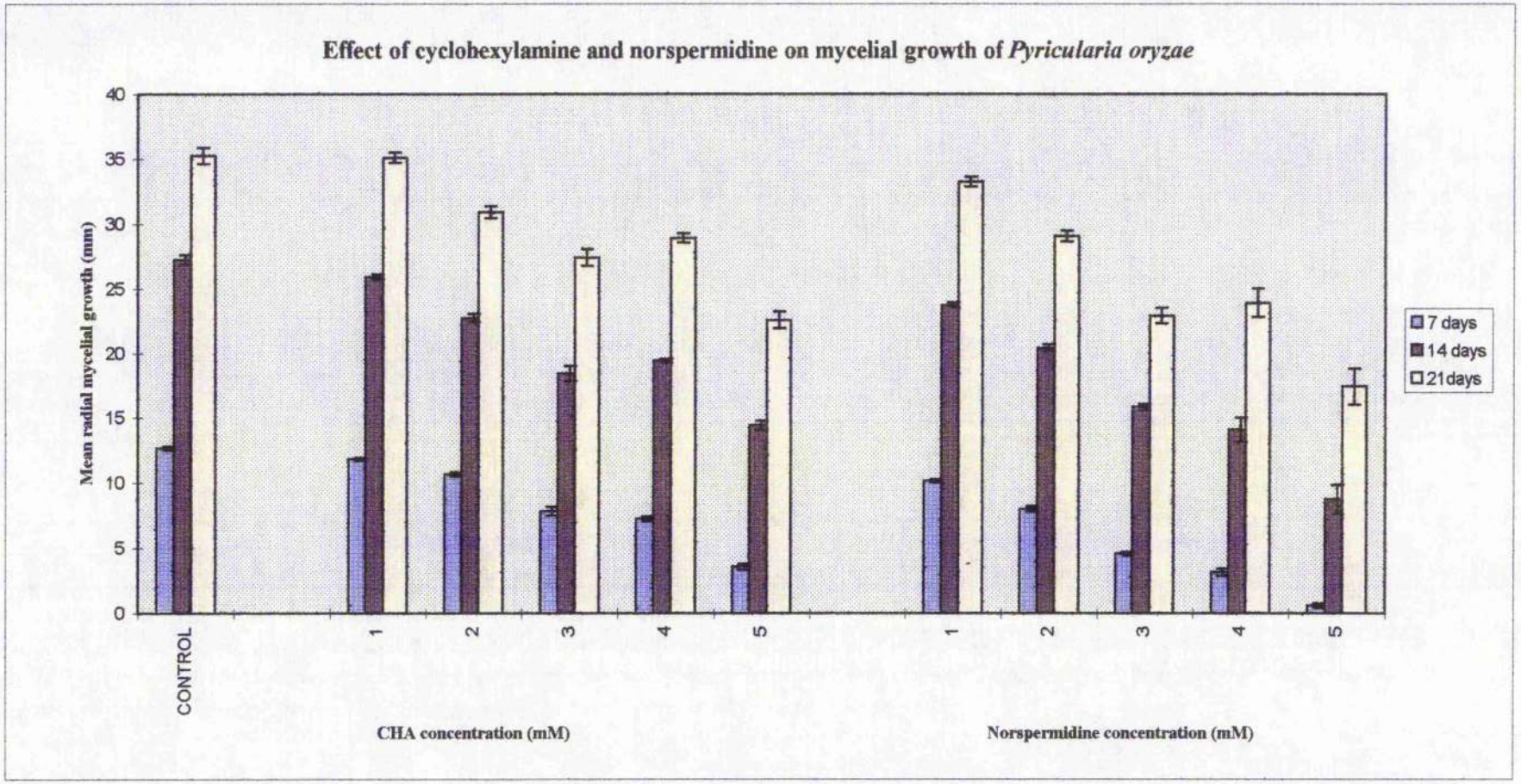


Figure 10. Effect of varying concentrations (1 - 5 mM) of CHA and norspermidine on mycelial growth of *Pyricularia oryzae* on solid media. Radial mycelial growth was measured 7, 14 and 21 days after inoculation of plates. Values are the means of 3 measurements from each of 4 replicates with standard errors of means indicated. Significant differences from control are indicated in Appendix 5.

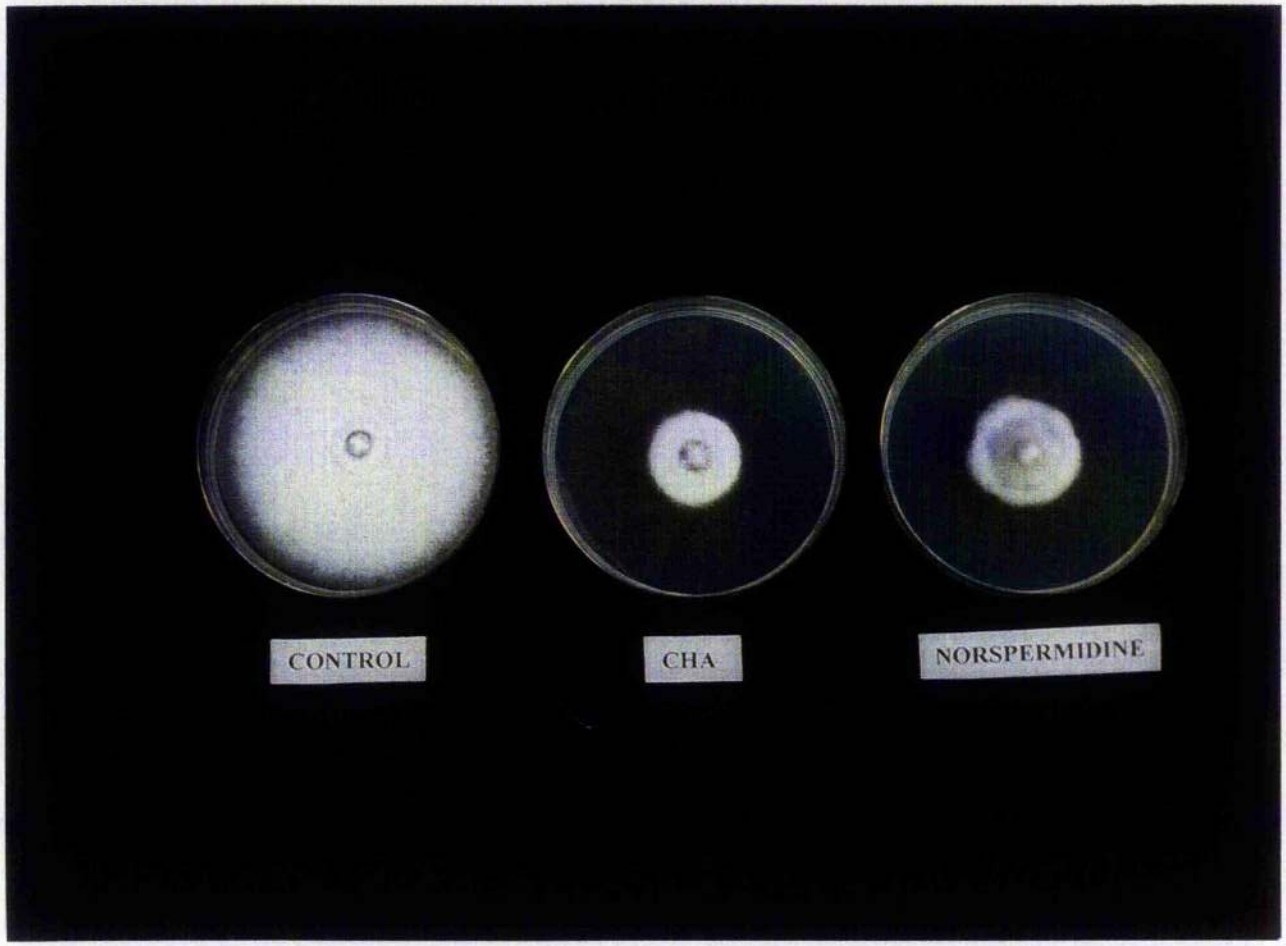


Plate 1. Effect of 2.0 mM CHA and 2.0 mM norspermidine on mycelial growth of *Pyrenophora avenae*, grown on solid media for eight days.

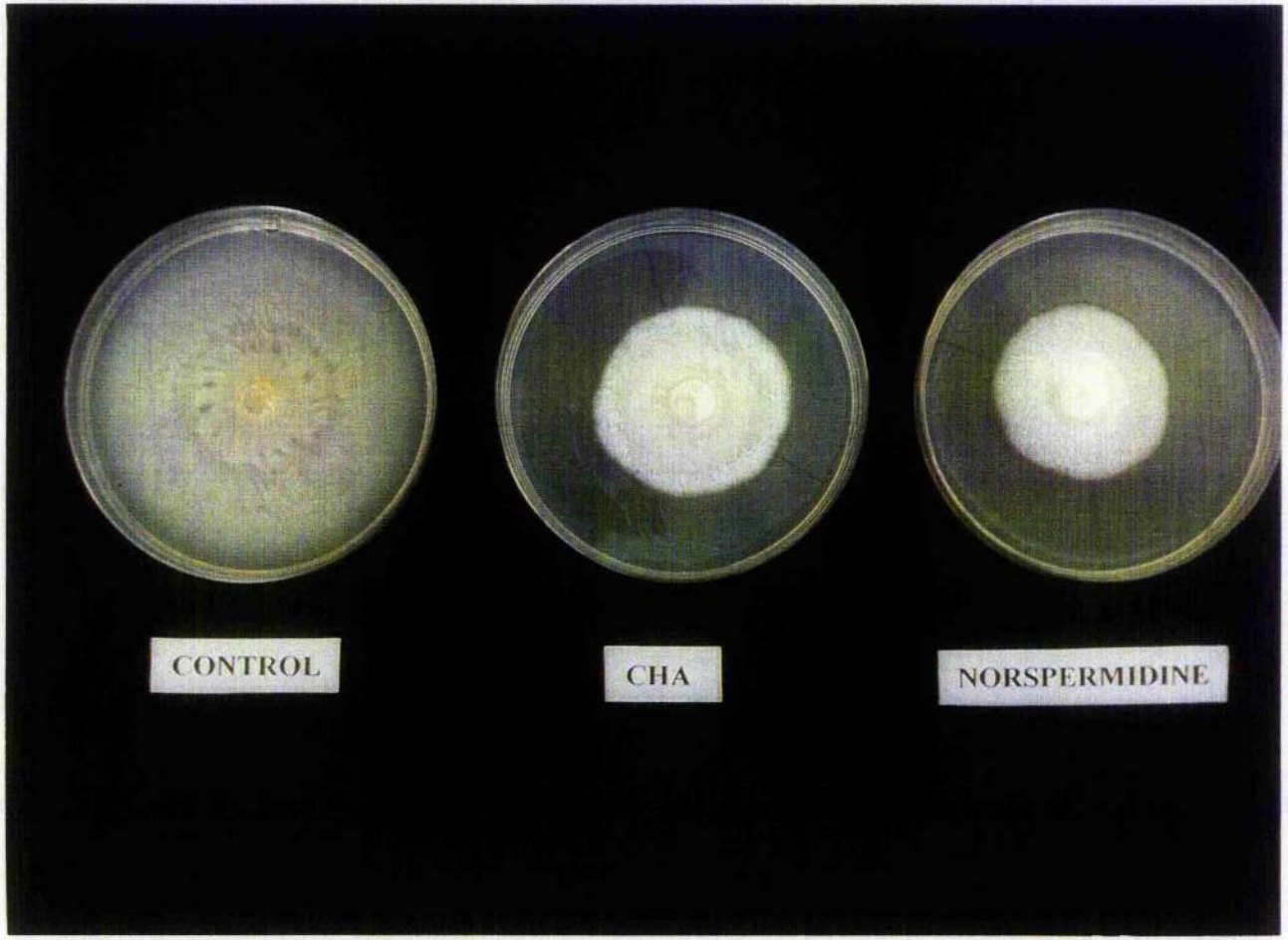


Plate 2. Effect of 2.0 mM CHA and 2.0 mM norspermidine on mycelial growth of *Pyricularia oryzae,* grown on solid media for eight days.

3.2 Effect of CHA and spermidine analogues on growth of Pyrenophora avenae in liquid culture

3.2.1 Introduction and Objectives

Because fungal growth on solid media is not always a reliable indicator of the effects of inhibitors added to the growth medium (Isaac & Jennings 1995), studies were carried out to determine the effect of CHA and the spermidine analogues on the growth of *P. avenae* in liquid culture. This would give a more realistic indication of the effects of the inhibitors on fungal growth, and would be a useful foundation for subsequent work on metabolic effects of the compounds. *P. avenae* was chosen as it had demonstrated an intermediate response to polyamine biosynthesis inhibitors, allowing collection of sufficient fungal material for biochemical analysis (Foster & Walters 1990; Havis *et al.* 1994a, c).

3.2.2 Materials and Methods

3.2.2.1 Maintenance of pathogen

P. avenae was maintained as described in section 3.1.2.1.

3.2.2.2 Growth of *Pyrenophora avenae* in inhibitor amended liquid media

Since mycelial growth of *P. avenae* had been reduced in the presence of 2.0 mM CHA, a preliminary experiment to examine the effect of CHA on growth of *P. avenae* in liquid culture was instigated, studying a range of concentrations (1.0 mM - 5.0 mM: data not shown). Fungal growth was markedly affected by 3.0 mM CHA and thus, concentrations ranging from 2.0 mM - 3.0 mM CHA were used to determine the effect of CHA upon growth of *P. avenae* in liquid culture.

Lower preliminary concentrations were chosen for norspermidine since greater fungal sensitivity had been exhibited towards norspermidine compared to CHA. Fungal growth was dramatically reduced in the presence of 0.5 mM norspermidine when concentrations ranging from 0.01 mM - 1.0 mM were used (data not shown). Thus, the effects of 0.1 mM - 0.5 mM concentrations of norspermidine on growth of *P. avenae* in liquid culture were examined.

The effect of 0.5, 1.0 and 2.0 mM concentrations of N^1- and N^8-acetylspermidine on *P. avenae* growth in liquid culture were also studied. Although such concentrations did not affect mycelial growth of *P. avenae*, it is well established that fungal growth in liquid culture is more sensitive towards inhibitors. Indeed, Havis *et al.* (1994c) found that mycelial growth of *P. avenae* on solid medium was unaffected by 1.0 mM E-TED, yet the same concentration of inhibitor completely inhibited growth of the fungus in liquid culture.

Filter-sterilised inhibitor solutions (10 ml) were added to 140 ml of sterile liquid Potato Dextrose Broth (PDB) in 250 ml conical flasks to obtain the desired final concentration of each inhibitor. Flasks were inoculated with a 10 mm disc of *P. avenae* mycelium and placed in a Gallenkamp orbital shaker (140 rev.min^{-1}) at 24°C. After 4 days growth, the fungus was washed with distilled water through a fine mesh seive and centrifuged at 16000 *g* at 0°C for 10 minutes before weighing. Control flasks contained 150 ml of sterile PDB only. Four replicates per treatment were used. Significance was assessed using Student's *t*-test and experiments were repeated with similar results obtained.

In order to carry out biochemical analyses, sufficient fungal material had to be amassed. It was desirable to study fungus after it had been growing for only a short time in the presence of the compounds, i.e. 2 days. Therefore, *P. avenae* was grown in liquid culture in the presence of lower concentrations of the compounds: 1.0 mM CHA, 0.01 mM norspermidine and 0.5 mM N^1- and N^8-acetylspermidine. Although such concentrations of inhibitors did not always cause a significant reduction in fungal fresh weight (Tables 7-8), growth changes were always apparent (Plates 3-4). Fungus grown in the presence of the compounds was darker than the control. Control flasks contained distinct balls of fungal mycelium, with the growing fungus giving those balls a spikey exterior look, growing outwards like needles from the fungal mass. Fungus grown in the presence of CHA and the spermidine analogues, however, was much less distinct and appeared to be more of a broken up mass. Given the growth changes, biochemical analyses were warranted. Havis *et al.* (1994c), similarly, used lower concentrations of E-TED to measure enzyme activity and polyamine content of *P. avenae* exposed to the inhibitor.

3.2.3 Results

Fungal growth was significantly inhibited by CHA over the range of concentrations, 2.25 mM - 3.00 mM (Table 5). A concentration of 3.0 mM CHA inhibited growth in liquid culture by 92.9 %. Fungal growth was also significantly inhibited by concentrations of norspermidine over the range 0.1 mM - 0.5 mM with the latter concentration completely inhibiting growth (Table 5). The fresh weight of fungus grown in liquid culture in the presence of N^1- and N^8-acetylspermidine was unaffected by concentrations as high as 2.0 mM (Table 6).

Table 5. Effect of varying concentrations of CHA and norspermidine on growth of *Pyrenophora avenae* in liquid culture for 4 days

Growth in liquid culture, measured as mean fresh weight (g)			
Concentration (mM)	CHA	Concentration (mM)	norspermidine
0	4.2 ± 0.37	0	5.2 ± 0.30
2.00	4.3 ± 0.16	0.1	1.8 ± 0.11 **
2.25	2.6 ± 0.06 *	0.2	1.3 ± 0.11 **
2.50	1.7 ± 0.19 **	0.3	0.7 ± 0.08 ***
2.75	0.7 ± 0.37 ***	0.4	0.3 ± 0.02 ***
3.00	0.3 ± 0.02 **	0.5	NG[a]

Note: Values are shown as the means ± SE of four replicates.

Significant differences from control are shown as: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

[a] NG: no growth

Table 6. Effect of 2.0 mM N^1- and N^8-acetylspermidine on growth of *Pyrenophora avenae* in liquid culture for 4 days

Treatment	Growth in liquid culture, measured as mean fresh weight (g)
Control	3.5 ± 0.01
N^1-acetylspermidine	3.3 ± 0.02
Control	3.4 ± 0.01
N^8-acetylspermidine	3.8 ± 0.10

Note: Values are shown as the means ± SE of four replicates.

Table 7. Effect of 1.0 mM CHA and 0.01 mM norspermidine on growth of *Pyrenophora avenae* in liquid culture for 2, 3 or 4 days

	Growth in liquid culture, measured as mean fresh weight (g)		
Days of growth	Control	CHA	norspermidine
2	0.6 ± 0.01	0.6 ± 0.01	0.6 ± 0.01
3	1.1 ± 0.02	1.2 ± 0.07	1.1 ± 0.04
4	3.4 ± 0.02	3.3 ± 0.41	2.2 ± 0.09

Note: Values are shown as the means ± SE of four replicates.

Table 8. Effect of 0.5 mM N^1- and N^8-acetylspermidine on growth of *Pyrenophora avenae* in liquid culture for 2, 3 or 4 days

	Growth in liquid culture, measured as mean fresh weight (g)		
Days of growth	Control	N^1-acetylspermidine	N^8-acetylspermidine
2	0.8 ± 0.05	1.0 ± 0.08	0.9 ± 0.09
3	1.5 ± 0.05	1.6 ± 0.06	1.2 ± 0.07
4	4.0 ± 0.07	4.1 ± 0.53	3.8 ± 0.23

Note: Values are shown as the means ± SE of four replicates.

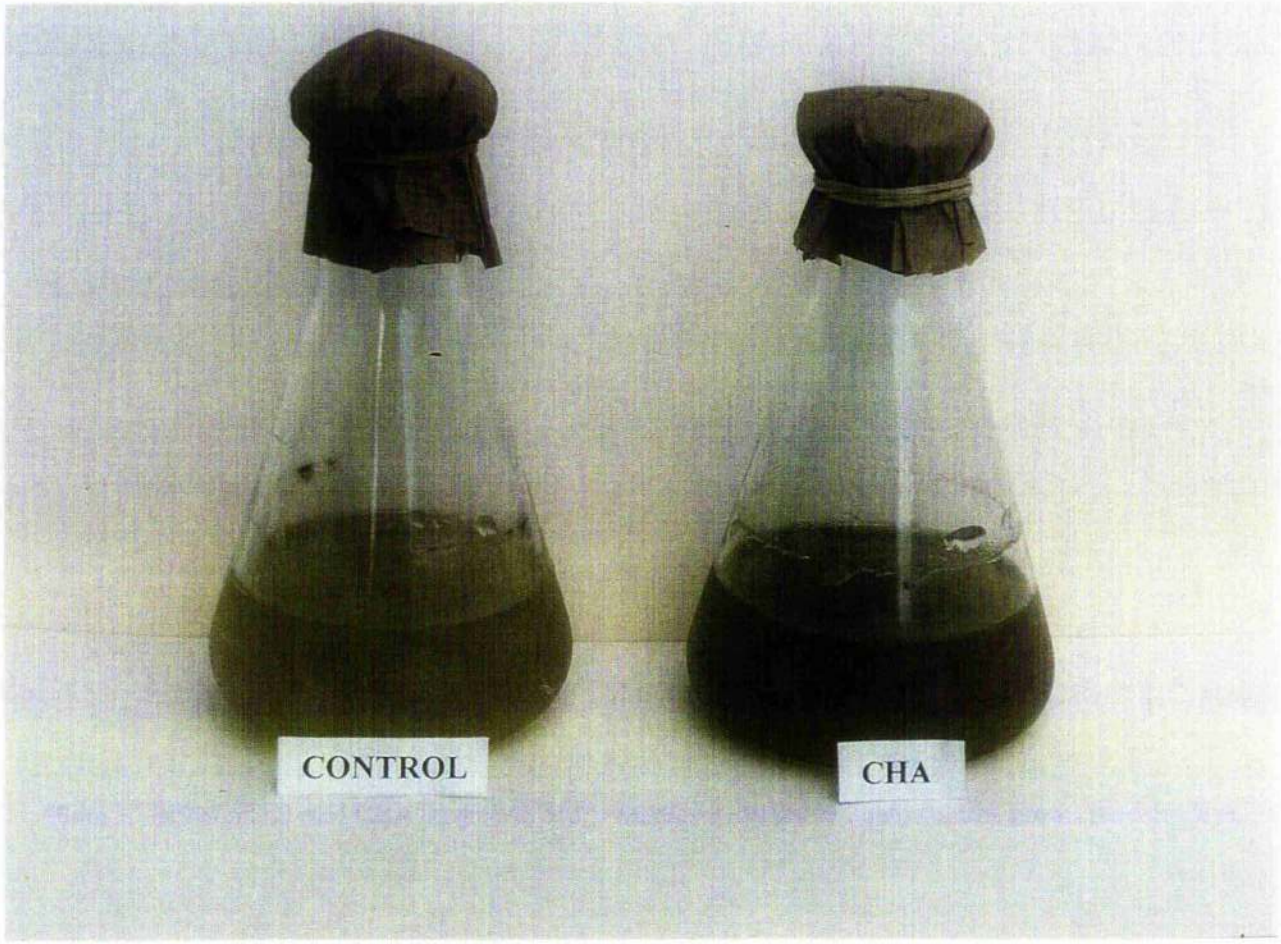


Plate 3. Effect of 1.0 mM CHA on growth of *Pyrenophora avenae* in liquid culture, grown for four days.

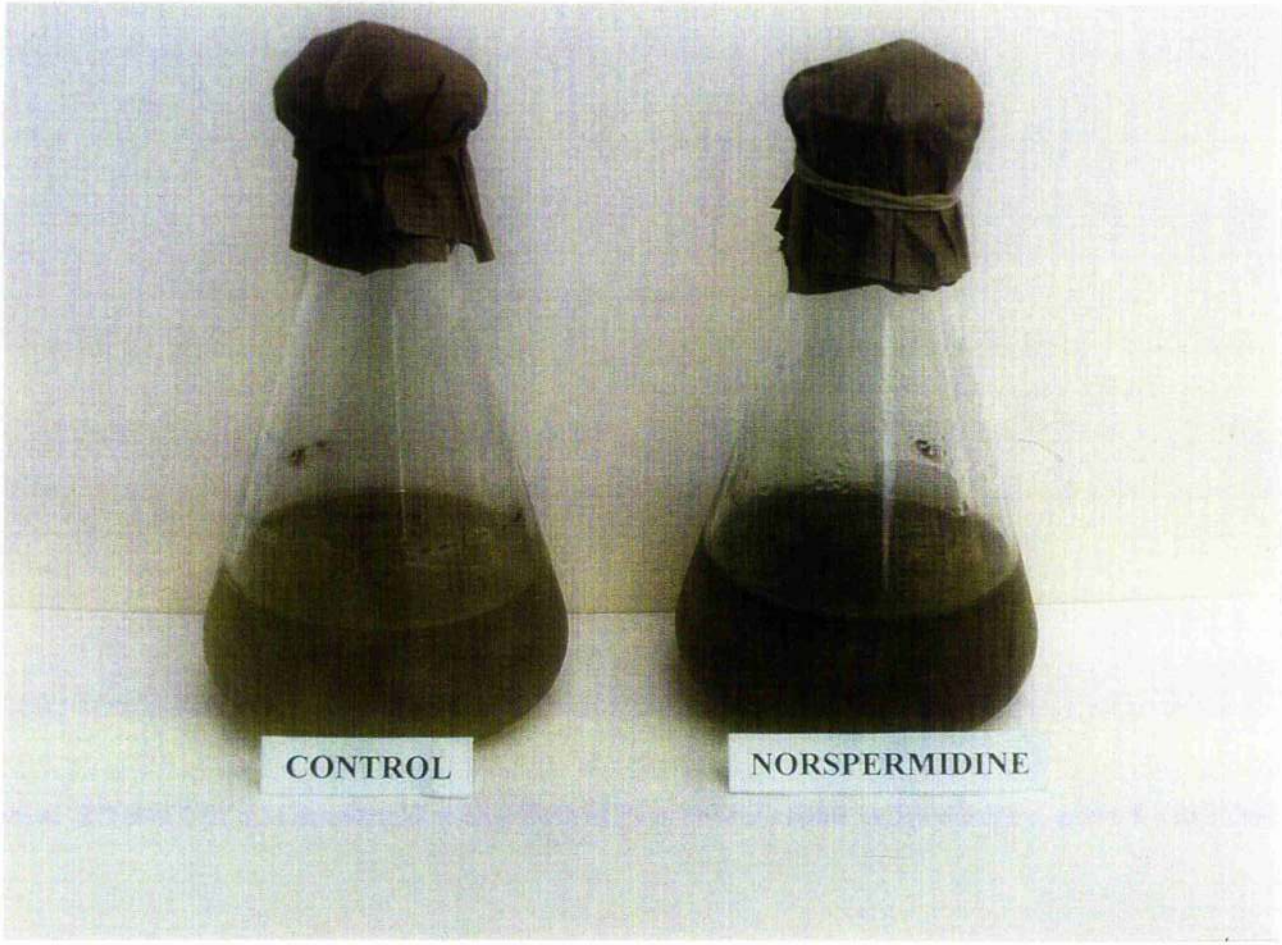


Plate 4. Effect of 0.01 mM norspermidine on growth of *Pyrenophora avenae* in liquid culture, grown for four days.

3.3 Effect of CHA and spermidine analogues on cell lengths of *Pyrenophora avenae* grown on solid media

3.3.1 Introduction and Objectives

Given the obvious changes in growth of *P. avenae* in liquid culture in the presence of 1.0 mM CHA, 0.01 mM norspermidine and 0.5 mM N^1- and N^8-acetylspermidine, although not always accompanied by a reduction in fungal fresh weight, microscopic examination of the fungus grown on solid media in the presence of the compounds was undertaken, in an attempt to detect morphological changes induced by the inhibitors. Mycelial growth on solid media was chosen for examination as it is inherently easier and more accurate than examination of fungus grown in liquid culture.

3.3.2 Materials and Methods

Fungus was grown on inhibitor amended media as described in section 3.1.2.2. Treatments examined were 0.05 mM - 2.0 mM CHA and norspermidine, and 2.0 mM N^1- and N^8-acetylspermidine. After 8 days growth, mycelium was scraped from the Petri dishes using a scalpel, spread onto microscope slides and stained with lactophenol cotton blue. Cell lengths were measured using a calibrated micrometer in an Olympus CH2 microscope at a magnification of 10 x (eye lens) and 100 x (objective oil lens). Ten measurements were taken from each of four replicates of each treatment, and significance was assessed using Student's *t*-test. The experiment was repeated and similar results obtained.

3.3.3 Results

Fungal cell lengths were quite variable within each treatment and so little significance was observed between the treatments, although all treatments did cause a reduction in fungal cell length (Table 9). The addition of 0.1 mM and 2.0 mM CHA, and 1.0 and 2.0 mM norspermidine to the growth medium resulted in a significant reduction in the lengths of *P. avenae* cells (Table 9). Neither 2.0 mM N^1- nor N^8-acetylspermidine had any significant effect on the fungal cell length (Table 9).

Table 9. Effect of varying concentrations of CHA and spermidine analogues on cell lengths in *Pyrenophora avenae* grown on solid media for 8 days

Treatment	Mean cell length (μm)
Control	60.98 ± 7.15
0.05 mM CHA	51.40 ± 4.69
0.1 mM CHA	43.60 ± 3.38 *
0.5 mM CHA	45.07 ± 4.53
1.0 mM CHA	52.29 ± 7.29
2.0 mM CHA	41.68 ± 2.85 *
0.05 mM norspermidine	44.92 ± 5.61
0.1 mM norspermidine	51.11 ± 6.36
0.5 mM norspermidine	52.58 ± 3.62
1.0 mM norspermidine	31.96 ± 4.40 **
2.0 mM norspermidine	38.88 ± 4.30 *
2.0 mM N^1-acetylspermidine	46.84 ± 3.17
2.0 mM N^8-acetylspermidine	47.42 ± 3.89

Note: Values are shown as the means ± SE of ten measurements from each of four replicates.

Significant differences from control are shown as: * $p \leq 0.5$; ** $p \leq 0.01$

3.4 DISCUSSION

This study found CHA and norspermidine to be effective in reducing the mycelial growth of two pathogenic fungi on solid media. Such findings are in agreement with those of West and Walters (1989) who found that CHA inhibited mycelial growth of *Gaeumannomyces graminis*. Little information exists on the fungicidal activity of norspermidine, although, antiproliferative activity has been demonstrated against experimental animal tumours (Prakash *et al.* 1988). This study has also shown, therefore, that norspermidine possesses growth inhibitory activity in fungal systems. Similarly, both compounds exhibited antifungal properties when *P. avenae* was grown in liquid culture.

No effect on mycelial growth was observed for N^1- or N^8-acetylspermidine at the concentrations examined, although growth changes were apparent when *P. avenae* was grown in the presence of the compounds in liquid culture. These findings are in contrast to the reported reduction of powdery mildew on barley in glasshouse trials by the spermidine analogues (S. A. Foster unpublished results). However, insensitivity of fungal mycelium towards inhibitors is not unusual and as discussed previously, Havis *et al.* (1994c) found that E-TED did not affect mycelial growth of *P. avenae* at a concentration of 1.0 mM, yet at the same concentration, it completely inhibited growth of the fungus in liquid culture. Mycelial growth is often regarded as less reliable as fungus can 'grow away' from inhibitor in the medium (Isaac & Jennings 1995).

Higher concentrations of CHA and norspermidine were required to achieve inhibition of *P. oryzae* on solid media compared to *P. avenae*. Birecka *et al.* (1986) suggested that differences between fungi in their response to a single inhibitor may be the result of differences between genera. Such a phenomenon has been discussed previously in relation to differences between fungi growing on plants and their response to CHA and norspermidine (section 2.5, page 72). Birecka *et al.* (1986) suggested that such differences may be due to variations in inhibitor uptake by the fungus or fungal enzyme insensitivity towards the inhibitor. Further work would, however, be necessary to elucidate exactly why *P. oryzae* is less sensitive than *P. avenae* to CHA and norspermidine.

Similarly, both species of fungus were more sensitive towards norspermidine than CHA. Norspermidine and CHA were more effective than N^1- and N^8-acetylspermidine against growth of *P. avenae* on solid media. Further study would be necessary to determine the cause of the differing sensitivity of the fungal species to the various inhibitors.

Growth changes were apparent when fungus in liquid culture was exposed to concentrations of CHA, norspermidine and N^1- and N^8-acetylspermidine that did not always affect fungal

fresh weight. Inhibitors of polyamine biosynthesis have been reported to cause morphological changes in fungus growing on solid media, for example, Boyle *et al.* (1988) found that abnormal hyphae and swollen cells were caused in species of *Microsporum* and *Trichophyton* exposed to DFMO. Clearly, CHA and the spermidine analogues affected growth of *P. avenae* although further microscopic analysis would be required to detect the morphological effects caused.

This study found that cell lengths of *P. avenae* grown on solid media were reduced by higher concentrations of CHA and norspermidine. Such findings are in agreement with those of West & Walters (1989) who found that CHA reduced cell lengths in *Pyrenophora teres*. However, these authors also observed fungal cells of *Septoria nodorum* to be unaffected by CHA (West & Walters 1989), and is therefore similar to the observed lack of effect on cell lengths of *P. avenae* exposed to 2.0 mM N^1- and N^8-acetylspermidine. Thus, *P. avenae* has been shown to respond quite differently to a number of inhibitors, resulting in differing effects on cell lengths following exposure to different inhibitors. This agrees with previous studies which report varying effects to be caused by different inhibitors on different fungal species.

Thus, CHA and norspermidine were shown to possess antifungal activity against two pathogenic fungal species on solid media and one in liquid culture. N^1- and N^8-acetylspermidine were less effective in reducing fungal growth but a degree of antifungal activity was observed.

Chapter 4

Effect of cyclohexylamine and spermidine analogues on biosynthesis and catabolism of polyamines in *Pyrenophora avenae*

4. EFFECT OF CYCLOHEXYLAMINE AND SPERMIDINE ANALOGUES ON BIOSYNTHESIS AND CATABOLISM OF POLYAMINES IN *PYRENOPHORA AVENAE* GROWN *IN VITRO*

4.1 INITIAL DETERMINATION OF THE EFFECTS OF CHA AND SPERMIDINE ANALOGUES ON POLYAMINE BIOSYNTHESIS IN *PYRENOPHORA AVENAE*

4.1.1 Introduction and Objectives

Given the observed fungicidal and antifungal effects of CHA and norspermidine *in vivo* and *in vitro*, studies were undertaken to determine the effect of those compounds on polyamine biosynthesis in *P. avenae* in an attempt to determine their fungicidal and antifungal mode(s) of action. Studies were also undertaken with N^1- and N^8-acetylspermidine, even though the present study had failed to demonstrate antifungal properties attributable to either of these compounds: the compounds had, however, been observed to reduce powdery mildew infection of barley in previous preliminary work (S. A. Foster unpublished results).

4.1.2 Materials and Methods

4.1.2.1 Growth of fungal material

As described in section 3.2.2.2 (page 85), *P. avenae* was grown in liquid culture amended with either 1.0 mM CHA, 0.01 mM norspermidine, 0.5 mM N^1-acetylspermidine or 0.5 mM N^8-acetylspermidine for 2, 3 or 4 days. The fungal pellet obtained after centrifugation was frozen at -20°C and used for enzyme and polyamine analyses as required. All results are the means of four replicates and statistical significance was assessed using Student's *t*-test. All analyses were repeated and similar results obtained.

4.1.2.2 ODC assay

Fungus (0.5 g) was ground using a pre chilled mortar and pestle with 1.75 ml of a buffer containing 10 mM potassium phosphate, 2 mM 1,4-dithiothreitol, 1 mM magnesium chloride, 0.1 mM ethylenediaminetetra-acetic acid (EDTA) and 0.1 mM pyridoxal-5-phosphate, adjusted to a pH of 7.6, as described by Stevens *et al.* (1976). The crude enzyme extract was sonicated on ice using a Soniprep 150 for 10 cycles of 10 seconds on/20 seconds off before being centrifuged at 24000 *g* for 15 minutes at 0°C. The supernatant (cytosolic fraction) was dialysed against 30 times its volume of buffer, overnight in the dark at 4°C, using dialysis tubing with a molecular weight cut off of 12000. Protein assays were carried out using the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) as the standard[2].

[2] Protein calibration curve used was as described by Havis, N. D. (1993). *Control of fungal plant pathogens using novel putrescine analogues*. Ph.D. Thesis, Glasgow University.

ODC activity was assayed by measuring the $^{14}CO_2$ released after incubation with [1-^{14}C] ornithine. Enzyme extract (0.1 ml) and 0.125 μCi of L-[1-^{14}C] ornithine hydrochloride (specific activity: 57 mCi.mmol^{-1}, Amersham International, UK) were added to 0.3 ml of a buffer containing 50 mM Tris/HCl, 0.05 mM L-ornithine monohydrochloride and 0.031 mM pyridoxal-5-phosphate, adjusted to a pH of 8.0, as described by Stevens *et al.* (1976). The assay was carried out in 100 mm glass test tubes fitted with silicone rubber stoppers and 35 mm long, 22 guage needles. A 25 mm^2 filter paper impregnated with 10μl of 2 M KOH was fitted to each needle to trap $^{14}CO_2$ released during the reaction. The test tubes were placed in a water bath at 37°C for 30 minutes, after which 0.2 ml of 6% perchloric acid (v/v) was added to each tube to stop the reaction before incubation for a further 30 minutes. The filter paper was removed and placed in 12 ml Emulsifier-Safe scintillant (Packard) and left overnight before counting the radioactivity using a Packard 1900 TR liquid scintillation counter. Values obtained as disintegrations per minute were converted and expressed as pmol $^{14}CO_2$ (mg protein)$^{-1}$ hour^{-1}.

4.1.2.3 AdoMetDC assay

The crude enzyme extract was prepared, sonicated and centrifuged as described in section 4.1.2.2. Ammonium sulphate (754 mg) was dissolved in the supernatant (cytosolic fraction) and the suspension centrifuged at 24000 *g* for 20 minutes at 0°C. The pellet obtained was redissolved in 1.75 ml buffer and dialysed as described in section 4.1.2.2. AdoMetDC activity was assayed by measuring the $^{14}CO_2$ released after incubation with S-adenosyl-[1-^{14}C] methionine. Enzyme extract (0.1 ml) and 0.025 μCi of S-adenosyl-L-[carboxyl-^{14}C] methionine (specific activity: 55 mCi.mmol^{-1}, Amersham International, UK) were added to 0.3 ml of a buffer containing 0.1 M sodium phosphate, 0.2 mM S-adenosyl-L-methionine and 1.0 mM putrescine, adjusted to a pH of 7.4, as described by Stevens *et al.* (1976). The remainder of the assay was carried out as described in section 4.1.2.2.

4.1.2.4 Incorporation of radio-labelled ornithine into polyamines

Several complications arise in attempting to assay spermidine synthase activity. The assay is known to be complex and time consuming, and a necessary substrate, decarboxylated AdoMet, is not commercially available (Stevens & Winther 1979). Thus, to examine spermidine synthase activity, radio-labelled ornithine is allowed to form polyamines and by determining the radioactivity in the end products, an indirect examination of spermidine synthase activity can be made.

An ODC assay was carried out as described in section 4.1.2.2, except that 0.125 μCi of L-[U-^{14}C] ornithine hydrochloride (specific activity: 242 mCi.mmol^{-1}, Amersham

International, UK) was used in the reaction mixture. After completion of the incubation process in the water bath, an aliquot (100 µl) of the reaction mixture was removed, 200 µl of saturated sodium carbonate solution and 400 µl dansyl chloride in acetone solution (30 mg per ml acetone) added, and the reaction mixtures incubated in darkness at 60°C for 25 minutes. L-proline solution (100 µl of a 100 mg per ml solution in distilled water) was added and the samples incubated in darkness for a further 10 minutes at room temperature to convert excess dansyl chloride to dansyl proline. Toluene (500 µl) was added and the reaction mixtures shaken for 20 seconds to extract the dansylated polyamines. Aliquots of extract (25 µl) were spotted onto silica-gel thin layer chromatography plates which had been activated in an oven at 110°C for 90 minutes. The plates were developed using a chloroform:triethylamine (12:1) (v/v) solvent mixture and the polyamines visualised under ultraviolet light and identified against standards. Spots were scraped off the plate into 10 ml of Emusifier-Safe scintillant (Packard) and the radioactivity counted using a Packard 1900 TR liquid scintillation counter. Values obtained as disintegrations per minute were converted and expressed as dpm g^{-1} fresh weight.

4.1.2.5 Analysis of free polyamines in fungal tissue

Fungus (0.5 g) was ground in a pre chilled mortar and pestle using 1 ml of 10 % perchloric acid. The suspension was centrifuged at 16000 *g* for 25 minutes at 0°C. 100 µl of the supernatant was used for polyamine analysis as described in section 4.1.2.4. The spots were scraped off the plate into 4 ml of ethyl acetate and fluorescence measured in a Perkin-Elmer LS5 luminescence spectrometer at excitation 350 nm, emission 540 nm. Values obtained as units of fluorescence were converted and expressed as µmol g^{-1} fresh weight[3] .

[3] Polyamine calibration curves used were as described by Havis, N. D. (1993). *Control of fungal plant pathogens using novel putrescine analogues*. Ph.D. Thesis, Glasgow University.

4.1.3 Results

4.1.3.1 ODC and AdoMetDC activities

Neither 1.0 mM CHA, 0.01 mM norspermidine, 0.5 mM N^1-acetylspermidine nor 0.5 mM N^8-acetylspermidine had any effect on the activities of ODC and AdoMetDC enzymes in *P. avenae* exposed to the inhibitors for 2, 3, or 4 days (Tables 10-11).

4.1.3.2 Incorporation of radio-labelled ornithine into polyamines

None of the compounds examined had any effect on the flux of labelled ornithine through to polyamines in fungus grown in the presence of the inhibitors for 2, 3, or 4 days (Table 12). No radio-label was found in putrescine (data not shown) with all of the label found in spermidine and spermine (Table 12). This indirect determination of spermidine synthase activity showed the enzyme to be unaffected by the treatments examined.

4.1.3.3 Analysis of free polyamines in fungal tissue

Exposure of fungal tissue to 1.0 mM CHA for 2, 3 or 4 days resulted in an increase in the amount of putrescine found in *P. avenae* (Table 13), e.g. a 74.8 % increase was found in fungus exposed to the inhibitor for 4 days. No other compound examined had any effect on the amounts of free polyamines in fungal tissue (Table 13).

Table 10. Effect of CHA and spermidine analogues on ODC activity in *Pyrenophora avenae* grown for 2, 3 or 4 days

	ODC activity [pmol CO_2 (mg protein)$^{-1}$hr^{-1}]		
Treatment	2 days	3 days	4 days
Control	42.6 ± 8.91	33.0 ± 3.47	48.2 ± 7.61
1.0 mM CHA	31.5 ± 7.55	39.2 ± 5.77	50.5 ± 7.40
Control	55.3 ± 8.61	68.2 ± 4.14	54.2 ± 8.13
0.01mM norspermidine	46.2 ± 5.57	50.6 ± 6.51	61.2 ± 6.69
Control	334 ± 27.3	312 ± 32.9	255 ± 12.5
0.5 mM N^1-acetylspermidine	330 ± 33.9	293 ± 23.8	233 ± 28.2
0.5 mM N^8-acetylspermidine	316 ± 25.8	271 ± 35.8	227 ± 24.2

Note: Values are shown as the means ± SE of four replicates.
Variability in control values of ODC activity may be the result of different growth rates of fungus measured during the different experiments (data not shown). Such an observation has also been noted by Havis and Walters (1992b).

Table 11. Effect of CHA and spermidine analogues on AdoMetDC activity in *Pyrenophora avenae* grown for 2, 3 or 4 days

	AdoMetDC activity [pmol CO_2 (mg protein)$^{-1}$hr^{-1}]		
Treatment	2 days	3 days	4 days
Control	61.5 ± 7.10	69.6 ± 4.32	92.5 ± 11.01
1.0 mM CHA	70.3 ± 7.32	57.3 ± 4.91	99.4 ± 10.32
Control	51.9 ± 5.03	58.9 ± 6.93	83.0 ± 2.06
0.01mM norspermidine	59.8 ± 2.10	49.7 ± 7.37	75.2 ± 6.66
Control	32.2 ± 8.64	28.8 ± 2.88	28.7 ± 3.51
0.5 mM N^1-acetylspermidine	33.3 ± 6.21	22.5 ± 5.49	39.6 ± 4.71
0.5 mM N^8-acetylspermidine	39.7 ± 6.91	39.2 ± 4.82	29.0 ± 6.32

Note: Values are shown as the means ± SE of four replicates.
Variability in control values of AdoMetDC activity may be the result of different growth rates of fungus measured during the different experiments (data not shown). Such an observation has also been noted by Havis and Walters (1992b).

Table 12. Effect of CHA and spermidine analogues on the incorporation of radio-labelled ornithine into polyamines in *Pyrenophora avenae* grown for 2, 3 or 4 days

	Radioactivity in polyamine (dpm g^{-1} FW)					
	Spermidine			Spermine		
Treatment	2 days	3 days	4 days	2 days	3 days	4 days
Control	43.9 ± 6.54	40.1 ± 7.31	78.0 ± 9.54	5.1 ± 1.39	3.1 ± 0.89	12.0 ± 2.88
1.0 mM CHA	50.3 ± 4.46	35.3 ± 3.85	56.6 ± 8.39	9.8 ± 2.88	7.1 ± 3.41	15.4 ± 2.19
0.01 mM norspermidine	47.5 ± 6.96	38.7 ± 3.77	60.3 ± 9.26	4.7 ± 1.93	2.7 ± 0.51	16.9 ± 4.21
Control	54.2 ± 0.01	58.4 ± 6.40	107.2 ± 3.76	4.5 ± 1.01	9.0 ± 0.74	8.8 ± 2.10
0.5 mM N^1-acetylspermidine	52.2 ± 3.13	39.7 ± 5.85	109.0 ± 5.10	8.2 ± 1.92	6.3 ± 1.46	10.8 ± 2.92
0.5 mM N^8-acetylspermidine	52.7 ± 6.57	39.0 ± 4.97	103.0 ± 7.04	6.6 ± 1.75	12.8 ± 1.26	12.4 ± 1.22

Note: Values are shown as the means ± SE of four replicates.

Variability in control values of radioactivity in polyamines may be the result of different growth rates of fungus measured during the different experiments (data not shown). Such an observation has also been noted by Havis and Walters (1992b).

Table 13. Effect of CHA and spermidine analogues on free polyamine levels in *Pyrenophora avenae* grown for 2, 3 or 4 days

	Polyamine concentation (µmol g^{-1} FW)											
	Putrescine			Cadaverine			Spermidine			Spermine		
Treatment	2 days	3 days	4 days	2 days	3 days	4 days	2 days	3 days	4 days	2 days	3 days	4 days
Control	200 ± 20.4	273 ± 14.0	369 ± 22.4	50 ± 7.0	115 ± 9.5	103 ± 8.3	143 ± 11.8	206 ± 16.6	270 ± 9.7	42 ± 3.7	75 ± 6.2	86 ± 4.1
1.0 mM CHA	467 ± 34.5 **	608 ± 26.7 ***	645 ± 29.1 **	59 ± 6.7	131 ± 12.3	126 ± 12.2	123 ± 8.5	179 ± 13.6	266 ± 10.7	51 ± 5.1	87 ± 5.3	97 ± 13.8
Control	200 ± 20.4	273 ± 14.0	523 ± 35.2	300 ± 18.0	258 ± 13.8	222 ± 15.1	171 ± 9.7	224 ± 31.2	419 ± 33.4	42 ± 3.7	80 ± 12.9	150 ± 18.6
0.01 mM norspermidine	220 ± 16.4	230 ± 29.2	587 ± 42.8	315 ± 27.4	234 ± 12.8	201 ± 16.6	174 ± 9.0	253 ± 11.1	457 ± 19.9	46 ± 5.2	89 ± 9.2	167 ± 14.6
Control	140 ± 8.3	185 ± 26.9	72 ± 3.4	70 ± 4.6	54 ± 5.0	45 ± 3.2	215 ± 17.9	334 ± 22.6	44 ± 1.8	86 ± 13.6	93 ± 15.5	32 ± 3.5
0.5 mM N^1-acetylspermidine	127 ± 12.0	197 ± 24.6	86 ± 4.3	69 ± 8.1	71 ± 10.1	49 ± 2.7	245 ± 14.3	327 ± 11.1	42 ± 2.0	84 ± 11.5	92 ± 3.5	39 ± 3.0
0.5 mM N^8-acetylspermidine	176 ± 11.0	197 ± 24.5	82 ± 3.1	65 ± 6.4	62 ± 8.4	45 ± 3.9	182 ± 19.8	358 ± 24.7	58 ± 3.7	89 ± 13.4	99 ± 8.7	34 ± 2.3

Note: Values are shown as the means ± SE of four replicates.

Significant differences from control are shown as: ** $p \leq 0.01$; *** $p \leq 0.001$

Variability in control values of polyamine concentrations may be the result of different growth rates of fungus measured during the different experiments (data not shown). Such an observation has also been noted by Havis and Walters (1992b).

4.2 FURTHER DETERMINATION OF THE EFFECTS OF CHA AND NORSPERMIDINE ON BIOSYNTHESIS AND CATABOLISM OF POLYAMINES IN *PYRENOPHORA AVENAE*

4.2.1 Introduction and Objectives

Given the increase in putrescine observed in fungal tissue exposed to 1.0 mM CHA for 2, 3 or 4 days, without an effect on ODC, AdoMetDC or the flux of labelled ornithine through to polyamines, further study was necessary to elucidate the mechanism(s) responsible for such an increase.

As reviewed in section 1.1.3.3 (page 14), organisms possess a variety of mechanisms to enable them to control intracellular polyamine levels. Polyamine catabolism, whereby polyamines are interconverted and degraded back to putrescine, is well characterised for mammalian cells, and many plants and microbes (Pegg 1988; Davis 1990). Generally, spermine can be converted to spermidine in a two step reaction, initially catalysed by the enzyme spermidine/spermine N^1-acetyltransferase (SSAT), to form an N^1-acetyl derivative of spermine before forming spermidine via the action of polyamine oxidase (PAO). Spermidine can similarly be converted to an N^1-acetyl derivative by SSAT before forming putrescine via PAO (Figure 3, page 16). Putrescine can then be degraded by diamine oxidase (DAO) (Pegg 1988, Large 1992). Further investigations carried out to study the cause of the observed increase in putrescine in CHA treated *P. avenae,* therefore, involved the determination of PAO and DAO activities, along with levels of N^1-acetylspermidine. It was decided to determine acetylated polyamine levels as a means of studying catabolism since the SSAT enzyme assay is inherently unreliable. N^1-acetylspermine was not determined as its commercial availability was restricted.

Levels of N^8-acetylspermidine and N-acetylputrescine were also determined in case either conjugation of polyamines, or breakdown of the conjugates was occurring, mechanisms which are present in mammalian cells and which may cause fluctuations in the free polyamine pool (Figure 3, page 16).

Acetylated polyamine levels were also determined for fungus exposed to 0.01 mM norspermidine. Although 0.01 mM norspermidine failed to affect levels of free polyamines in *P. avenae,* intracellular polyamine levels are often regulated by conjugation, selective uptake and excretion of polyamines (Davis 1990). An effect on free polyamine levels may have been masked by such processes and therefore, acetylated polyamine levels were studied. Since no effect on free polyamine levels had been observed, a comprehensive examination of the catabolic enzymes, (i.e. including determination of PAO and DAO activities), was not thought to be necessary and the determination of acetylated polyamine

levels would indicate if further study of those enzymes was desirable for norspermidine treated fungus.

Due to limited amounts of chemicals available, no further study of the effects of the spermidine analogues, N^1- and N^8-acetylspermidine, on fungal polyamine biosynthesis was carried out.

4.2.2 Materials and Methods

4.2.2.1 Growth of fungal material

As described in section 3.2.2.2 (page 85), *P. avenae* was grown in the presence of 1.0 mM CHA or 0.01 mM norspermidine for 2, 3 or 4 days. The pellet obtained was used to assay the activities of PAO and DAO, and to determine acetylated polyamine levels. All results are the means of four replicates and statistical significance was assessed using Student's *t*-test. All analyses were repeated and similar results obtained.

4.2.2.2 PAO assay

Fungus (0.6 g) was ground using a mortar and pestle with 4 ml of a buffer containing 100 mM potassium phosphate and 2 mM 1,4-dithiothreitol, adjusted to a pH of 8.0, as described by Okuyama and Kobayashi (1961). The crude enzyme extract was centifuged at 20000 *g* for 20 minutes at 4°C. Supernatant (0.5 ml) and 0.15 μCi of [^{14}C] spermidine trichloride (specific activity: 108 mCi.mmol^{-1}, Amersham International, UK) were added to 0.5 ml of a buffer containing 100 mM potassium phosphate, 1 mM spermidine and 30 μg catalase, adjusted to a pH of 8.0, as described by Okuyama and Kobayashi (1961). The assay was carried out in 100 mm glass test tubes fitted with silicone rubber stoppers. Test tubes were incubated in a water bath at 37°C for 30 minutes before the reaction was stopped by adding 4 M sodium hydroxide solution (1 ml) and shaking. Enzymic products were extracted into toluene by adding toluene (2 ml) and vortexing the mixture for 10 seconds before leaving to stand for 30 minutes. An aliquot (1 ml) of the upper toluene phase was added to 10 ml Emusifier-Safe scintillant (Packard) before counting the radioactivity using a Packard 1900 TR liquid scintillation counter. Values obtained as disintegrations per minute were converted and expressed as pmol product (mg protein)$^{-1}$hour^{-1}. Protein concentrations were determined using the method of Lowry *et al.* (1951) with BSA as the standard.

4.2.2.3 DAO assay

DAO activity was determined as described for PAO activity in section 4.2.2.2. However, the reaction mixture consisted of 0.5 ml enzyme extract, 0.15 μCi [1,4-^{14}C] putrescine dihydrochloride (specific activity: 108 mCi.mmol^{-1}, Amersham International, UK) and

0.5 ml of a buffer containing 100 mM potassium phosphate, 1 mM putrescine and 30 μg catalase, adjusted to a pH of 8.0, as described by Okuyama and Kobuyashi (1961).

4.2.2.4 Analysis of acetylated polyamines in fungal tissue

Acetylated polyamines were determined as described in section 4.1.3.3 for free polyamines. The tlc plates, however, were developed using a chloroform : isopropyl alcohol (propan-2-ol) (95:5) (v/v) solvent mixture as described by Libby (1978). The different developing solvent was required since the use of chloroform : triethylamine (12:1) (v/v) resulted in incomplete separation of the acetylated amines and putrescine. Rf values for the common mixture constituents are detailed in Table 14. Calibration curves were calculated using standards with N-acetylputrescine (100 μg/ml) used to calibrate the fluorimeter on every occasion (Appendices 6-7). During recovery of the amines from the tlc plates, N^1-acetylspermidine and N^8-acetylspermidine were pooled together as their separation was not totally distinct.

Table 14: Rf values of various compounds, developed on tlc plates using chloroform : isopropyl alcohol (95:5) (v/v)

Compound	Rf value
Putrescine	0.76
Cadaverine	0.79
Spermidine	0.85
Spermine	0.88
N^1-acetylspermidine	0.41
N^8-acetylspermidine	0.37
N-acetylputrescine	0.15
CHA	0.84
Norspermidine	0.66

4.2.3 Results

4.2.3.1 PAO and DAO activites

Neither PAO nor DAO activity was affected when *P. avenae* was exposed to 1.0 mM CHA for 2, 3, or 4 days (Table 15).

4.2.3.2 Analysis of acetylated polyamines in fungal tissue

Little changes in levels of acetylated polyamines were detected in fungus exposed to 1.0 mM CHA and 0.01 mM norspermidine for 2, 3 or 4 days (Table 16).

Table 15. Effect of 1.0 mM CHA on PAO and DAO activities in *Pyrenophora avenae* grown for 2, 3, or 4 days

	Enzyme activity [pmol product (mg protein)$^{-1}$hr^{-1}]			
	Polyamine Oxidase		Diamine Oxidase	
Days	Control	1.0 mM CHA	Control	1.0 mM CHA
2	58.1 ± 5.04	77.6 ± 11.2	144 ± 15.6	167 ± 5.3
3	94.1 ± 11.9	82.7 ± 9.33	164 ± 10.2	143 ± 4.3
4	94.8 ± 10.9	75.3 ± 8.35	176 ± 8.7	162 ± 12.4

Note: Values are shown as the means ± SE of four replicates.

Table 16: Effect of 1.0 mM CHA and 0.01 mM norspermidine on acetylated polyamine levels in *Pyrenophora avenae* grown for 2, 3, or 4 days

		Polyamine concentration (μmol g^{-1}FW)	
	Treatment	N-acetylputrescine	N-acetylspermidines
2 day exposure	Control	45.5 ± 5.14	22.5 ± 5.34
	CHA	64.2 ± 3.85	26.4 ± 1.99
	norspermidine	43.0 ± 4.82	29.3 ± 1.82
3 day exposure	Control	54.5 ± 3.24	56.1 ± 7.58
	CHA	61.8 ± 3.86	59.0 ± 6.51
	norspermidine	55.1 ± 4.87	55.6 ± 6.80
4 day exposure	Control	46.4 ± 6.81	55.8 ± 2.85
	CHA	57.9 ± 1.53	59.6 ± 6.19
	norspermidine	46.8 ± 4.05	50.9 ± 7.68

Note: Values are shown as the means ± SE of four replicates.

4.3 Determination of the effects of higher concentrations of CHA and norspermidine on polyamine biosynthesis in *Pyrenophora avenae*

4.3.1 Introduction and Objectives

The study has so far shown that 0.01 mM norspermidine, 0.5 mM N^1- and 0.5 mM N^8-acetylspermidine have no effect on ODC and AdoMetDC activities, the flux of labelled ornithine to polyamines or free polyamine levels in *P. avenae* exposed to the compounds for 2, 3 or 4 days. Exposure of fungus to 1.0 mM CHA caused an increase in free putrescine, a phenomenon which does not appear to be the result of catabolic mechanisms.

Given that fungal growth was more markedly affected by increased concentrations of the above compounds, experiments were carried out to eliminate the possibility of an effect on fungal polyamine biosynthesis occurring when fungus was exposed to higher concentrations of CHA and norspermidine. For such studies, fungus had to be exposed to the inhibitors for 4 days in order to amass sufficient material for analysis. Another possibility is that an early effect on polyamine biosynthesis could occur and yet be nullified later on, preventing detection. Experiments were therefore designed to allow the study of fungus which had only been exposed to the inhibitors for one day.

4.3.2 Materials and Methods

As described in section 3.2.2.2 (page 85), *P. avenae* was grown in liquid culture amended with higher concentrations of compounds: either 2.75 mM CHA or 0.2 mM norspermidine, for 4 days. Concentrations chosen were the maximum which permitted growth to occur.

Fungus was also exposed to these higher concentrations of inhibitors for 1 day. Fungus was grown in culture medium only for two days before being harvested and transferred to inhibitor amended medium for one further day. The pellet obtained thus contained sufficient material for analysis yet had only been exposed to the inhibitor for one day.

Fungus obtained was used to study the incorporation of [U-^{14}C] ornithine into polyamines and to determine free polyamine levels. ODC and AdoMetDC assays were not thought to be necessary, given that an examination of the flux from labelled ornithine through to polyamines would also indicate if ODC or AdoMetDC activities had been affected. Analyses were carried out as described previously in sections 4.1.2.4 and 4.1.2.5. Results are the means of four replicates with all analyses repeated and similar results obtained. Significance was assessed using Student's *t*-test.

4.3.3 Results

4.3.3.1 Effect of 4 days exposure to higher concentrations of CHA and norspermidine on growth of *Pyrenophora avenae*

Exposure of fungus to 2.75 mM CHA and 0.2 mM norspermidine for 4 days resulted in substantial reductions in fungal fresh weight, similar to those results presented in Table 5 (section 3.2.3, page 87). A reduction in fungal fresh weight of 83 % was caused by 2.75 mM CHA while 0.2 mM norspermidine reduced growth by 75 % (Table 5).

4.3.3.2 Effect of 1 day exposure to higher concentrations of CHA and norspermidine on growth of *Pyrenophora avenae*

When fungus was exposed to higher concentrations of CHA or norspermidine for 1 day only, by transferring 2 day old mycelium into fresh medium, the fresh weight of the fungus decreased (Table 17). Fresh weight of control fungus increased by 118 % while fungus exposed to 2.75 mM CHA decreased in weight by 10 % and that exposed to 0.2 mM norspermidine decreased by 44 % (Table 17). The exact mechanism by which the compounds affected the fungus, causing a reduction in fresh weight as opposed to simply preventing further fungal growth, is unclear. It is possible that CHA and norspermidine affected the composition of the fungal cells so that, for example, the treated cells contained less water.

4.3.3.3 Incorporation of radio-labelled ornithine into polyamines

No effect was observed for the incorporation of radio-labelled ornithine into polyamines in fungus exposed to higher concentrations of CHA and norspermidine for 1 or 4 days (Table 18). Little radioactivity was found in putrescine (data not shown) with most being detected in spermidine and spermine (Table 18).

4.3.3.4 Analysis of free polyamines in fungal tissue

When fungus was exposed to higher concentrations of the inhibitors for 1 day, spermidine content in tissue exposed to 2.75 mM CHA decreased by 56.6 % (Table 19). Spermine content increased, although this result should be discounted as CHA co-chromatographed with spermine. No effect on putrescine was observed. Exposure to 0.2 mM norspermidine led to an increase in spermidine, although this should also be discounted since norspermidine co-chromatographs with spermidine (Table 19).

Exposure of fungus to higher concentrations of CHA and norspermidine for 4 days caused quite different effects. Thus, 2.75 mM CHA led to a 364 % increase in putrescine and a 20 % reduction in spermidine (Table 20). Again, the observed increase in spermine levels should be discounted. Little effect was caused by exposure of fungus to 0.2 mM norspermidine since the increase in spermidine should be discounted as indicated above (Table 20). The increase in spermine levels was probably the result of norspermidine co-chromatographing with spermidine, the high level of spermidine/norspermidine causing some to be detected along with spermine which is found very close to spermidine on the tlc plates.

Such instances of co-chromatography were indeed apparent in initial investigations into the effects of 1.0 mM CHA and 0.01 mM norspermidine on fungal free polyamine levels, albeit, infrequently. However, these later studies have used increased concentrations of CHA and norspermidine and it is possible that these compounds and not the polyamines, account for the increases in fluorescence observed at the 'spermine' and 'spermidine' spots respectively.

Table 17: Effect of 1 day exposure to higher concentrations of CHA and norspermidine on growth of *Pyrenophora avenae*

Treatment	Mean fresh weight after 2 days growth (g)	Mean fresh weight after 1 further days growth (g)	Percentage change wrt 2 day old fungus
Control	0.86 ± 0.012	1.88 ± 0.072	+ 118
2.75 mM CHA	0.91 ± 0.034	0.82 ± 0.152	- 10
0.2 mM norspermidine	0.93 ± 0.017	0.52 ± 0.011	- 44

Note. Values are shown as the means ± SE of four replicates.

Table 18. Effect of 1 or 4 day exposure to higher concentrations of CHA and norspermidine on the incorporation of radio-labelled ornithine into polyamines in *Pyrenophora avenae*

Treatment	Radioactivity in polyamine (dpm g^{-1} FW)	
Exposure for 4 days	Spermidine	Spermine
Control	83.8 ± 9.73	22.1 ± 3.6
2.75 mM CHA	89.1 ± 9.28	22.9 ± 1.55
0.2 mM norspermidine	98.4 ± 10.92	25.5 ± 5.53
Exposure for 1 day	Spermidine	Spermine
Control	184 ± 18.2	32.0 ± 1.77
2.75 mM CHA	177 ± 10.4	36.1 ± 1.90
0.2 mM norspermidine	197 ± 12.1	45.0 ± 5.07

Note: Values are shown as the means ± SE of four replicates.

Table 19. Effect of 1 day exposure to higher concentrations of CHA and norspermidine on free polyamine levels in *Pyrenophora avenae*

	Polyamine concentration (μmol g^{-1} FW)		
	Control	CHA	norspermidine
Putrescine	273 ± 12.5	280 ± 25.9	256 ± 17.9
Cadaverine	75.2 ± 5.65	84.3 ± 9.59	85.9 ± 13.40
Spermidine	318 ± 3.8	138 ± 13.7 **	594 ± 41.2 **
Spermine	134 ± 7.7	192 ± 12.2 **	188 ± 27.9

Note: Values are shown as the means ± SE of four replicates.
Significant differences from control are shown as: ** $p \leq 0.01$

Table 20. Effect of 4 day exposure to higher concentrations of CHA and norspermidine on free polyamine levels in *Pyrenophora avenae*

	Polyamine concentration (μmol g^{-1} FW)			
	Control	CHA	Control	norspermidine
Putrescine	77 ± 11.2	357 ± 19.7 ***	137 ± 5.0	152 ± 6.8
Cadaverine	26.8 ± 1.88	25.8 ± 2.54	32.5 ± 2.07	42.3 ± 4.14
Spermidine	118 ± 5.7	94 ± 5.2 *	150 ± 2.8	667 ± 15.9 ***
Spermine	37.1 ± 5.00	67.6 ± 6.61 *	49.1 ± 4.22	64.6 ± 3.76 *

Note: Values are shown as the means ± SE of four replicates.
Significant differences from control are shown as: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

4.4 FURTHER DETERMINATION OF THE EFFECTS OF HIGHER CONCENTRATIONS OF CHA AND NORSPERMIDINE ON BIOSYNTHESIS AND CATABOLISM OF POLYAMINES IN *PYRENOPHORA AVENAE*

4.4.1 Introduction and Objectives

Polyamine biosynthetic enzymes appeared to be unaffected in fungus exposed to higher concentrations of CHA and norspermidine as measured by examining the flux of radio labelled ornithine through to polyamines. Exposure to 2.75 mM CHA for 4 days did, however, cause an increase in fungal free putrescine levels. A slight decrease in spermidine levels was also detected. Interestingly, exposure of fungus to 2.75 mM CHA for one day failed to produce an increase in putrescine but caused a substantial decrease in spermidine, suggesting that the fluctuations in spermidine and putrescine levels were connected. Further work was therefore carried out to clarify these results.

The changes in free polyamine levels observed may have been caused by one or more of the numerous possible mechanisms. As reviewed earlier, (section 1.1.3.3, page 14), catabolism processes known for mammalian cells include the degradation of spermidine to an acetylated derivative which is in turn acted upon by PAO to yield putrescine. This process may account for the increase in putrescine detected and the reduction in spermidine levels observed. Thus, PAO activity was determined.

PAO may have acted upon conjugated N^1-acetyl derivatives of spermine and spermidine; i.e. derivatives that exist in their own right and not as products of SSAT. This would have led to the formation of spermidine and putrescine, and if catabolism of that spermidine to putrescine occurred, would account for the highly elevated levels of putrescine detected. N^8-acetylspermidine and N-acetylputrescine may have been hydrolysed to spermidine and putrescine to add to the free polyamine pool, or indeed, spermidine and putrescine removed by conjugation. Detection of acetylated amines was therefore carried out with the exception of N^1-acetylspermine whose availability was restricted.

Such acetyl derivatisation of spermidine, removing free spermidine from the polyamine pool and leading to reductions in spermidine levels warranted further investigation. Formation of acetylated amines from labelled ornithine was therefore studied.

Fluctuations in polyamine levels may result from excretion of amines, thus, this process was also studied. Similarly, uptake of amines can often account for fluctuations in the free polyamine pool, and this process will be considered in chapter 7.

Although the higher concentration of norspermidine did not cause any effects on free polyamine levels in fungus exposed for 1 or 4 days, acetylated amine levels were determined in case excretion or uptake of amines had masked a perturbation of polyamine content. As previously described, (section 4.2.1), this would indicate whether further enzymatic studies were necessary.

4.4.2 Materials and Methods

4.4.2.1 PAO activity

PAO activity was determined as described in section 4.2.2.2.

4.4.2.2 Analysis of acetylated polyamines in fungal tissue

Levels of acetylated polyamines were determined as described in section 4.2.2.4.

4.4.2.3 Formation of acetylated polyamines

Study of the formation of acetylated amines was attempted as follows. Fungus was grown in control medium for 2 days before being transferred into medium (50 ml), either unamended or amended to give a concentration of 2.75 mM CHA. 4 µl of [U-^{14}C] ornithine was added to each flask and the flasks incubated at 24°C for 24 hours in a Gallenkamp orbital shaker (140 rpm). Fungus was harvested and analysed for free and acetylated polyamines as described previously except that amines were scraped from the tlc plates and placed into scintillant and radioactivity counted.

4.4.2.4 Determination of amine excretion

As described previously, fungus was exposed to 2.75 mM CHA for 1 or 4 days. After harvesting, fungal mycelium was placed into 10 ml of an amine free buffer. The buffer used was a Citric acid - Na_2HPO_4 (McIlvaine) buffer at pH 4[4] . This buffer was chosen since fresh PDB has a pH of 4.85 and it is known to acidify in the presence of growing fungus. Fungal mycelium was mixed gently in the buffer using a rotamixer and left to stand at room temperature for 6 hours before harvesting the fungus by centrifugation. The buffer was retained and aliquots (100 µl) were analysed for free polyamine presence as described in section 4.1.2.5.

[4] 100 ml of buffer contained 61.45 ml of 0.1 M citric acid monohydrate and 38.55 ml of 0.2 M Na_2HPO_4, as described in "Data for Biochemical Research", (1986), (eds., Dawson, R. M. C., Elliott, D. C., Elliot, W. C. & Jones, K. M.), 3rd edition, Claredon Press, Oxford.

4.4.3 Results

4.4.3.1 PAO activity

Little difference was observed in PAO activity in fungus exposed to 2.75 mM CHA for 1 or 4 days (Table 21).

4.4.3.2 Analysis of acetylated polyamines in fungal tissue

Pooled acetylated spermidine levels were reduced upon exposure of fungus to 2.75 mM CHA, for 1 or 4 days, although the reduction for 1 day exposure was not always significant (Table 22). N-acetylputrescine levels were unaltered in fungus exposed to 2.75 mM CHA for either 1 or 4 days. Exposure of fungus to 0.2 mM norspermidine did not affect acetylated polyamine levels at either of the timings examined (Table 22).

4.4.3.3 Formation of acetylated polyamines

Determination of acetylated polyamines from labelled ornithine was largely unsuccessful. Results showed that despite using a large amount of radio-labelled ornithine, radioactivity was found largely in free spermidine and spermine with little or no radioactivity being detected in acetylated amines (data not shown). It is conceivable that acetylated amines may be formed from a spermidine pool quite distinct from the spermidine pool formed from ornithine. Different pools of free polyamines may exist in fungal cells, as they do in plants (Davis 1990).

4.4.3.4 Determination of amine excretion

Little differences were observed in amines excreted from fungus exposed to 2.75 mM CHA for 1 or 4 days compared to the control fungus (Table 23). Spermine was not detected (data not shown).

Table 21: Effect of 1 or 4 day exposure to 2.75 mM CHA on PAO activity in *Pyrenophora avenae*

	Enzyme activity [pmol product (mg protein)$^{-1}$hr^{-1}]	
Exposure	Control	CHA
1 day	17.6 ± 1.61	23.3 ± 2.21
4 days	31.1 ± 5.60	33.0 ± 1.72

Note: Values are shown as the means ± SE of four replicates.

Table 22: Effect of 1 or 4 day exposure to higher concentrations of CHA and norspermidine on acetylated polyamine levels in *Pyrenophora avenae*.

		Polyamine concentration (μmol g^{-1} FW)	
	Treatment	N-acetylputrescine	N-acetylspermidines
1 day exposure	Control	109 ± 7.4	116 ± 13.4
	2.75 mM CHA	125 ± 19.7	61 ± 2.5
	0.2 mM norspermidine	116 ± 18.9	110 ± 23.0
4 day exposure	Control	67.1 ± 1.23	65.3 ± 8.28
	2.75 mM CHA	58.1 ± 5.12	20.6 ± 1.23 *
	Control	75.4 ± 2.88	59.9 ± 8.14
	0.2 mM norspermidine	68.0 ± 4.36	46.6 ± 4.38

Note: Values are shown as the means ± SE of four replicates.
Significant differences from control are shown as: * $p \leq 0.05$

Table 23: Effect of 1 or 4 day exposure to 2.75 mM CHA on polyamine excretion from *Pyrenophora avenae*.

	Polyamine concentration (μg / ml buffer)			
	Control	CHA	Control	CHA
	1 day exposure		4 day exposure	
spermidine	11.45 ± 0.846	15.30 ± 1.780	12.36 ± 0.466	16.82 ± 1.580
putrescine	9.01 ± 0.530	11.56 ± 1.140	9.07 ± 0.460	8.62 ± 1.340

Note: Values are shown as the means ± SE of four replicates.

4.5 Discussion

The present study found that 1.0 mM CHA increased the level of free putrescine in fungal tissue exposed to the inhibitor. Increased putrescine and decreased spermidine levels are the widely reported results of spermidine synthase inhibition. Such CHA-induced changes in polyamine levels have been observed in *Helianthus tuberosus* extracts (Torrigiani *et al.* 1987), pine cotyledons (Biondi *et al.* 1986) and cultured animal cells (Mitchell *et al.* 1985). However, in the absence of an effect on ODC and AdoMetDC activities or the flux of radio labelled ornithine into polyamines, indicating that spermidine synthase was unaffected, the increase in putrescine cannot be attributed to inhibition of spermidine synthase.

In mammalian cells, putrescine can be formed by a 'back-conversion' pathway involving the action of spermidine/spermine N^1-acetyltransferase and polyamine oxidase on spermine and spermidine (Pegg 1988). However, levels of N^1-acetylspermidine and PAO activity were unaltered, thus, increased catabolism of amines was unlikely to be the cause of the putrescine increase. Such non correlation between the elevated putrescine level and SSAT activity is in agreement with the reports that SSAT activity is not induced by putrescine (Pegg 1988).

The increase in putrescine does not appear to be the result of reduced diamine oxidase activity, the enzyme degrading putrescine. No increase in N^8-acetylated spermidine or N-acetylated putrescine levels was detected, and thus, the increase in free putrescine cannot be accounted for by the breakdown of acetylated polyamine derivatives. Although such conjugation and breakdown pathways have been characterised for mammalian cells only (Seiler 1988), deacetylase enzymes have been reported in fungi (Haywood & Large 1986), therefore suggesting that breakdown of conjugated derivatives is indeed possible in fungi.

Neither 0.01 mM norspermidine, 0.5 mM N^1- nor 0.5 mM N^8-acetylspermidine had any effect on the polyamine biosynthetic enzymes examined or the levels of free polyamines in *P. avenae*. Little information exists on the effects of such analogues on spermidine synthase activity. Masse and co - workers (1985, 1988) did indicate that norspermidine-induced growth effects on maize roots and potatoes were due to the perturbation of spermidine biosynthesis. Similarly, the spermidine analogues N^1, N^8-bis-(ethyl)spermidine (BES) and N^1, N^8-bis(propyl)spermidine (BPS), not only inhibited growth of cultured L1210 cells, but altered polyamine biosynthesis (Porter *et al.* 1985). The results of the present study are, therefore, in contrast to previous findings.

However, a norspermidine-induced effect on cultured HeLa cells was attributed both to effects on polyamine biosynthesis and mitotic activity (Sunkara *et al.* 1988), while Porter *et al.* (1985) found an additional spermidine analogue inhibited L1210 cell growth, but were unable to determine its mode(s) of action. The results of the present study, therefore, agree with those findings, although exactly why differences exist in the effects of the analogues in different organisms is unclear.

The higher (0.2 mM) concentrations of norspermidine had similar effects on *P. avenae:* no effects on the flux of radio labelled ornithine through to polyamines was observed. Free and acetylated polyamine levels were also unaltered. Thus, norspermidine appeared to be exerting its antiproliferative effects without an effect on polyamine biosynthesis.

The higher concentration of CHA (2.75 mM) failed to produce an effect on the flux of radio labelled ornithine through to polyamines in *P. avenae*, suggesting that spermidine synthase activity was unaffected by the compound. However, after 1 days exposure to the compound, spermidine levels were depleted while after 4 days exposure to CHA, spermidine levels were still depleted although, less so, and putrescine levels were elevated substantially. The absence of an initial effect on putrescine and the later smaller depletion of spermidine, suggests that the two phenomena are related. Had catabolism occurred to deplete spermidine and increase putrescine, this would have accounted for the observed results. However, no effect on PAO activity was detected. No effect on acetylated putrescine levels was found, thus breakdown of conjugated polyamines does not account for the increase in the free putrescine pool.

Thus, with both 1.0 mM and 2.75 mM CHA, an increase in putrescine levels in fungus exposed to the inhibitors remains unaccounted for. Exactly why spermidine levels decrease is also unclear. Conjugation would account for a reduction in the free spermidine pool, although, levels of acetylated spermidines also decreased. It should be noted that such reductions in acetylated spermidines may have accounted for the increase in putrescine but without an effect on PAO activity, this is unlikely.

Nevertheless, the above discussion of catabolism is based largely upon mechanisms characterised for mammalian cells. Knowledge of catabolism in many microorganisms is fragmentary (Large 1992), and indeed, little is known of such pathways in plant pathogenic fungi (Walters 1995). Large (1992) reports how two main catabolic pathways exist for many microorganisms, the N-acetylated route and an alternative route, involving Δ^1-

pyrroline as an intermediate. Thus, it may be possible that an as yet uncharacterised catabolic pathway exists in *P. avenae*.

Regulation of intracellular polyamines may also involve selective uptake of polyamines, and this is considered later in this thesis. Excretion of polyamines may also be associated with abberant intracellular polyamine levels, although no evidence of such a mechanism was detected. Release of bound polyamines from, for example, macromolecular binding sites, may account for observed increases in putrescine content while stimulated binding of polyamines would account for the reduction in free spermidine. Indeed, Paulus *et al.* (1993) noted how the majority of polyamines in *Neurospora crassa* were bound to cell constituents. Further work would be necessary to determine bound polyamine levels in *P. avenae*.

It should be noted that an accumulation of putrescine is a widely reported response to stress in an organism, particularly plants (for example, Foster & Walters 1991). Bound putrescine could, therefore, have been released in an attempt to off-set the stress caused by exposure of fungus to CHA. A similar phenomenon was observed by Havis *et al.* (1996b), with spermine being displaced from binding sites following the exposure of *Phytophthora infestans* to E-TED. Exactly how elevated putrescine regulates stress is unknown but it has been associated with increasing membrane stability (Marton & Morris 1987), maintenance of cellular pH (Smith & Sinclair 1967), increased protein synthesis (Kaur-Sawhney *et al.* 1980) and synthesis of protective alkaloids (Hartmann *et al.* 1988).

In contrast to the hypothesis that elevated putrescine occurs to enable the fungus to withstand the effects of CHA, the antifungal activity of the compound may also be associated with the increase in putrescine concentration. An accumulation of putrescine was suggested by Davis & Ristow (1991) to be responsible for the reduction in growth of a mutant of *N. crassa*. Excess putrescine has been shown to be toxic in the cyanobacterium *Anabaena* (Guarino & Cohen 1979). Also, in spite of Dumbroff (1991) suggesting that polyamines may act as free radical scavengers, DiTomaso *et al.* (1989) hypothesised that catalytic breakdown of excess putrescine by DAO to hydrogen peroxide and free radicals, caused membrane damage of maize roots.

The findings for CHA are thus in contrast to many which indicate that CHA inhibits spermidine synthase from a variety of sources. Examples include spermidine synthase from mammalian cells, trypanosomes, certain bacteria (Pegg & Williams-Ashman 1987) and from Chinese cabbage leaves (Sindhu & Cohen 1984). However, work on other polyamine biosynthetic enzymes has shown that enzymes will react differently upon exposure to an

inhibitor *in vitro* and *in vivo* (Walters *et al.* 1995a). Such a phenomenon could, therefore, explain the lack of effect by CHA on spermidine synthase in fungi. Nevertheless, CHA is known to inhibit spermidine synthase in protoplasts extracted from Chinese cabbage leaves (Greenberg & Cohen 1985). Other authors, however, have observed CHA to cause an inhibitory growth effect on potatoes (Masse *et al.* 1988) and in tobacco callus culture systems (Tiburcio *et al.* 1987), but have been unable to fully account for this effect by perturbation of polyamine biosynthesis, and indeed, suggest that spermidine synthase inhibition is unlikely.

Previous work with CHA, norspermidine and spermidine analogues in other organisms aside, the findings of the present study with fungi were not entirely unexpected. Indeed, work using putrescine analogues had concluded that observed effects on polyamine biosynthesis in fungi grown *in vitro* were unlikely to have been wholly responsible for reductions in fungal growth (Havis *et al.* 1994a,c, 1997). Havis *et al.* (1996a) also found cyclic diamines possessed fungicidal activity against powdery mildew on barley yet failed to affect polyamine biosynthesis of *P. avenae* grown *in vitro*. Further work is therefore necessary to elucidate the mechanism(s) by which CHA and spermidine analogues exert their antifungal effects.

Future work will be discussed in the conclusion of this thesis. There are, however, some specific areas associated with the work reported in this chapter which may be worthy of future exploration.

CHA was found to co-chromatograph with spermine whilst norspermidine co-chromatographed with spermidine. Such a phenomenon meant that fluctuations in these polyamines were generally ignored, especially when dealing with fungus exposed to the higher concentrations of CHA and norspermidine. Development of a more accurate method of polyamine analysis would be prudent since 'discounting' such results may mean that fluctuations in free polyamine levels are undetected. It should also be noted that APC and 3APC, higher cadaverine homologues, also co-chromatograph with spermidine and spermine respectively (Zarb & Walters 1994b). Production of these homologues may also affect amine detection. Indeed, Alhonen-Hongisto & Jänne (1980) reported how tumour cells in which polyamine biosynthesis had been perturbed, produced APC and 3APC. APC and 3APC formation has been reported for *P. avenae* and mycorrhizal species (Zarb & Walters 1994b) as well as *N. crassa* starved of polyamines (Paulus *et al.* 1982). However, cadaverine levels were unaltered in *P. avenae* exposed to CHA and spermidine analogues, thus, biosynthesis of the higher homologues was unlikely to have occurred. Interestingly,

CHA inhibited formation of the higher homologues in extracts of *P. avenae* (Zarb & Walters 1994b).

Determination of amine excretion was attempted by placing fungus, which had been grown for 1 or 4 days in the presence of CHA, into an amine free buffer for some hours before analysing the buffer for polyamine presence. However, this failed to account for those polyamines already excreted over the four day growing period. Excretion is likely to occur via a passive diffusion mechanism once intracellular polyamine levels become too high (Davis 1990). Thus, excretion may have occurred before its determination was attempted in this study. Polyamines are present in the growing medium of fungus, thus, taking aliquots of the growing medium for analysis throughout the growth period of the fungus is not desirable. However, given a sensitive analytical method for determining polyamines, this process would give a clearer impression of the effects of inhibitors on excretion of amines by a growing fungus.

As discussed, excess putrescine may regulate stress by stabilising membranes but may also cause inhibition of growth by damaging membranes. Further work examining ion efflux from fungus grown in the presence of inhibitors may indicate if cellular membranes are damaged.

Chapter 5

Effect of novel spermidine analogues on *in vitro* growth and polyamine biosynthesis in *Pyrenophora avenae*

5. EFFECT OF NOVEL SPERMIDINE ANALOGUES ON *IN VITRO* GROWTH AND POLYAMINE BIOSYNTHESIS OF *PYRENOPHORA AVENAE*

5.1 INTRODUCTION AND OBJECTIVES

Given the antifungal and fungicidal activity of commercially available spermidine analogues, albeit with their mode(s) of action as yet undetermined, various novel spermidine analogues were synthesised and their antifungal activity determined by examining their effect on the growth of *P. avenae* on solid media. This work was part of an on-going collaborative research programme with Professor David Robins of the Chemistry Department at Glasgow University, who synthesised the novel analogues. Solid media studies were chosen over a study of the effects in liquid culture since limited amounts of each chemical were available, even though effects on growth on solid media can be misleading. Analogues which possessed significant antifungal activity were then examined further, determining their effect on growth of *P. avenae* in liquid culture and on fungal polyamine biosynthesis by carrying out biochemical analyses.

5.2 EFFECT OF NOVEL SPERMIDINE ANALOGUES ON MYCELIAL GROWTH OF *PYRENOPHORA AVENAE* ON SOLID MEDIA

5.2.1 Materials and Methods

P. avenae was maintained as described in section 3.1.2.1 (page 76). Fungus was grown on solid media amended individually with eight novel spermidine analogues (Table 24), to give final concentrations between 0.05 mM and 2.0 mM. The concentrations were chosen on the basis of previous work using CHA and norspermidine which utilised such concentrations (section 3.1.2.2, page 76). Four replicates were used for each treatment with significance being assessed using Student's *t*-test. All experiments were repeated and similar results obtained.

5.2.2 Results

Mycelial growth was successfully inhibited by two of the novel spermidine analogues, LAS 2/12 and LAS 2/28, at concentrations of 2.0 mM (Figures 11-14; Appendix 8). LAS 2/12 reduced fungal growth by 47.3 %, while LAS 2/28 reduced growth by 34.5 %, eight days after inoculation ($p \leq 0.001$). None of the other compounds had any great effect on mycelial growth of *P. avenae* on solid media at the concentrations examined (Figures 11-14; Appendix 8).

Table 24: Structures of novel spermidine analogues

Analogue	Structure
C59	$H_2N(CH_2)_3NH(CH_2)_4NHC_2H_5$
C73	$H_2N(CH_2)_3NH(CH_2)_5NHC_2H_5$
LAS 2/11	$H_2N(CH_2)_3NHCH_2CH{=}CHCH_2N(C_2H_5)C_2H_5$
LAS 2/12	$H_2N(CH_2)_3NH(CH_2)_4N(C_2H_5)C_2H_5$
LAS 2/26	$H_2N(CH_2)_3NH(CH_2)_3N(CH_3)CH_3$
LAS 2/27	$H_2N(CH_2)_3NH(CH_2)_4N(CH_3)CH_3$
LAS 2/28	$H_2N(CH_2)_3NH(CH_2)_3N(C_2H_5)C_2H_5$
LAS 3/8	$H_2N(CH_2)_3NHCH_2CH{=}CHCH_2N(CH_3)CH_3$

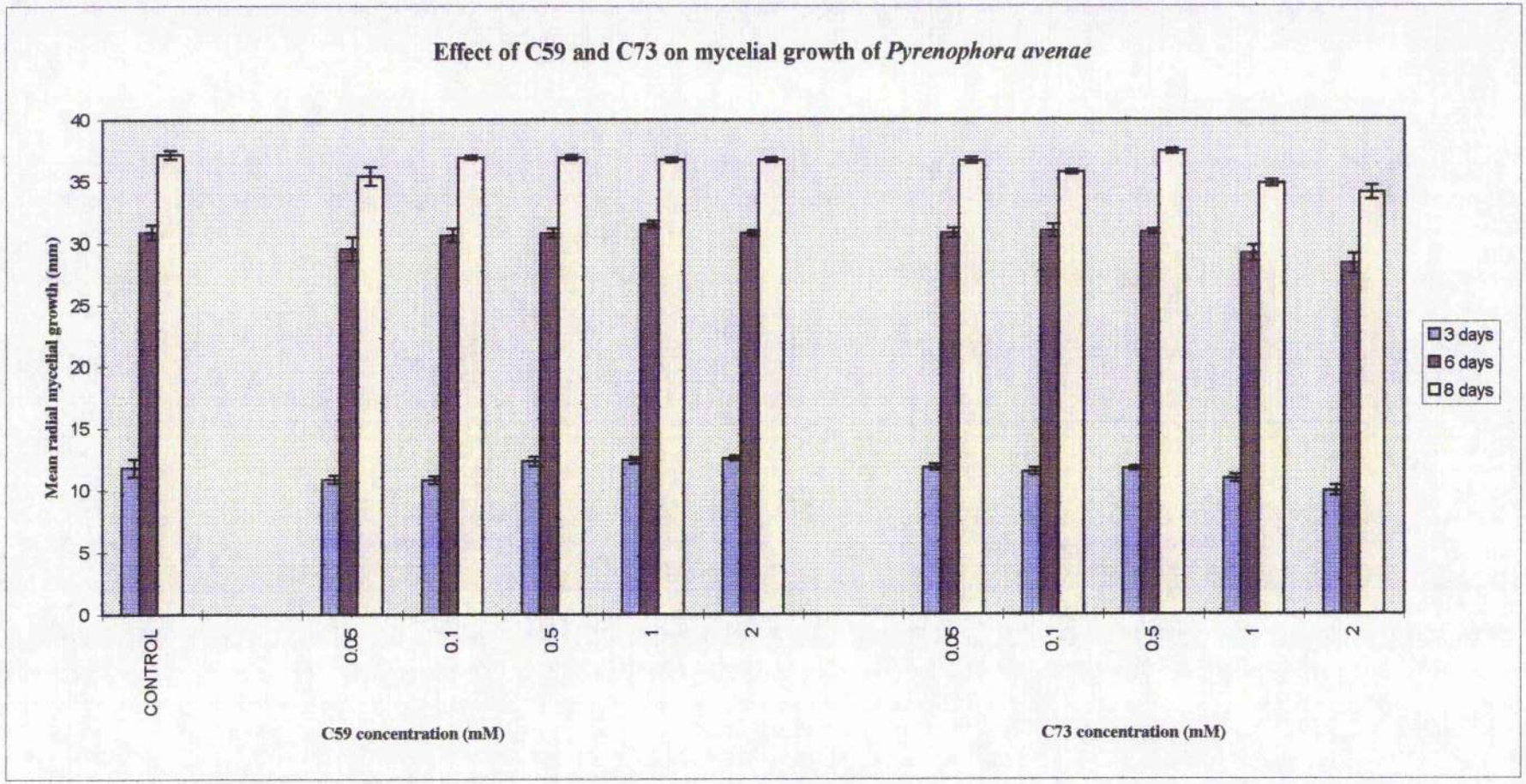


Figure 11. Effect of varying concentrations (0.05 - 2 mM) of the novel spermidine analogues C59 and C73 on mycelial growth of *Pyrenophora avenae* on solid media. Radial mycelial growth was measured 3, 6 and 8 days after inoculation of plates. Values are the means of 3 measurements from each of 4 replicates with standard errors of means indicated. Significant differences from control are indicated in Appendix 8a.

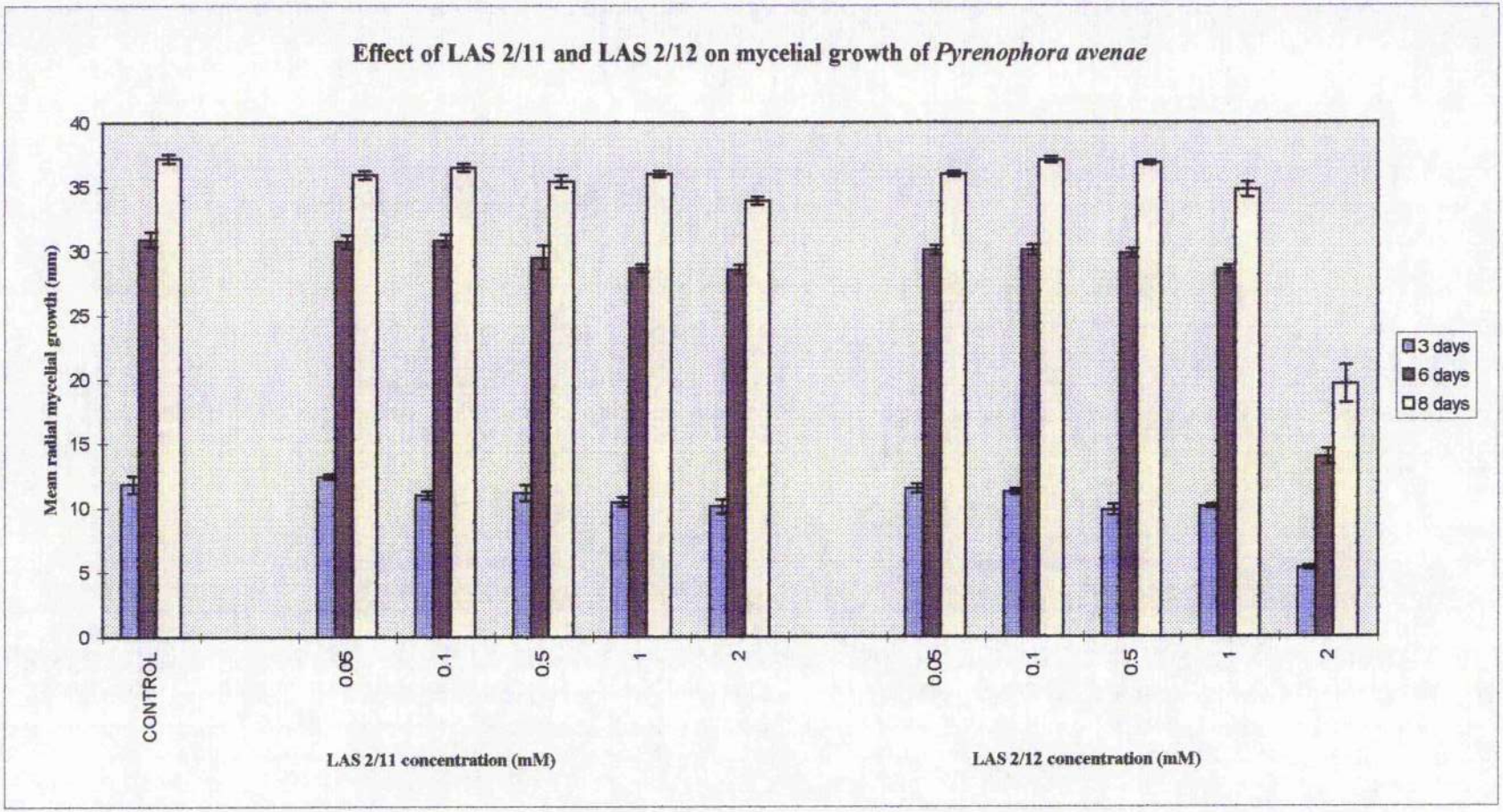


Figure 12. Effect of varying concentrations (0.05 - 2 mM) of the novel spermidine analogues LAS 2/11 and LAS 2/12 on mycelial growth of *Pyrenophora avenae* on solid media. Radial mycelial growth was measured 3, 6 and 8 days after inoculation of plates. Values are the means of 3 measurements from each of 4 replicates with standard errors of means indicated. Significant differences from control are indicated in Appendix 8a.

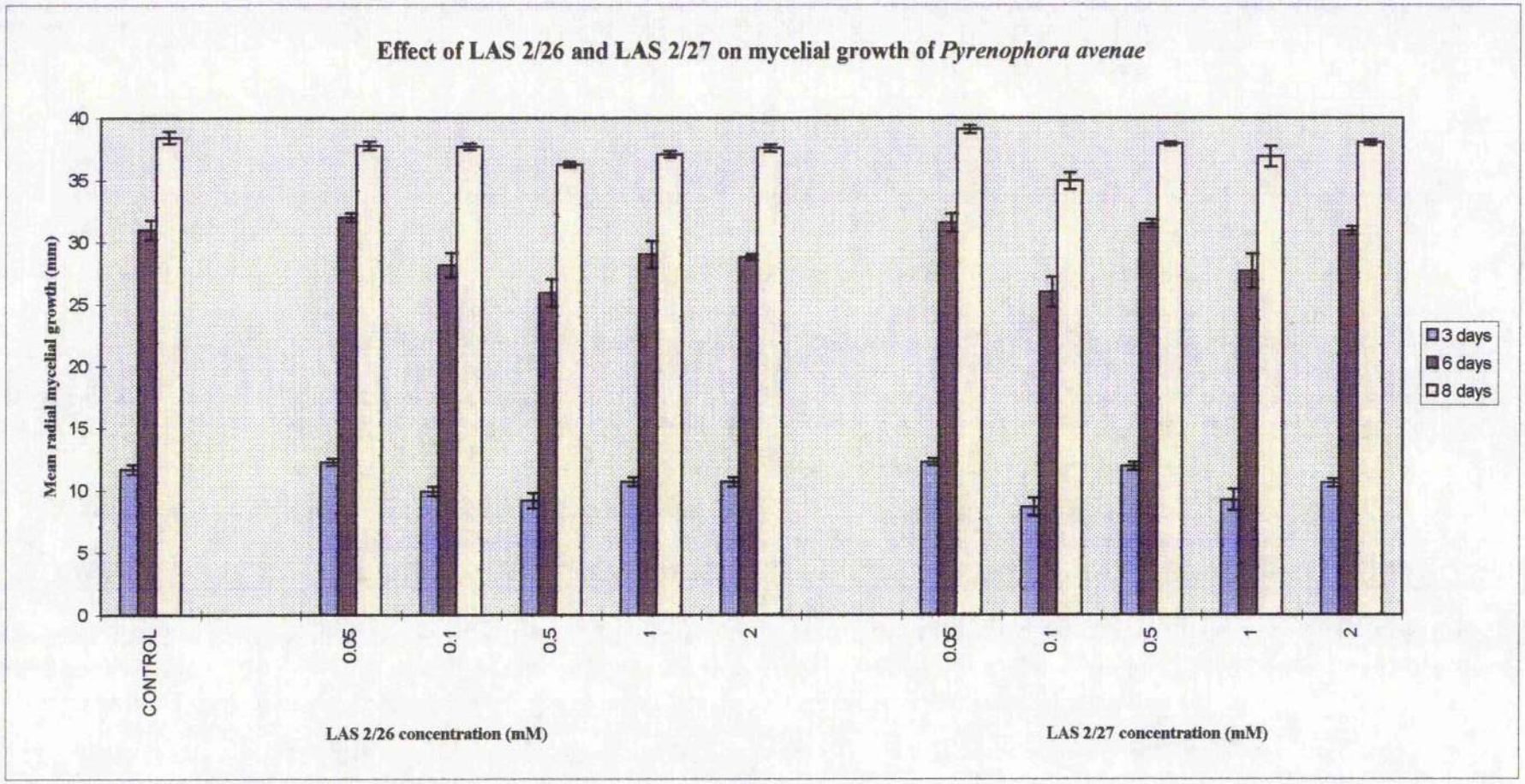


Figure 13. Effect of varying concentrations (0.05 - 2 mM) of the novel spermidine analogues LAS 2/26 and LAS 2/27 on mycelial growth of *Pyrenophora avenae* on solid media. Radial mycelial growth was measured 3, 6 and 8 days after inoculation of plates. Values are the means of 3 measurements from each of 4 replicates with standard errors of means indicated. Significant differences from control are indicated in Appendix 8b.

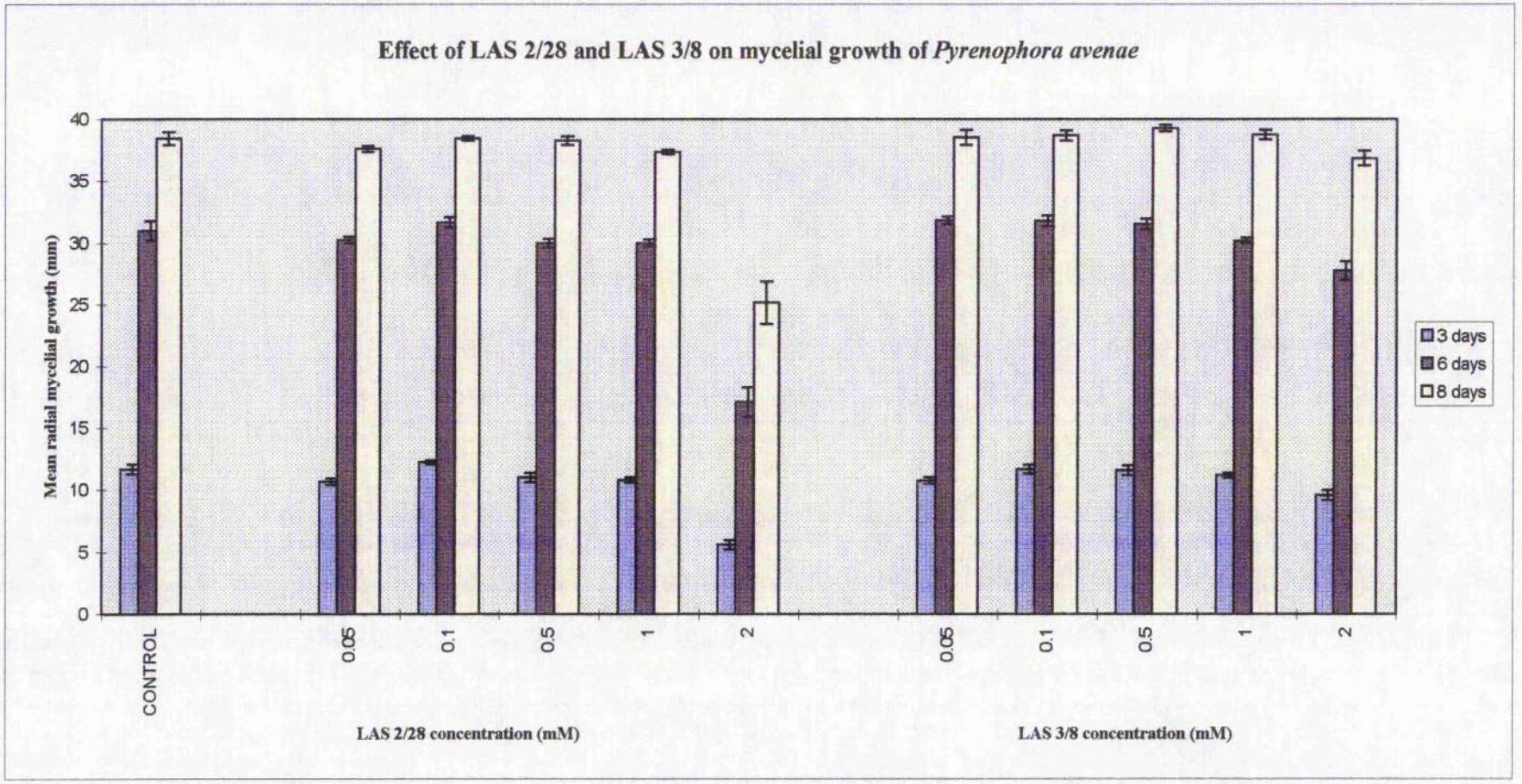


Figure 14. Effect of varying concentrations (0.05 - 2 mM) of the novel spermidine analogues LAS 2/28 and LAS 3/8 on mycelial growth of *Pyrenophora avenae* on solid media. Radial mycelial growth was measured 3, 6 and 8 days after inoculation of plates. Values are the means of 3 measurements from each of 4 replicates with standard errors of means indicated. Significant differences from control are indicated in Appendix 8b.

5.3 Effect of novel spermidine analogues on growth of *Pyrenophora avenae* in liquid culture

5.3.1 Materials and Methods

P. avenae was grown in liquid culture as described in section 3.2.2.2 (page 85), in the presence of either 1.0 mM LAS 2/28, C59, C73 or 0.5 mM LAS 2/12. A concentration of 1.0 mM was chosen for those compounds since previous work had shown that CHA affected growth of *P. avenae* in liquid culture at that concentration. 0.5 mM was chosen as the concentration of LAS 2/12 to be studied since a preliminary experiment found that 1.0 mM LAS 2/12 completely inhibited growth of *P. avenae* (data not shown).

LAS 2/12 and LAS 2/28 were chosen for further investigation given their successful inhibition of mycelial growth of *P. avenae* on solid media. Although C59 and C73 failed to affect mycelial growth of *P. avenae* on solid media, previous glasshouse experiments had shown that these compounds possessed fungicidal activity against powdery mildew on barley, rust on broad bean and powdery mildew on apple (N. D. Havis unpublished results). Therefore, further investigation of these compounds was also appropriate.

Since the amounts of these compounds was limited, fungus was only grown in liquid culture for 4 days. Previous work with the commercially available spermidine analogues had found little differences in *P. avenae* exposed to the compounds for 2, 3 or 4 days, thus examination of 4 day old fungus only was deemed appropriate. Limited amounts of chemical meant that only two replicates were used. Significance was assessed using Student's *t*-test. Experiments were repeated and similar results were obtained.

5.3.2 Results

All of the compounds examined reduced fungal growth of *P. avenae* in liquid culture 4 days after inoculation. 0.5 mM LAS 2/12 reduced growth by 92.4 %, while 1.0 mM concentrations of C59, C73 and LAS 2/28 reduced fungal growth by 56.0, 65.5 and 87.1 % respectively (Table 25). Only growth in LAS 2/28 was significantly different from the control since a small number of replicates were used.

5.4 Effect of novel spermidine analogues on the incorporation of radio-labelled ornithine into polyamines in *Pyrenophora avenae* grown in liquid culture

5.4.1 Materials and Methods

The incorporation of radio-labelled ornithine into polyamines was carried out as described in section 4.1.2.4 (page 97). Such an analysis was chosen to determine if spermidine formation was affected in *P. avenae* grown in liquid culture in the presence of the novel spermidine analogues. Due to the limited amount of fungal material available, this was the only analysis carried out, although, it would also provide an indication of the activities of ODC and AdoMetDC. All results are the means of four replicates with significance being assessed using Student's *t*-test. All experiments were repeated with similar results obtained.

5.4.2 Results

Neither 0.5 mM LAS 2/12, 1.0 mM LAS 2/28, 1.0 mM C59 or 1.0 mM C73 had any effect on the incorporation of radio-labelled ornithine into polyamines. All radioactivity was found in spermidine or spermine with none in putrescine (Table 26).

Table 25. Effect of novel spermidine analogues on growth of *Pyrenophora avenae* in liquid culture for 4 days

Treatment	Growth of fungus, measured as mean fresh weight (g)
Control	4.6 ± 0.19
1.0 mM LAS 2/28	0.6 ± 0.02 *
Control	5.6 ± 0.87
1.0 mM C59	2.5 ± 0.16
1.0 mM C73	1.9 ± 0.12
0.5 mM LAS 2/12	0.4 ± 0.01

Note: Values are shown as the means ± SE of two replicates.
Significant difference from control is as: * $p \leq 0.05$

Table 26. Effect of novel spermidine analogues on the incorporation of radio-labelled ornithine into polyamines in *Pyrenophora avenae* grown for 4 days

	Radioactivity in polyamine (dpm g-^{1} FW)	
Treatment	Spermine	Spermidine
Control	8.6 ± 3.04	284 ± 11.0
1.0 mM LAS 2/28	17.4 ± 1.7	253 ± 18.2
Control	6.4 ± 0.35	160 ± 13.4
0.5 mM LAS 2/12	11.8 ± 2.29	135 ± 17.7
1.0 mM C59	7.2 ± 1.87	194 ± 11.1
1.0 mM C73	11.4 ± 2.35	172 ± 26.2

Note: Values are shown as the means ± SE of four replicates.

5.5 Discussion

The observed inhibition of fungal growth by novel spermidine analogues agrees with previous work by Porter *et al.* (1985) who showed that analogues of spermidine inhibited growth of L1210 cultured cells. Also, the findings are in agreement with the previous work carried out in this study, when a commercially available analogue, norspermidine, was observed to possess antifungal properties against pathogenic fungi *in vitro*.

However, growth of *P. avenae* was insensitive towards many of the inhibitors, although this may be indicative of the unreliability of studies of fungal growth on solid media (Isaac & Jennings 1995). As discussed previously, 1.0 mM E-TED was ineffective against *P. avenae* on solid media yet completely inhibited growth of the fungus in liquid culture (Havis *et al.* 1994c). Thus, compounds which did not affect mycelial growth may possess antifungal activity. Limited amounts of the compounds prevented further work, although this may an area for future interest.

On the other hand, the compounds may be truely insensitive towards *P. avenae*. Growth of some fungal species on solid media was reported to be unaffected by CHA (West & Walters 1989). There may be a number of reasons for this phenomenon, as already discussed in previous chapters. Briefly, Birecka *et al.* (1986) suggested that differences in fungal sensitivity to inhibitors may be due to varying uptake of inhibitor by the fungus or differences in fungal enzyme sensitivity to the inhibitors. Another possibility for fungal insensitivity towards the analogues may be rapid metabolism of the inhibitors, as suggested by Khan & Minocha (1989a). Further work would be required to establish reasons for the observed differences between the analogues in their effect on *P. avenae* growth. It should be noted that even stereo-isomers of the same compound, for example E-BED and Z-BED (Havis *et al.* 1994a) and trans BAD and cis BAD (Havis *et al.* 1996a) produce very different effects on growing fungus.

The four compounds examined did not appear to affect the flux of radio labelled ornithine through to polyamines. This indirect examination of spermidine synthase activity demonstrates that the analogues do not inhibit fungal growth *in vitro* through an effect on the enzyme. Given the results of the study so far, this finding is not unexpected. In other work, novel putrescine analogues did indeed perturb polyamine metabolism yet this phenomenon was not suggested as the main cause of the observed antifungal effects (Havis *et al.* 1994a,c). These workers also went onto demonstrate how some cyclic putrescine analogues perturbed polyamine metabolism while others had no effect and, as with the aliphatic analogues, any effect on polyamine biosynthesis was not thought to wholly account for the antifungal properties of the analogues (Havis *et al.* 1996a, 1997).

In their original hypothesis, Porter & Sufrin (1986) suggested inhibition of a polyamine biosynthetic enzyme to be only one potential mode of action by which analogues may exert antiproliferative activity. Alternative suggested mechanisms of effect include regulation of enzymes, binding to sites associated with cellular proliferation and binding to macromolecules and disrupting their structure and/or function. Thus, the novel spermidine analogues showed potential as antifungal agents although further work is necessary to elucidate their mode(s) of action.

Chapter 6

Field studies

6. FIELD STUDIES

6.1 Introduction and Objectives

It has already been demonstrated in this study that CHA and norspermidine possess fungicidal activity against a number of plant pathogens (Chapter 2). While glasshouse experiments are important for preliminary screening, such studies are not always true reflections of the behaviour of a compound in the field. For compounds to be of any use agriculturally, they must show activity against fungal pathogens in the field. Thus, despite the mode of action of the compounds being undetermined, small scale field trials were carried out to evaluate CHA and norspermidine as novel fungicides against powdery mildew on spring barley.

6.2 Materials and Methods

6.2.1 1995 Trial

Spring barley (cv. Prisma) was sown at a row spacing of 12 cm with a seed rate of 220 $kg.ha^{-1}$. Plots (4.0 m x 2.0 m) were arranged in a randomised block design with four replicates. With the exception of disease control, normal husbandry practices were employed throughout the growth of the crop. 500 $kg.ha^{-1}$ of a 20:10:10 compound fertiliser was applied at sowing (100:50:50 $kg.ha^{-1}$ NPK) and a mixture of two broad spectrum herbicides was applied at GS 15 (Zadoks growth stage: five leaves unfolded). Metsulfuron-methyl (Ally, Du Pont) and fluroxypyr + ioxynil + bromoxynil (Advance, Zeneca) control many broad leaved weeds including charlock, chickweed, cleavers, hemp-nettle, knotgrass, redshank and deadnettles.

To evaluate CHA and norspermidine as novel fungicides against powdery mildew, various treatments were applied at GS 32 (Zadoks growth stage: second node detectable), when mildew was initially observed. CHA and norspermidine were both applied at concentrations of 1.0 mM and 5.0 mM along with mixtures of 5.0 mM CHA + 5.0 mM E-TED[5] and 5.0 mM norspermidine + 5.0 mM E-TED. Jänne *et al.* (1985) suggested that by using an inhibitor of AdoMetDC along with one of ODC, complete depletion of putrescine, spermidine and spermine in mammalian cells should be possible. DFMO often failed to deplete cells of all polyamines and those remaining amines were thought to be responsible for sustained cellular growth. The combination of two analogues, proposed to affect spermidine synthase and ODC, was thus thought to be a useful additional investigation in this field trial.

[5] E-TED was provided by Professor D. J. Robins of Glasgow University. Synthesis of the compound is described by Havis *et al.* (1994a).

The treatments were applied using an AZO compressed air sprayer at a rate of 300 $l.ha^{-1}$. Treatments were made up in water with Agral (alkyl phenol ethylene oxide, Zeneca) at a rate of 0.5 $l.ha^{-1}$. A commercial fungicide, a mixture of flutriafol + carbendazim, (Early Impact, Zeneca; 118 g and 188 g $a.i.ha^{-1}$ respectively) was also evaluated. Ten plants per plot were assessed 7 and 14 days after treatment by estimating the percentage mildew infection on the third leaf using a standard area diagram.

All plots except those treated with the mixture of 5.0 mM CHA + 5.0 mM E-TED were sprayed a second time at GS 59 (Zadoks growth stage: emergence of inflorescence) and the percentage mildew infection on the flag leaves assessed 7, 14 and 21 days later. Limited amounts of E-TED restricted use of this chemical. Since preliminary results had shown the initial CHA + E-TED spray to be less effective in controlling powdery mildew infection than the other treatments, it was omitted from the second spray programme.

Ten plants and 100 ears per plot were harvested and measurements made of plant height, plant dry weight and grain dry weight at 14.5 % moisture content.

Disease assessment results were analysed using an analysis of variance of square root transformed data. Data were transformed to acquire the normal distribution necessary for statistical analysis to be carried out. Plant height, plant dry weight and grain dry weight results were simply analysed using an analysis of variance.

6.2.2 1996 Trial

The 1996 field trial was carried out as described for the 1995 trial in section 6.2.1 with the exception of the herbicide application, where metsulfuron-methyl (Ally, Du Pont) was applied at GS 32. Fungicidal evaluation was carried out with sprays at GS 32 and GS 59 and two and three disease assessments after the respective sprays. CHA (1.0 mM and 5.0 mM) and norspermidine (1.0 mM and 5.0 mM), and the mixture of flutriafol + carbendazim (Early Impact, Zeneca), were re-evaluated. Additional compounds were also evaluated: tebuconazole (Folicur, Bayer; 250 $a.i.ha^{-1}$), a half dose of the fungicide and mixtures of a half dose of the fungicide + 1.0 mM CHA or 1.0 mM norspermidine. Strategies for combatting the onset of fungicide resistance are vital to extend the life of the decreasing number of active ingredients available. Mixtures of fungicides with different modes of action appear to be one way of counteracting the development of resistance (Bolton and Smith 1988). Thus, it was decided to evaluate mixtures of CHA or norspermidine with a known fungicide. The lower 1.0 mM concentrations of CHA and norspermidine were chosen since the 1995 field trial failed to differentiate between the two concentrations (i.e. 1.0 mM and 5.0 mM) with respect to mildew control.

6.2.3 1996 Mini plots

Surplus areas sown with spring barley for the 1996 field trial were sub-divided to give a randomised block design with 14 plots (2.0 m x 1.0 m) (four replicates). Treatments in the large field trials were limited to 1.0 mM and 5.0 mM CHA and norspermidine: thus, 1.0, 2.0, 3.0, 4.0, and 5.0 mM concentrations of the compounds were evaluated using the mini-plots. The combination of a putrescine analogue with CHA or norspermidine was investigated in the 1995 field trial. Since plots were unavailable for such an evaluation in 1996, mini plots were used to examine the effect of an alternative ODC inhibitor, DFMO[6] , on spring barley alone, and in combination with 1.0 mM concentrations of CHA or norspermidine. 1.0 mM concentrations were again chosen since the 1995 field trial had found no difference in fungicidal activity of 1.0 mM and 5.0 mM concentrations of the compounds. The mini-trial was carried out almost in the same manner as the 1996 field trial as described in section 6.2.2. However, treatments were made up in water with 0.01 % Tween 20 (v/v) to act as a surfactant and were applied to the plots using hand held compressed air sprayers. Since the treatments were applied with a reduced pressure compared to the AZO compressed air sprayer, only infection on the flag leaf was assessed. Measurements of plant height, plant dry weight and grain dry weight were not made for the mini plots.

[6] DFMO was kindly provided by Marion Merrell Dow Pharmaceuticals.

6.3 Results

6.3.1 1995 Trial

All treatments significantly reduced mildew levels on 19 June except the mixture of 5.0 mM CHA and 5.0 mM E-TED (Table 27). All treatments significantly reduced infection on the second and fourth assessments. No significant differences were observed between any of the treatments at the third assessment, and was probably due to the low levels of mildew present. The reduction in mildew observed with 5.0 mM CHA and the mixture of 5.0 mM CHA and 5.0 mM E-TED at the fifth assessment (19 July) was not significant. All other treatments significantly reduced the infection level on this date.

Little differences were observed between the treatments. Although the commercial fungicide was significantly better than 5.0 mM CHA on both the first and final assessments, generally, the treatments were no less effective than the mixture of flutriafol and carbendazim in controlling mildew infection. No differences were observed between the lower and higher concentrations of either CHA or norspermidine. Similarly, the addition of a putrescine analogue to CHA and norspermidine did not enhance the disease control conferred (Table 27).

No significant differences were observed between any of the treatments in either plant height, plant dry weight or grain dry weight (Table 28).

An estimation of the grain yield per plot was made to give a more meaningful value for the field trial yield data. The number of stems (equivalent to the number of ears) in a 1 m row were counted at 20 random locations over the field trial area. Thus, the mean value was calculated and with 14 rows per plot, the number of ears per plot was estimated. The known grain weight of 100 ears in each plot was then converted to give the grain weight per plot. Statistical analysis was carried out on the unconverted data since the value of grain weight per hectare is only an estimation and assumes equal plant establishment over the trial area.

Table 27: Effect of CHA, norspermidine, E-TED and a flutriafol and carbendazim mixture on powdery mildew infection of spring barley: 1995 Trial

Treatment	Mean percentage powdery mildew infection				
	19 June	26 June	5 July	12 July	19 July
untreated	19.6	38.4	8.8	24.6	28.8
flutriafol and carbendazim	7.9	28.1	6.1	10.8	9.5
1.0 mM CHA	12.2	25.6	5.9	12.6	11.5
5.0 mM CHA	12.4	25.1	7.3	11.5	19.5
1.0 mM norspermidine	11.4	27.4	6.1	8.9	9.6
5.0 mM norspermidine	10.0	27.1	5.4	11.2	14.6
5.0 mM CHA + 5.0 mM E-TED	19.3	28.5	7.4	16.0	19.6
5.0 mM norspermidine + 5.0 mM E-TED	8.6	22.8	5.5	12.4	13.4
LSD ($p \leq 0.05$)	4.28	4.81	ns	6.22	8.37
sed *	0.287	0.224	ns	0.355	0.530

Note: Values are shown as the means of ten measurements from each of four replicate plots.

ns no significant difference between treatments

* of transformed data

Table 28: Effect of CHA, norspermidine, E-TED and a flutriafol and carbendazim mixture on plant height, plant dry weight and grain dry weight of spring barley: 1995 Trial

Treatment	Mean plant height (cm)	Mean plant dry weight (g)	Grain dry weight of 100 ears (g)	Estimated grain yield per plot (kg)
untreated	70.51	0.97	107.08	4.92
flutriafol and carbendazim	70.25	1.02	111.92	5.14
1.0 mM CHA	66.98	0.91	107.64	4.94
5.0 mM CHA	71.08	0.97	108.32	4.97
1.0 mM norspermidine	68.58	0.98	107.10	4.92
5.0 mM norspermidine	67.20	0.95	107.39	4.93
5.0 mM CHA + 5.0 mM E-TED	70.19	0.93	106.00	4.87
5.0 mM norspermidine + 5.0 mM E-TED	69.84	1.02	107.97	4.96

Note: Values are shown as the means of ten measurements from each of four replicate plots.

ANOVA f value probabilities were > 0.05 (0.403, 0.149 and 0.556 respectively for plant height, plant dry weight and grain dry weight of 100 ears)

6.3.2 1996 Trial

In all of the assessments, all treatments significantly reduced powdery mildew infection of spring barley (Table 29). Little differences were observed between the mixture of flutriafol and carbendazim and the CHA and norspermidine treatments. Similarly, little differences were observed between the tebuconazole (full dose) treatment, tebuconazole (half dose) and the mixtures of half doses of tebuconazole and 1.0 mM CHA or 1.0 mM norspermidine.

Tebuconazole (full dose) was more effective in controlling disease than the mixture of flutriafol and carbendazim, and the CHA and norspermidine (1.0 mM and 5.0 mM) treatments, although only significantly so at the second assessment.

No differences were found between the heights, dry weights and grain weights of plants harvested from the plots (Table 30).

Table 29. Effect of CHA, norspermidine, a flutriafol and carbendazim mixture and tebuconazole on powdery mildew infection of spring barley: 1996 Trial

Treatment	Mean percentage powdery mildew infection				
	12 June	19 June	15 July	22 July	29 July
untreated	27.8	38.8	9.9	16.7	24.9
flutriafol and carbendazim	16.2	17.5	2.6	4.5	8.7
1.0 mM CHA	18.1	24.5	2.8	2.9	5.8
5.0 mM CHA	18.8	22.2	2.4	3.0	7.0
1.0 mM norspermidine	17.5	22.9	2.2	4.2	12.0
5.0 mM norspermidine	18.2	22.8	4.5	2.6	9.1
tebuconazole	14.8	10.5	1.6	3.6	8.1
1/2 dose tebuconazole	14.4	13.8	1.6	2.2	7.4
1/2 dose tebuconazole plus 1.0 mM CHA	17.5	16.3	2.2	4.0	9.7
1/2 dose tebuconazole plus 1.0 mM norspermidine	14.0	10.7	1.6	2.3	9.7
LSD ($p \leq 0.05$)	5.10	7.21	4.20	5.10	5.52
sed *	0.302	0.475	0.449	0.385	0.350

Note: Values are shown as the means of ten measurements from each of four replicate plots.

* of transformed data

Table 30. Effect of CHA, norspermidine, a flutriafol and carbendazim mixture and tebuconazole on plant height, plant dry weight and grain dry weight of spring barley: 1996 Trial

Treatment	Mean plant height (cm)	Mean plant dry weight (g)	Grain dry weight of 100 cars (g)	Estimated grain yield per plot (kg)
untreated	70.26	0.77	93.4	4.29
flutriafol and carbendazim	72.27	0.87	83.2	3.82
1.0 mM CHA	72.11	0.81	74.1	3.40
5.0 mM CHA	67.45	0.70	86.9	3.99
1.0 mM norspermidine	70.19	0.80	83.7	3.84
5.0 mM norspermidine	69.64	0.79	87.7	4.03
tebuconazole	69.95	0.82	82.5	3.79
1/2 dose tebuconazole	69.40	0.83	88.1	4.05
1/2 dose tebuconazole plus 1.0 mM CHA	73.00	0.82	78.3	3.60
1/2 dose tebuconazole plus 1.0 mM norspermidine	70.35	0.88	90.3	4.15

Note: Values are shown as the means of ten measurements from each of four replicate plots.

ANOVA f value probabilities were > 0.05 (0.630, 0.117 and 0.460 respectively for plant height, plant dry weight and grain dry weight of 100 cars)

6.3.3 1996 Mini plots

As discussed in section 6.2.3, only the flag leaves on the mini plot plants were assessed for mildew infection. Consequently, only three assessments were made, 7, 14 and 21 days after the second spray. On the dates of the two assessments after the first spray, no disease was present on the flag leaves.

All of the treatments examined reduced powdery mildew infection significantly on all three assessment dates (Table 31). Little differences were apparent between the treatments and none were observed between the CHA treatments themselves, the norspermidine treatments or the DFMO ± CHA / norspermidine treatments.

Table 31. Effect of CHA, norspermidine and DFMO on powdery mildew infection of spring barley: 1996 Mini plots

Treatment	Mean percentage powdery mildew infection		
	19 July	26 July	2 August
untreated	10.1	14.3	26.7
1.0 mM CHA	4.0	5.6	12.6
2.0 mM CHA	3.9	4.7	10.6
3.0 mM CHA	2.3	3.8	11.9
4.0 mM CHA	2.0	3.7	10.3
5.0 mM CHA	2.6	4.9	10.2
1.0 mM norspermidine	2.8	4.5	12.4
2.0 mM norspermidine	4.6	4.0	12.9
3.0 mM norspermidine	2.4	3.8	12.7
4.0 mM norspermidine	3.1	2.6	11.4
5.0 mM norspermidine	2.2	3.5	12.6
1.0 mM DFMO	3.1	3.6	13.6
1.0 mM DFMO plus 1.0 mM CHA	2.3	3.3	10.1
1.0 mM DFMO plus 1.0 mM norspermidine	2.6	3.2	10.9
LSD ($p \leq 0.05$)	2.26	2.06	4.59
sed *	0.319	0.423	0.318

Note: Values are shown as the means of ten measurements from each of four replicate plots.

* of transformed data

6.4 Discussion

Little difference was observed between the 1.0 mM and 5.0 mM CHA and norspermidine treatments, and the mixture of flutriafol and carbendazim in years 1995 and 1996, suggesting that the compounds controlled mildew infection as effectively as the commercial fungicide. For example, both the mixture of flutriafol and carbendazim and 1.0 mM norspermidine reduced disease by 67 % on the final assessment of the 1995 trial. Such results are in agreement with West and Walters (1988) who showed that CHA controlled powdery mildew on barley in the glasshouse. No comparable data exist for norspermidine but the compound has demonstrated antiproliferative activity against animal tumours (Prakash *et al.* 1988), and thus, the fungicidal properties of the compound are in agreement with previous findings. The results for CHA and norspermidine also compare favourably with DFMO and the novel putrescine analogue E-BED, which both provided early season control of powdery mildew in the field when compared with the same commercial fungicide (Havis & Walters 1992a; Havis *et al.* 1994b).

No additional control was conferred by increasing the concentration of the spermidine analogues. This suggested that the optimum dose of CHA and norspermidine was less than 5.0 mM. Such results were also observed in the mini plots where no difference was seen in mildew control afforded by the range of 1.0, 2.0, 3.0, 4.0 and 5.0 mM CHA and norspermidine. This further suggested that the optimum field dose of each analogue was 1.0 mM.

In the 1995 field trial, no additional control was observed through the combined use of a putrescine and spermidine analogue although the later control afforded by 5.0 mM CHA and 5.0 mM E-TED was interesting since the plots were only sprayed once. The combination of 1.0 mM CHA or norspermidine with 1.0 mM DFMO on the mini plots yielded a similar result with no additional control over DFMO alone observed. A synergistic effect was expected with the combined use of an ODC inhibitor and a spermidine synthase inhibitor. Jänne *et al.* (1985) suggested that the combined use of an AdoMetDC and an ODC inhibitor would lead to complete depletion of putrescine, spermidine and spermine in mammalian cells. Thus, if a plant pathogenic fungus possessed only one route to putrescine, the combined use of polyamine biosynthesis inhibitors should give more effective control. Indeed, West and Walters (1988) found various combinations of MGBG and DFMO reduced powdery mildew infection of barley more consistently than either compound alone in glasshouse experiments. CHA acted as a synergist for DFMO-mediated growth reduction of *Botrytis cinerea* on solid media (Smith *et al.* 1990b) and Pfaller *et al.* (1988) reported how a sequential treatment of DFMO followed by CHA provided increased control over CHA alone against growth of the yeast *Candida albicans*. However, spermidine content of

carrot cell suspension cultures treated with a combination of cyclohexylammonium phosphate and DFMO was unchanged (Minocha & Khan 1991). These authors suggested that the two inhibitors had counteracted the effects of each other (Minocha & Khan 1991). This would, therefore, account for the lack of synergism observed in this study, although a number of other mechanisms may be possible.

ODC is a rate limiting enzyme of polyamine biosynthesis and inhibition of the enzyme often leads to compensatory mechanisms enabling the conservation of cellular polyamine levels (Pegg 1988). Spermidine synthase is not as well regulated (Pegg 1988), but ODC regulation may be responsible for the lack of increased response through the combined use of an ODC inhibitor and a spermidine synthase inhibitor. Additional environmental pressures may also have attributed to the lack of synergism observed, as discussed below.

Despite the substantial control of mildew in both field trials, no significant differences in the final yield were found between the treatments. This is in contrast to previous field studies with inhibitors of polyamine biosynthesis whereby DFMO and E-BED caused an increase in grain yield over untreated controls (Havis & Walters 1992a; Havis *et al.* 1994b). In the present study, even commercial fungicides did not increase grain yields although reductions in disease were apparent. It is well documented that net photosynthesis increases in healthy leaves of barley otherwise infected with powdery mildew (Ayres 1984), suggesting that partial control of infection will result in a yield benefit. However, the 1995 season was particularly dry and this may have influenced the results. Ayres (1984) suggests that the ability of a plant to cope with an infection may be seriously diminished by the simultaneous occurrence of an abiotic stress, for example drought, which could minimise the chances of compensatory growth and activity occurring in uninfected tissues. Indeed, Johnson (1995) reported how winter wheat had been affected in the 1994/5 season. She suggested that it was drought rather than disease which had limited canopy life and thus responses to fungicides were lower than would normally have been expected. On a similar note, the observed level of *Rhynchosporium secalis* infection was much higher in the 1996 field trial compared to the previous year. The incidence of this additional pathogen may have reduced the ability of the plants to compensate for the occurrence of powdery mildew, perhaps by reducing the amount of healthy tissue available for photosynthesis. This may have contributed to the lack of fungicide response in the 1996 season.

Tebuconazole treatments in 1996 controlled mildew more effectively than the CHA and norspermidine treatments and the mixture of flutriafol and carbendazim. The increase in efficacy over flutriafol and carbendazim is not surprising since tebuconazole has been developed more recently for mildew control. It should also be noted when comparing CHA

and norspermidine to tebuconazole, that the experimental compounds are not formulated as commercial fungicides but only combined with a surfactant.

No additional disease control was observed through combining CHA or norspermidine with a reduced dose of tebuconazole in the 1996 field trial. Indeed, the reduced dose was as effective as the full dose of the fungicide. Wale *et al.* (1993) reported how powdery mildew of barley can be effectively controlled using low doses of fungicides under a range of optimum conditions which include the timing of fungicide application, disease pressure and resistance of the cultivar. However, rather than an effective low dose, it may be possible that the full dose did not achieve its potential as a result of additional environmental pressures on the crop, as already discussed.

CHA and norspermidine were thus shown to possess fungicidal activity in the field, an important phenomenon when attempting to determine potential compounds for crop disease control.

Chapter 7

Effect of cyclohexylamine and norspermidine on uptake of amino acids, polyamines and sugars by *Pyrenophora avenae*

7. EFFECT OF CYCLOHEXYLAMINE AND NORSPERMIDINE ON UPTAKE OF AMINO ACIDS, POLYAMINES AND SUGARS BY *PYRENOPHORA AVENAE*

7.1 INTRODUCTION

7.1.1 Review of literature

7.1.1.1 Transport mechanisms: an introduction

Nutrients which are essential for cells to survive generally enter those cells by either passive diffusion or carrier mediated transport. Passive diffusion is dependent upon the concentration of the nutrient on either side of the cell membrane. Thus, if the difference in intracellular and extracellular concentrations increases, the rate of diffusion increases and vice versa. Passive diffusion is non-specific and nutrients taken up by this mechanism usually do so such that their concentrations on either side of the membrane become equal (Jenkins 1992).

Nutrients taken up by carrier mediated transport do so by binding to proteins of the cell membrane, and indeed, most nutrients are transported by such systems. In order to be transported across the cell membrane, each substrate reversibly binds to a specific site of a carrier protein. With a limited number of binding sites on a protein, saturability of the transport system can occur, and the rate of transport across the membrane will reach a maximum limit. Such processes can therefore, like enzymes, be described by Michaelis-Menten kinetics, the equation of which is shown below (Jenkins 1992).

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

V is the transport rate; V_{max}, the maximum transport rate; [S], the concentration of substrate to be transported and K_m, the Michaelis-Menten constant, which is equal to the substrate concentration taken up at half the maximum rate of transport. K_m is a measure of the affinity of the carrier site for the substrate. Thus, low K_m values reflect a high affinity while high K_m values correspond to lower affinity or binding strength between the carrier site and the substrate molecule (Jenkins 1992).

The specificity of such transport processes allows inhibition to occur, which may be competitive or non-competitive. Competitive inhibition occurs with substances closely related to the substrate. The amount of inhibition depends on the ratio of substrate and inhibitor present and the affinity of each of these substances for the carrier sites. Thus, the

effect of a competitive inhibitor decreases as its concentration is reduced. Kinetic effects of competitive inhibition are therefore observed as an increase in K_m with no change in V_{max} (Jenkins 1992).

Non-competitive inhibition can also occur with substances unrelated to the substrates being transported. Such inhibition is not related to the concentration of the inhibitor. Kinetic effects of non-competitive inhibition are therefore observed as a reduction in V_{max} with no change in K_m (Jenkins 1992).

Different types of carrier mediated transport occur and further distinction can be made, for example, with active and passive transport. Active transport requires energy while passive transport, also known as facilitated diffusion, does not. The energy for active transport can be provided by the proton motive force, generated as a result of cellular metabolism where H^+ ion gradients arise across membranes. H^+ ions are thus known as coupling ions and combined movement of those ions and substrate molecules is used to force movement of the substrate molecules across membranes. This situation where both H^+ ions and the substrate molecule are moved in the same direction is known as a symport transport system. Antiport transport systems occur when the two entities are being moved in opposite directions while uniport transport refers to movement of a single ion in response to the particular electrostatic gradient between the exterior and interior of the cell (Jenkins 1992).

Many more uptake mechanisms exist, reflecting the diversity of organisms and individual substrates. For example, proteins can form channels in cellular membranes, allowing movement of small molecules and ions. No binding to the proteins occurs and movement is regarded as a faster form of passive diffusion, with Michaelis-Menton kinetics not applicable (Jenkins 1992).

7.1.1.2 Transport mechanisms in fungi

Much work has studied transport of cations, anions, sugars and amino acids into fungal cells. However, only uptake of the later two shall be reviewed. Much more work on the uptake of polyamines has been carried out in other organisms and thus, polyamine uptake by fungi will be examined along with that associated work.

7.1.1.2.1 Transport of sugars

Sugars are transported into fungal cells via carrier mediated transport mechanisms, either passively or actively, depending upon the fungus, substrate and growth conditions. Comparisons between different organisms is thus difficult, as are comparisons between the same organism grown under different conditions (Griffen 1994).

Furthermore, tremendous diversity exists in fungal transport systems. Symport transport with hydrogen ions was implicated in active uptake of glucose by *Neurospora crassa* and many different yeast species (Deak 1978). *N. crassa* was, however, reported to possess the ability to take up glucose via passive transport when grown in high glucose concentrations (Scarborough 1970). Interestingly, H^+ symport transport was associated with uptake of maltose by *Saccharomyces cerevisiae* via passive transport (Okada & Halvorson 1964). This phenomenon was indeed unusual and symport transport with hydrogen ions has been widely accepted as the main active transport system for fungal uptake of sugars (Griffen 1994). Active transport has also been reported for sugar uptake into *Rhodotorula gracilis* and *Aspergillus nidulans* (Griffen 1994).

Glucose transport into *S. cerevisiae* occurred via passive transport (Heredia *et al.* 1968; Novak *et al.* 1990). Two systems were apparent, a low affinity system and a high affinity system (Heredia *et al.* 1968; Novak *et al.* 1990). Such uptake processes are known as biphasic. At low concentrations of substrate, high affinity between the substrate and the carrier site is necessary to move the substrate across the membrane. However, when the concentration of substrate increases, less affinity is required and the low affinity process predominates. Such biphasic uptake of sugars by fungus is well documented (McDermott & Jennings 1976; Sheard & Farrar 1987; Walters *et al.* 1996). It has also been suggested that the low affinity system does not operate over the concentrations at which the high affinity system operates (Reinhold & Kaplan 1984).

Biphasic sugar uptake in *Pyrenopeziza brassicae* appeared to occur wholly via passive transport (Walters *et al.* 1996). However, work on *Phytophthora palmivora* suggested that active uptake of sugars took place for the high affinity system while the low affinity system operated via passive transport (Sheard & Farrar 1987).

In studying sugar uptake into *P. brassicae*, Walters and co-workers (1996) found that the kinetics for glucose and fructose uptake were similar. K_ms for the high affinity system for glucose and fructose were 3.5 μM and 4.6 μM respectively and 69 μM and 75 μM for the low affinity system. Such similar values and studies whereby glucose and fructose were taken up together, suggested that glucose and fructose shared a common carrier (Walters *et al.* 1996). This phenomenon was also found in *P. palmivora* for glucose and fructose uptake (Sheard & Farrar 1987). Kinetic parameters for sucrose uptake in *P. brassicae* were lower and competition studies where uptake of sucrose was unaffected by extracellular concentrations of glucose and fructose suggested a separate uptake carrier system existed for sucrose. Interestingly though, high invertase activity was detected (Walters *et al.* 1996).

Such an enzyme is known to hydrolyse sucrose to glucose prior to uptake, eliminating the need for a separate carrier in *P. palmivora* (Sheard & Farrar 1987).

7.1.1.2.2 Transport of amino acids

As with sugar transport into fungus, amino acid transport is extremely complex not least because of the large variety of different substrates as well as diversity of fungal organisms. Also, the stage of development of the fungus, the age of the culture and growth medium contents are all known to greatly influence the properties of the fungal transport system, and thus, different studies are not generally comparable (Griffen 1994).

Amino acid uptake is carrier mediated, however, differences are apparent in the specific mechanisms of such transport. Hunter & Segel (1971) reported that uptake of amino acids into *Penicillium chrysogenum* was sensitive towards inhibitors of fungal respiration, suggesting that transport was energy dependent. Similarly, Jones (1963) suggested active transport was responsible for amino acid uptake into *Botrytis fabae*. Active transport of amino acids was reported to occur via H^+ symport transport in yeast (Seaston *et al.* 1973; Eddy 1980; Cooper 1982).

Walters and co-workers (1997) however, suggested that uptake into *Pyrenopeziza brassicae* of glutamine and glutamic acid was independent of energy and likely to occur by passive transport. Interestingly, these authors found uptake of amino acids into the fungus to be a biphasic process. Kinetic parameters for the two amino acids were similar: K_ms for the high affinity system were 4.0 μM and 4.4 μM for glutamic acid and glutamine respectively and 580 μM and 560 μM for the low affinity system. Such similar parameters and inhibition of uptake of glutamic acid by glutamine and vice versa, suggested a single carrier protein to operate for both compounds. Studies using other amino acids; ornithine, arginine, lysine and asparagine suggested further that glutamic acid and glutamine were not transported using carrier systems associated with these other amino acids (Walters *et al.* 1997).

Similarly, in experiments with *Neurospora crassa*, *Aspergillus nidulans* and *P. chrysogenum*, competitive inhibition was observed between certain amino acids while little or no inhibition was observed among others (Griffen 1994). Thus, the existence of multiple systems for fungal amino acid uptake was suggested and studies in *N. crassa* found that five systems existed for such uptake. Aromatic and aliphatic amino acids were proposed to share a carrier system as were aromatic, aliphatic and basic amino acids. Three more systems were proposed to exist for basic amino acids, acidic amino acids and methionine

(Whitaker 1976). It can thus be seen that such multiple systems have overlapping specificities (Griffen 1994).

An interesting phenomenon of amino acid uptake is transinhibition, whereby large intracellular concentrations of an amino acid prevent further uptake of that compound. Inhibition is primarily non competitive, thus the maximum rate of transport is affected but K_m values unaltered. Transinhibition is system specific and only amino acids which compete for carrier binding sites are capable of transinhibition of each other. It has been suggested that the internal amino acid concentration may reduce the mobility of the carrier thus reducing the number of binding sites available for the external amino acid (Pall & Kelly 1971).

7.1.1.3 Transport of polyamines

As mentioned in section 1.1.3.3 (page 14), cells possess a large number of mechanisms enabling them to control their intracellular polyamine pools. Uptake of polyamines is one such process available to cells. A wide range of mammalian cells contain uptake pathways for polyamines (Porter & Sufrin 1986; Pegg 1987). Knowledge of these pathways is fragmentary, however, their importance in regulating intracellular levels of polyamines is demonstrated by the increased uptake of polyamines into cells in which amine synthesis is prevented through treatment with, for example, DFMO (Alhonen-Hongisto *et al.* 1980; Porter & Jänne 1987). Further studies using CHO cells in which a mutation had caused the transport system to be inactivated, showed how exogenous application of putrescine and spermidine failed to stimulate cells to grow in the presence of DFMO, demonstrating the transport system to be vital should cellular polyamine biosynthesis be prevented (Pegg 1988). Thus, the polyamine uptake system has been implicated in the ineffective treatment of cancer patients with, for example, DFMO. Polyamines may have been available from the diet and their uptake induced to compensate for drug related losses of intracellular amines (Pegg 1988).

Mammalian polyamine transport is thought to be specific for polyamines and distinct from amino acid uptake (Pegg 1988). Indeed, CHO cells were shown to possess a transport system specific for polyamines (Byers & Pegg 1989). This system was saturable and energy dependent, and thus suggested to be an active carrier-mediated transport system (Byers & Pegg 1989). Various mammalian cell types were used to study uptake of spermidine (Khan *et al.* 1990). Spermidine uptake was inhibited by putrescine and spermine, suggesting that the three polyamines shared the same carrier. Also, pre loading of cells with amino acids such as ornithine and lysine did not affect spermidine uptake, suggesting that different transport systems existed for the polyamines and amino acids.

Polyamine uptake has also been studied in other organisms, for example, putrescine uptake by maize root cells operated via a single saturable component (DiTomaso *et al.* 1992), as did putrescine uptake into *Saintpaulia* petals (Bagni & Pistocchi 1985). In contrast, uptake of the diamine into carrot cell cultures was biphasic, with two saturable components existing (Pistocchi *et al.* 1987).

Studies on the uptake of polyamines into *Aspergillus nidulans* revealed that a single component saturable uptake system was operating (Spathus *et al.* 1982). The authors concluded that putrescine and spermidine appeared to be transported by different carrier systems, the presence of one amine inhibiting uptake of the other (Spathus *et al.* 1982). In contrast, uptake into *Neurospora crassa*, revealed that putrescine, spermidine and spermine were likely to share a transport system (Davis & Ristow 1988). These authors also found polyamine uptake to be biphasic (Davis & Ristow 1988). Similarly, putrescine and spermidine uptake in the fungus *Fusarium culmorum* was biphasic (West & Walters 1991b). K_m values for the high affinity system were 0.17 mM and 0.34 mM for putrescine and spermidine respectively while values for the low affinity system were 32.73 mM and 42.17 mM. Interestingly, uptake of each of the polyamines was not inhibited by the presence of the other amine in the uptake medium, suggesting that different carrier systems existed for each compound. Uptake was inhibited by sodium azide, suggesting that active carrier mediated transport occurred (West & Walters 1991b). Growth of the fungus in the presence of DFMO resulted in a slight increase in uptake of the amines, suggesting that polyamine depletion caused a commitant increase in uptake from extracellular sources. Interestingly though, growth in the presence of MGBG decreased uptake of the polyamines. This was, however, suggested to be the result of other effects of MGBG such as mitochondrial damage affecting the polyamine uptake systems (West & Walters 1991b).

7.1.1.4 Transport of other substances

Many studies have examined uptake of polyamine biosynthetic inhibitors. Uptake of DFMO into the fungus *Fusarium culmorum* was linear and non saturable, suggesting a passive diffusion mechanism. However, uptake was inhibited by ornithine, putrescine and spermidine, suggesting that DFMO shared a carrier with the amino acid and polyamines. Also, uptake was inhibited in the presence of sodium azide, implicating the occurrence of active carrier mediated transport. The authors thus suggested that two processes were responsible for uptake of DFMO into *F. culmorum*, passive diffusion and carrier mediated transport (West & Walters 1991a).

Passive diffusion was reported to be responsible for DFMO uptake in mouse fibroblasts (Erwin & Pegg 1982) and *Trypanosoma brucei brucei* (Bitonti *et al.* 1986a) since

extracellular amino acids and polyamines did not affect the uptake processes (Erwin & Pegg 1982; Bitonti *et al.* 1986). In contrast, transport of DFMO into *T. b. brucei* was suggested by Phillips & Wang (1987) not to be entirely due to passive diffusion.

DFMO uptake into barley seedlings was inhibited by ornithine, suggesting that DFMO may utilise an amino acid carrier (Walters & Kingham 1990). Active transport was also implicated by the energy requirement for such uptake which was biphasic (Walters & Kingham 1990). DFMA and DFMO were indeed suggested by Slocum & Galston (1987) to utilise the amino acid transport system in plants and animals.

Interestingly, the total herbicide paraquat is taken up into cells utilising the polyamine transport system (Byers *et al.* 1987).

7.1.1.5 Polyamine analogues

The advantages of using analogues of polyamines in chemotherapeutic strategies has already been discussed in the thesis (section 1.1.4.6, page 20). Porter & Sufrin (1986) suggested that polyamine analogues may be able to utilise the polyamine uptake system to be transported into cells and since rapidly proliferating cells take up polyamines faster than normal cells, a degree of selectivity towards cancerous tissues would be possible. Once inside the cell, the analogues could exert an antiproliferative effect.

Indeed, although the polyamine transport system appears to be specific for polyamines, polyamine analogues are known to utilise the system. MGBG, a spermidine analogue, is taken up into cells via the same carrier as spermidine (Porter & Sufrin 1986; Porter & Jänne 1987). Such uptake was implicated in the antiproliferative success of MGBG with prevention of spermidine uptake through competition for carrier binding sites occurring rather than an inhibition of spermidine formation (Porter & Sufrin 1986; Porter & Jänne 1987).

Byers & Pegg (1989) reported how various bis(ethyl) polyamine analogues were taken into CHO cells while other polyamine analogues, for example, N^1-N^4-diacetylputrescine and N^1,N^{12}-diacetylspermine inhibited spermidine uptake, probably by competing for binding sites, in a range of mammalian cells (Khan *et al.* 1990).

Recently, Walters & McPherson (1997) reported how uptake of methionine, ornithine, putrescine, spermidine, spermine, fructose and glucose into *Pyrenophora avenae* was reduced when the fungus had been grown in the presence of the novel putrescine analogue, E-BED. Exposure of *P. avenae* to the related analogue, E-TED, also reduced uptake of

methionine into the fungus. Competition studies showed that uptake of the compounds into *P. avenae* was reduced in the presence of E-BED, suggesting E-BED shared the transport systems of the amino acids, polyamines and sugars (Walters & McPherson 1997).

Kinetic studies were carried out for uptake of two of the compounds, methionine and fructose. In both cases, uptake was biphasic. The authors found that exposure of fungus to E-BED did not affect the K_m values of methionine or fructose uptake but reduced V_{max} values, suggesting non competitive inhibition. E-BED was thus suggested to inhibit uptake of methionine and fructose by perhaps binding to carrier sites associated with methionine and fructose.

7.1.1.5 Factors affecting transport processes

Many factors affect the uptake of substances into cells including the structure of the compound and the individual organism into which uptake is to occur (Griffen 1994). The pH of the external medium also influences uptake by various mechanisms. It can determine the charge of the substance to be carried and the charge on the carrier protein, thus affecting binding. If active transport operates, the pH of the external medium can influence the proton gradient across the membranes. Thus, for a given external concentration of substrate, the rate of uptake will vary with the pH of the external medium (Whitaker 1976). Putrescine uptake in *Saintpaulia* petals varied with external pH and two different optima were determined for low and high external concentrations of putrescine (Bagni & Pistocchi 1985). Similarly, two optima were reported for glutamine and glutamic acid uptake by *Pyrenopeziza brassicae* (Walters *et al.* 1997). Temperature affects transport of substances into cells as it affects all chemical reactions. Thus, the rate of carrier mediated uptake transport will increase with temperature to a point at which protein denaturation will occur, and uptake fall off rapidly (Hunter & Segal 1971). Cellular energy production will also affect the uptake process, if active transport is involved (Seaston *et al.* 1973). Clearly, transport studies are complex, however, uptake of amino acids, polyamines and sugars into cells is essential for such cell viability.

7.1.2 Aim of studies

Porter & Sufrin (1986) speculated that polyamine analogues may be able to utilise the polyamine transport system to enter cells before exerting their antiproliferative effects by one or more of various mechanisms. Antifungal and fungicidal activity have been demonstrated by norspermidine although no specific mode of action has been determined. Khan *et al.* (1990) reported how various analogues inhibited uptake of spermidine into mammalian cells. Since spermidine is the major polyamine in fungi (Stevens & Winther 1979), it seemed prudent to examine the uptake of norspermidine by fungus in an attempt to

establish the cause of the antifungal properties of norspermidine in the absence of an effect on polyamine biosynthesis.

Also, the cause of the increase in fungal putrescine caused by exposure to CHA is unknown. Exposure of fungus to E-BED also increased putrescine levels (Havis *et al.* 1994a), and recent work by Walters and McPherson (1997) has reported that E-BED caused substantial reductions in uptake of amino acids, polyamines and sugars in *Pyrenophora avenae*. Whether the E-BED induced reductions in uptake were responsible for the antifungal effects of the compound is not known. However, given these findings, it seemed prudent to examine the effect of CHA on the uptake of various compounds into *P. avenae*.

Experiments thus sought to determine the effects of CHA and norspermidine on fungal uptake of various compounds in an attempt to establish the cause of the antifungal properties of the compounds in the absence of polyamine biosynthetic inhibition.

7.2 Materials and Methods

7.2.1 Experiment 1: Effect of CHA and norspermidine on the uptake of compounds by *Pyrenophora avenae* during a four hour period

P. avenae was maintained and grown in liquid culture amended with inhibitors to give final concentrations of 1.0 mM CHA and 0.01 mM norspermidine as described previously in sections 3.1.2.1 and 3.2.2.2 (pages 76 & 85). Two day old fungus (100 mg) was placed in a vial with 5 ml PDB and 3.7 kBq of a radio-labelled substance added. The compounds studied were [^{3}H] methionine (2608.5 GBq mmol^{-1}, NEN Research Products, UK), [1-^{14}C] ornithine (2.11 GBq mmol^{-1}, Amersham International, UK), [^{14}C] putrescine, [^{14}C] spermidine (both 4.0 GBq mmol^{-1}, Amersham International, UK), [^{14}C] spermine (4.1 GBq mmol^{-1}, Amersham International, UK), D-[U-^{14}C] glucose, D-[U-^{14}C] fructose (both 10.5 GBq mmol^{-1}, Amersham International, UK) and [U-^{14}C] sucrose (23.3 GBq mmol^{-1}, Amersham International, UK). The vials were incubated for 4 h in a shaking water bath at 27°C after which the mycelium was removed by centrifugation at 16000 *g* for 10 min at 0°C. The pellet obtained was washed twice with PDB (8 ml) by re-suspending the mycelium and centrifuging before depositing it in a vial with 2 ml Soluene-350 (Packard), followed by shaking on a rotamixer and incubating at 60°C for 3 h. Vials were then left at room temperature overnight before 10 ml of Hionic Fluor (Packard) was added and the radioactivity counted using a Packard 1900 TR scintillation spectrometer.

To examine the competitive effect of CHA and norspermidine on uptake of compounds, fungus grown only in culture medium for 3 days was used. Experiments were carried out as described above with one exception; fungus was incubated for 4 h in PDB amended with either CHA or norspermidine to give concentrations of 1.0 mM and 0.01 mM respectively. Controls used unamended PDB.

As with all experiments detailed in this chapter, values obtained as disintegrations per minute were converted and expressed as nmol g^{-1} hour^{-1}. Results are the means of four replicates and statistical significance was assessed using Student's *t*-test. Experiments were repeated and similar results obtained.

7.2.2 Experiment 2: Effect of prolonged exposure to CHA and norspermidine on the uptake of compounds by *Pyrenophora avenae* during a four hour period

To examine the possibility of an effect on uptake of compounds by fungus exposed to inhibitors for longer than two days, uptake of putrescine and spermidine were examined by

fungus exposed to 1.0 mM CHA or 0.01 mM norspermidine for 4 days. Experiments were carried out as described above. Putrescine and spermidine were chosen as representative compounds of those available.

7.2.3 Experiment 3: Effect of higher concentrations of CHA and norspermidine on the uptake of compounds by *Pyrenophora avenae* during a 15 minute period

Further studies were carried out to examine the possibility that fungal uptake may be affected by exposure of the fungus to higher concentrations of the inhibitors. Thus, the effect of 2.75 mM CHA and 0.2 mM norspermidine on uptake processes was investigated. Studies were also carried out to determine the effect of CHA and norspermidine on fungal uptake of compounds over a short period. Uptake effects may occur quickly yet be masked if detection is delayed.

Two compounds, methionine and spermidine, were chosen as representative compounds of the original eight. Fungus was exposed to 2.75 mM CHA or 0.2 mM norspermidine for 4 days so as to gain enough fungal material for analysis. Fungal uptake of the compounds was allowed to proceed for 15 minutes as described above. The competitive nature of CHA and norspermidine was also examined by exposing 3 day old fungal mycelium, grown in culture medium only, to the inhibitors for the 15 minute uptake period.

Experiments were repeated 3-4 times with similar results obtained.

7.2.4 Experiment 4: Determination of uptake kinetics

To examine effects on the kinetics of the uptake process, fungus was grown in the presence of 2.75 mM CHA or 0.2 mM norspermidine for 4 days in order to amass sufficient fungal material. *P. avenae* was then allowed to take up varying levels of methionine (0.01 mM - 128 mM, containing different levels of radioactive methionine, 0.25 µl - 8 µl) for a 15 minute period as in the procedure described above. Since smaller amounts of radioactive methionine could not be added precisely, the lower concentrations of unlabelled substrate had proportionately more radioactivity than was desired. However, this was accounted for in later calculations. Controls used fungus that was grown in culture medium only. Data were analysed using regression analysis from Eadie-Hofstee plots.

7.3 RESULTS

7.3.1 Experiment 1: Effect of CHA and norspermidine on the uptake of compounds by *Pyrenophora avenae* during a four hour period

Neither 1.0 mM CHA nor 0.01 mM norspermidine had any significant effect on the uptake of amino acids, polyamines or sugars by *P. avenae* when the fungus was exposed to the inhibitors for 2 days (Table 32). Exposure to CHA caused a decrease in uptake of spermine while exposure to norspermidine caused an increase in the uptake of fructose, when fungus was exposed to CHA or norspermidine for the four hour uptake period only (Table 33).

7.3.2 Experiment 2: Effect of prolonged exposure to CHA and norspermidine on the uptake of compounds by *Pyrenophora avenae* during a four hour period

Exposure of fungus to 1.0 mM CHA or 0.01 mM norspermidine for 4 days had no effect on uptake of putrescine or spermidine (Table 34).

7.3.3 Experiment 3: Effects of higher concentrations of CHA and norspermidine on the uptake of compounds by *Pyrenophora avenae* during a 15 minute period

Various effects on the uptake of methionine and spermidine by *P. avenae* were detected when fungus was exposed to the inhibitors for 4 days and the uptake process allowed to proceed for 15 minutes. Exposure to 2.75 mM CHA caused an increase in the uptake of methionine, while exposure to 0.2 mM norspermidine resulted in a reduction in uptake of the compound. Treatment of fungus with norspermidine also caused an increase in uptake of spermidine although this was not always significant (Table 35).

CHA, however, failed to affect uptake when placed in competition with the compounds, i.e. when *P. avenae* was only exposed to the inhibitor for 15 minutes. Nevertheless, the presence of norspermidine for 15 minutes along with methionine or spermidine, increased uptake of methionine and decreased that of spermidine (Table 35).

Table 32. Uptake of methionine, ornithine, polyamines and sugars by *Pyrenophora avenae* exposed to 1.0 mM CHA or 0.01 mM norspermidine for 2 days

	Uptake (nmol g^{-1} h^{-1})		
Compound	Control	CHA	norspermidine
Methionine	1.0 ± 0.11	0.8 ± 0.06	1.0 ± 0.03
Ornithine	46.4 ± 6.32	54.6 ± 2.79	57.9 ± 3.61
Putrescine	124.8 ± 8.15	103.5 ± 5.91	119.4 ± 13.67
Spermidine	1754 ± 117.4	1483 ± 156.2	2322 ± 210.0
Spermine	2158 ± 177.6	2415 ± 151.7	2655 ± 34.1
Glucose	18.4 ± 1.46	16.7 ± 1.42	19.3 ± 1.08
Fructose	71.1 ± 3.62	84.6 ± 10.26	81.0 ± 5.23
Sucrose	12.7 ± 1.66	13.8 ± 2.24	11.1 ± 0.87

Note: Values are shown as the means ± SE of four replicates.

Table 33. Uptake of methionine, ornithine, polyamines and sugars by *Pyrenophora avenae* exposed to 1.0 mM CHA or 0.01 mM norspermidine for 4 hours

	Uptake (nmol g^{-1} h^{-1})		
Compound	Control	CHA	norspermidine
Methionine	1.2 ± 0.06	1.4 ± 0.18	1.3 ± 0.10
Ornithine	89.1 ± 11.76	61.6 ± 12.35	103.1 ± 3.39
Putrescine	202.3 ± 7.63	182.3 ± 10.54	217.3 ± 35.60
Spermidine	2211 ± 241.7	1556 ± 235.3	1293 ± 127.9
Spermine	3412 ± 186.3	2215 ± 101.6 **	3604 ± 181.0
Glucose	23.0 ± 0.89	20.4 ± 0.92	19.5 ± 1.82
Fructose	89.1 ± 8.89	149.9 ± 20.93	209.6 ± 9.75 **
Sucrose	29.2 ± 2.45	28.9 ± 1.74	30.7 ± 2.09

Note: Values are shown as the means ± SE of four replicates.
Significant differences from control are shown as: ** $p \leq 0.01$

Table 34. Uptake of polyamines by *Pyrenophora avenae* exposed to 1.0 mM CHA or 0.01 mM norspermidine for 4 days

	Uptake (nmol g^{-1} h^{-1})		
Compound	Control	CHA	norspermidine
Putrescine	91.3 ± 8.67	71.3 ± 5.99	81.5 ± 2.61
Spermidine	382 ± 57.3	202 ± 23.2	371 ± 18.8

Note: Values are shown as the means ± SE of four replicates.

Table 35. Uptake of methionine and spermidine by *Pyrenophora avenae* exposed to 2.75 mM CHA or 0.2 mM norspermidine for 4 days or 15 minutes

	Uptake (nmol $g^{-1}h^{-1}$) in fungus exposed to inhibitors for 4 days		
Compound	Control	CHA	norspermidine
Methionine	3.78 ± 0.296	12.26 ± 0.095 **	0.66 ± 0.087 **
Spermidine	8224 ± 349	7243 ± 420	11350 ± 823
	Uptake (nmol $g^{-1}h^{-1}$) in fungus exposed to inhibitors for 15 minutes		
Compound	Control	CHA	norspermidine
Methionine	0.87 ± 0.010	0.87 ± 0.087	1.32 ± 0.091 *
Spermidine	11671 ± 912	11792 ± 1052	4507 ± 832 **

Note: Values are shown as the means ± SE of four replicates.

Significant differences from control are shown as: * $p \leq 0.05$; ** $p \leq 0.01$

7.3.4 Experiment 4: Determination of uptake kinetics

The kinetics of uptake of methionine by *P. avenae* exposed to 2.75 mM CHA or 0.2 mM norspermidine for 4 days were calculated from the classical equation relating to enzyme-catalysed reactions, the Michaelis-Menton equation, as described in section 7.1.1.1.

$$V_o = \frac{V_{max}\,[S]}{[S] + K_m}$$

The initial velocity (V_o) of carrier mediated uptake varies with the concentration of substrate supplied [S]. A plot of the initial velocity against the substrate concentration gives a rectangular hyperbolic curve, demonstrating that at low substrate concentrations, the initial velocity of the reaction is directly proportional to the substrate concentration but at higher concentrations, the initial velocity reaches a maximum. The initial velocity at that point is denoted as V_{max}.

Since the reciprocal of the equation of a rectangular hyperbola is the equation of a straight line, the equation can be re-arranged to take the form of an Eadie - Hofstee equation as shown below.

$$V_o = -K_m \times \frac{V_o}{[S]} + V_{max}$$

In accordance with the equation, plots of V_o against $V_o/[S]$ will produce straight lines of gradient equal to $-K_m$ and which intercept the y-axis at V_{max}. Thus, values of K_m and V_{max} for the uptake process were established.

Exposure of fungus to 2.75 mM CHA for 4 days caused a reduction in the uptake of methionine by *P. avenae* at all concentrations of methionine investigated. Exposure of fungus to 0.2 mM norspermidine reduced uptake of methionine only at the lowest concentrations examined. At concentrations of 16 - 128 mM, no effect on methionine uptake was observed (Table 36).

The plot of V_o against $V_o/[S]$ for the control fungus was best fitted by two straight lines, revealing the presence of a biphasic uptake system (Figure 16). Linear regression analysis of system 1 (high affinity) found V_{max} and K_m to have values of 0.87 nmol g^{-1} hr^{-1} and

3.84 mM respectively. Values for system 2 (low affinity) were 27.3 nmol g^{-1} hr^{-1} and 520 mM.

The Eadie-Hofstee plot for fungus exposed to 2.75 mM CHA for 4 days revealed that the two phases were less defined (Figure 17). Analysis of the two phases failed to give significant regressions and a plot of V_o against $V_o/[S]$ using the same scale as the control demonstrated that uptake had indeed been dramatically altered by the presence of CHA in the growing medium of the fungus (Figure 18).

The effect of norspermidine on fungal uptake of methionine resulted in complete disruption of the two uptake phases (Figure 19). Examination of the Eadie-Hofstee plot using the same scale as the control fungus further demonstrated this result (Figure 20).

Table 36. Uptake of methionine by *Pyrenophora avenae* exposed to 2.75 mM CHA or 0.2 mM norspermidine for 4 days

	Uptake (nmol g^{-1} h^{-1})		
Methionine conc. (mM)	Control	CHA	norspermidine
0.01	0.0026 ± 0.00011	0.0003 ± 0.00006 ***	0.0006 ± 0.00013 ***
0.1	0.020 ± 0.0027	0.002 ± 0.0004 **	0.006 ± 0.0012 **
1	0.153 ± 0.0109	0.016 ± 0.0011 **	0.052 ± 0.0087 ***
2	0.29 ± 0.022	0.02 ± 0.003 **	0.09 ± 0.008 **
4	0.44 ± 0.017	0.06 ± 0.002 ***	0.20 ± 0.040 **
8	0.58 ± 0.042	0.08 ± 0.004 **	0.35 ± 0.049 *
16	0.80 ± 0.079	0.15 ± 0.016 **	1.08 ± 0.198
32	1.59 ± 0.200	0.35 ± 0.016 **	1.53 ± 0.037
64	3.05 ± 0.356	0.53 ± 0.033 **	3.88 ± 0.338
128	5.36 ± 0.434	0.96 ± 0.150 **	7.45 ± 1.590

Note: Values are shown as the means ± SE of four replicates.

Significant differences from control are shown as: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

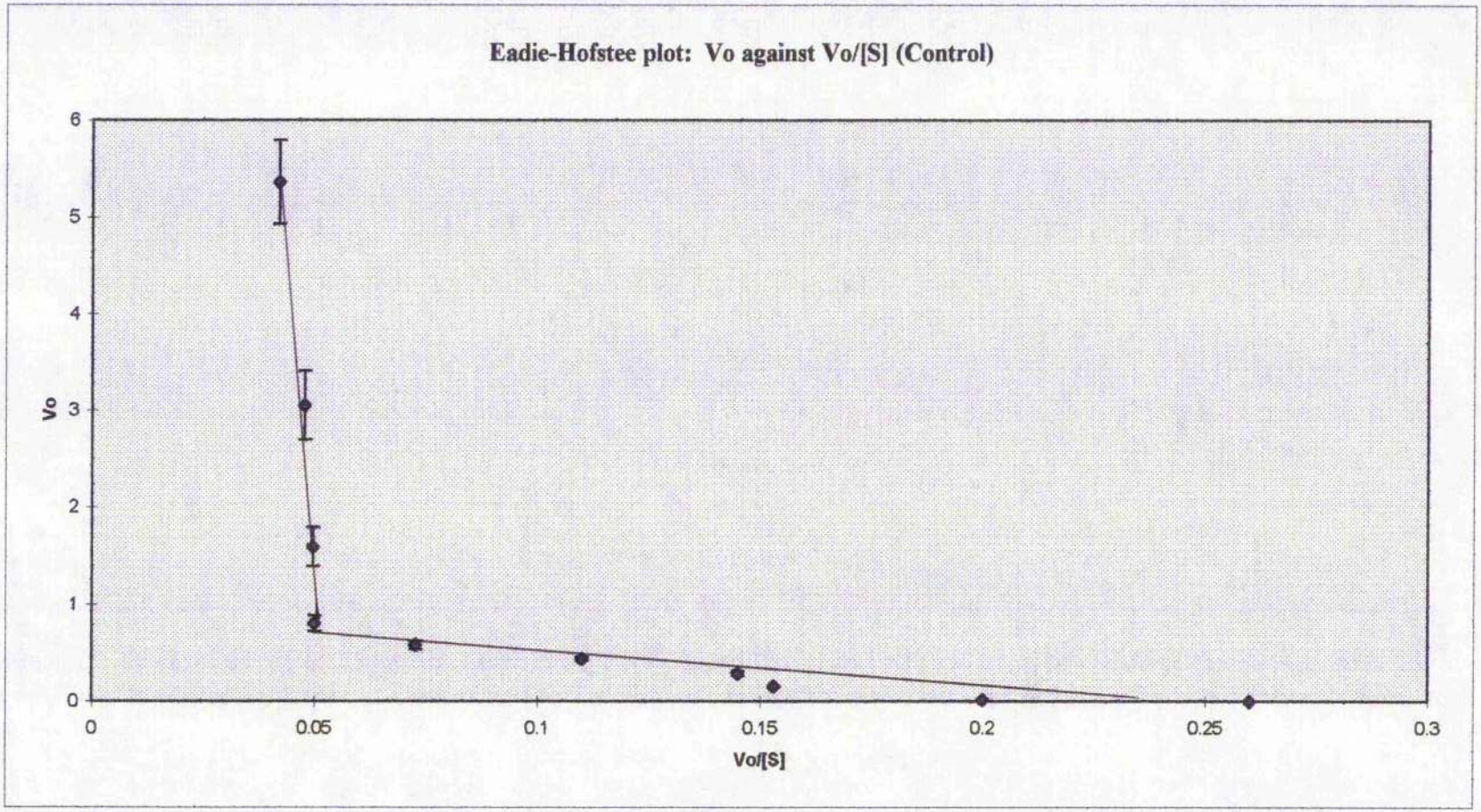


Figure 15. Uptake of varying concentrations of methionine (0.01 - 128 mM) by *Pyrenophora avenae* , grown as an experimental control. Uptake was allowed to proceed for 15 mins. Results are the means of four replicates and values for Vmax and Km defined from the graph using regression analysis.

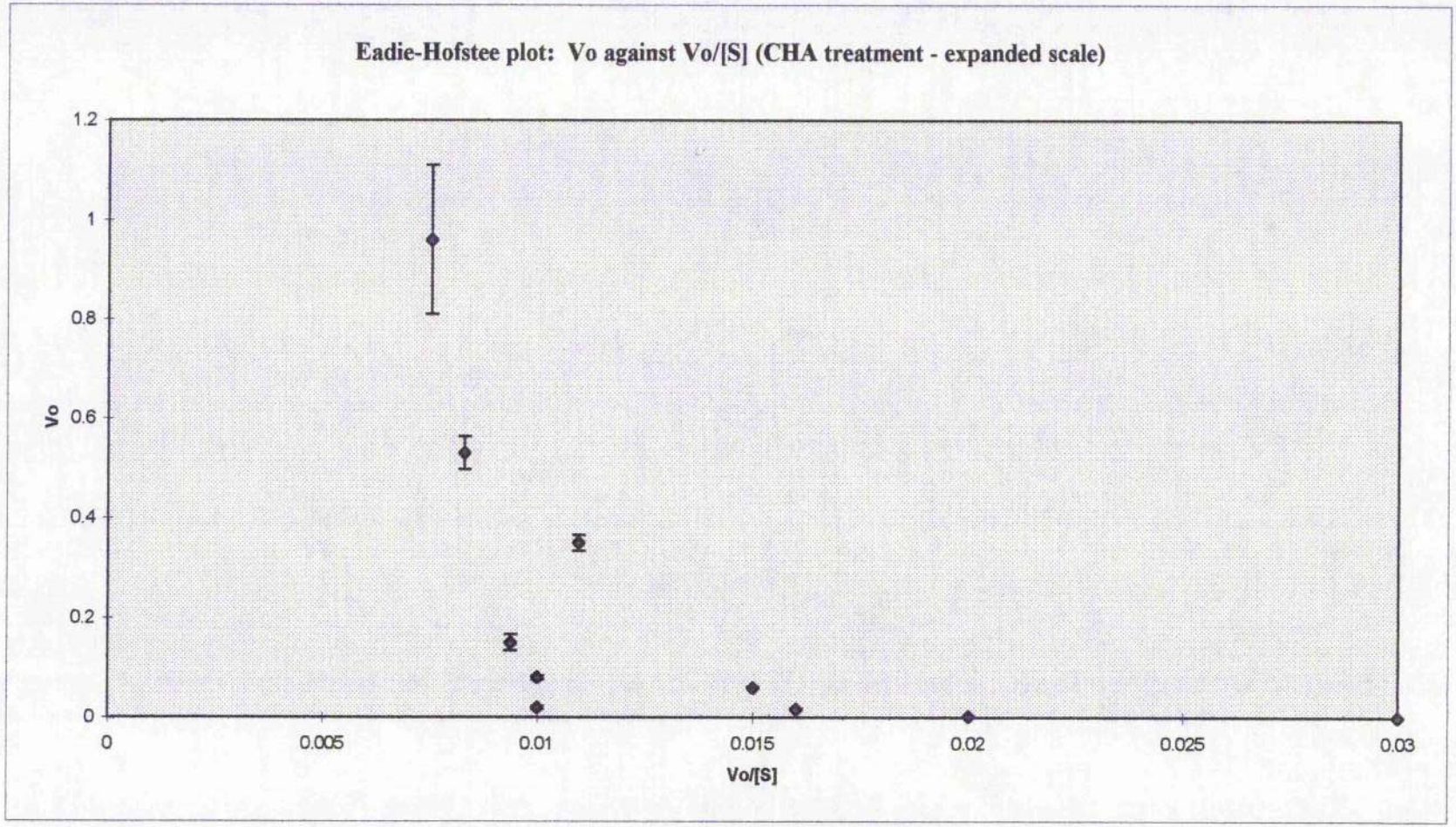


Figure 16. Uptake of varying concentrations of methionine (0.01 - 128 mM) by *Pyrenophora avenae* , exposed to 2.75 mM CHA for 4 days. Uptake was allowed to proceed for 15 mins. Results are the means of four replicates and the data were analysed using regression analysis.

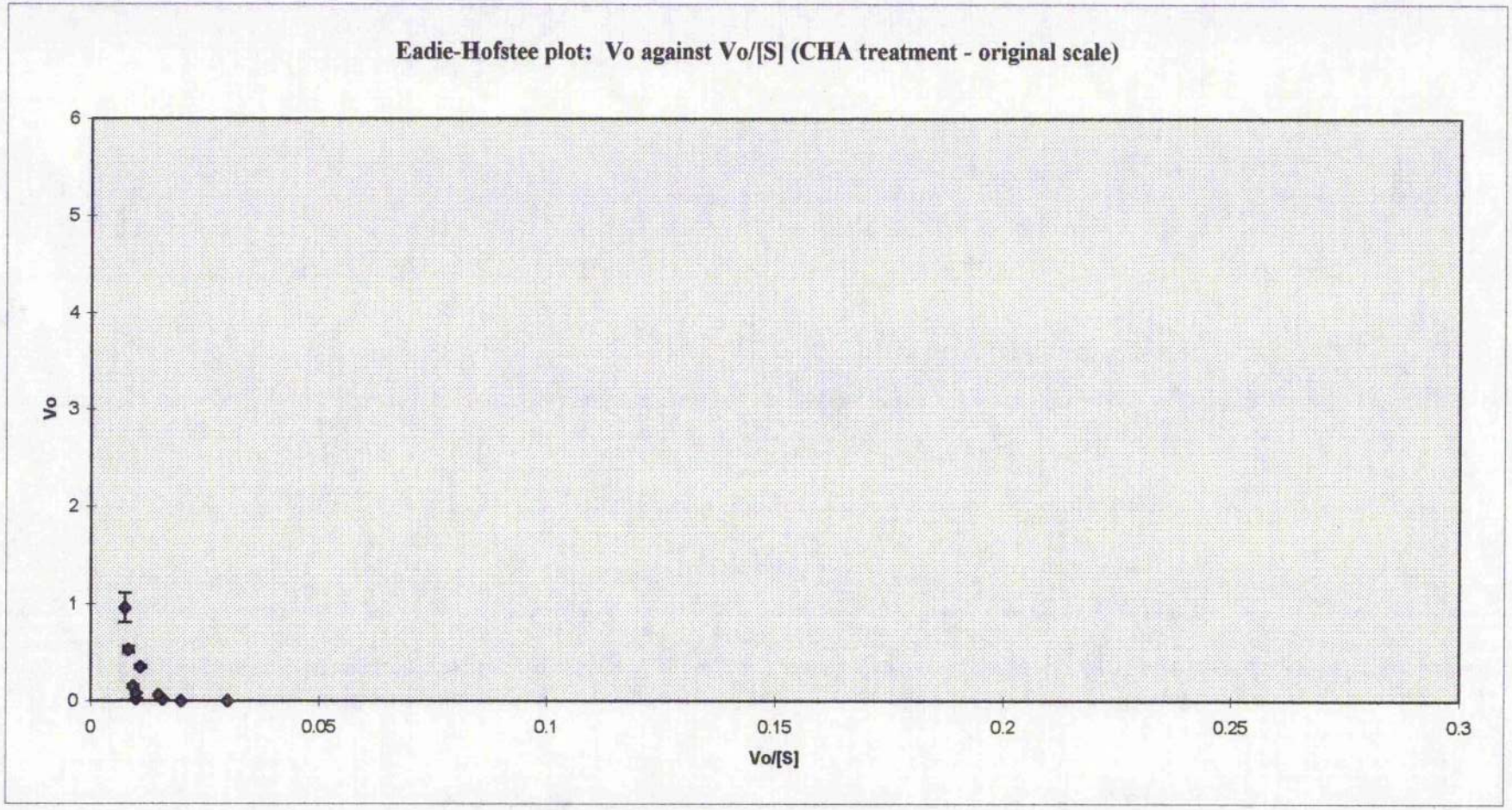


Figure 17. Uptake of varying concentrations of methionine (0.01 -128 mM) by *Pyrenophora avenae* , exposed to 2.75 mM CHA for 4 days. Uptake was allowed to proceed for 15 mins. Results are the means of four replicates and the data were analysed using regression analysis.

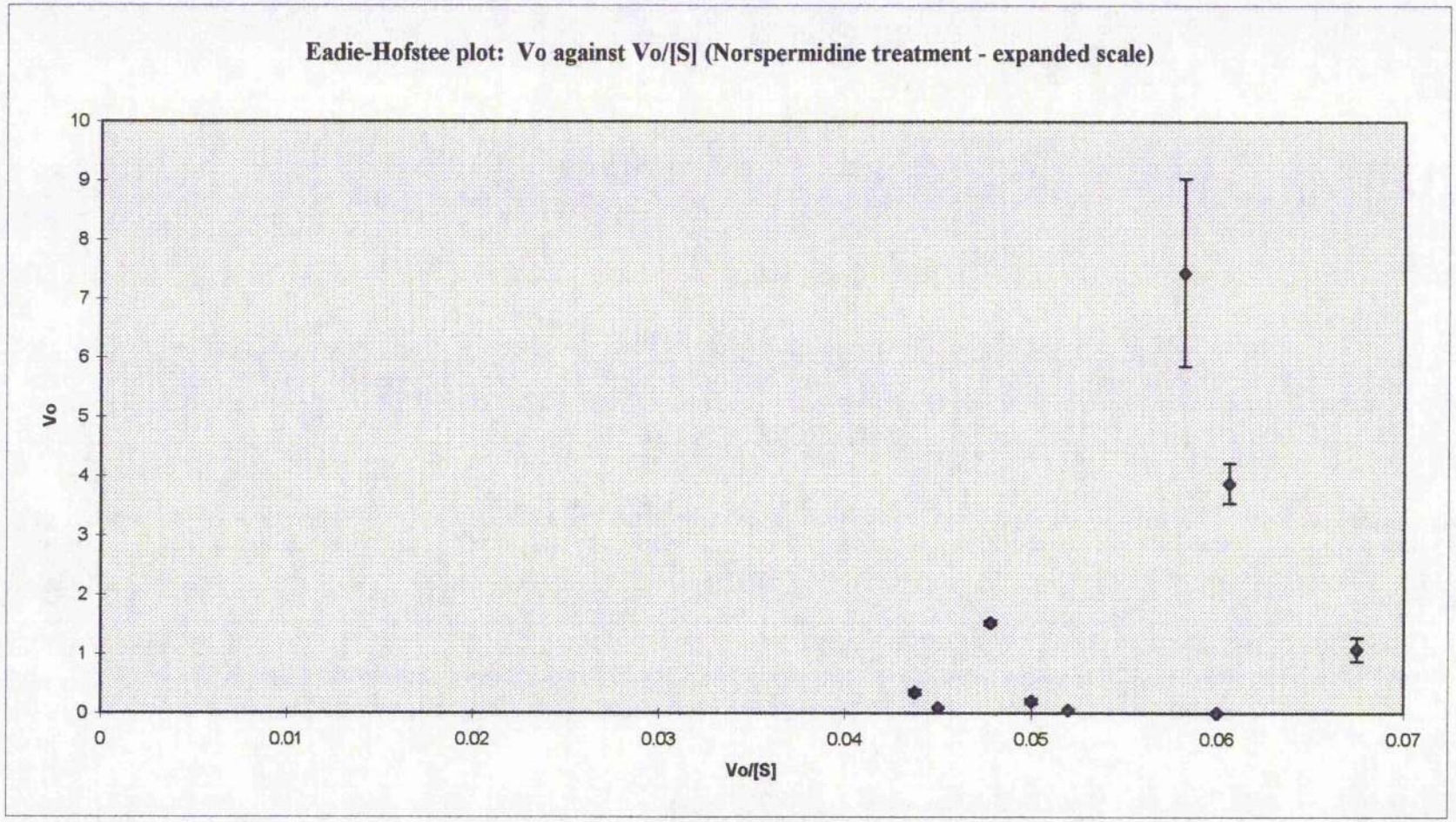


Figure 18. Uptake of varying concentrations of methionine (0.01 - 128 mM) by *Pyrenophora avenae* , exposed to 0.2 mM norspermidine for 4 days. Uptake was allowed to proceed for 15 mins. Results are the means of four replicates and the data were analysed using regression analysis.

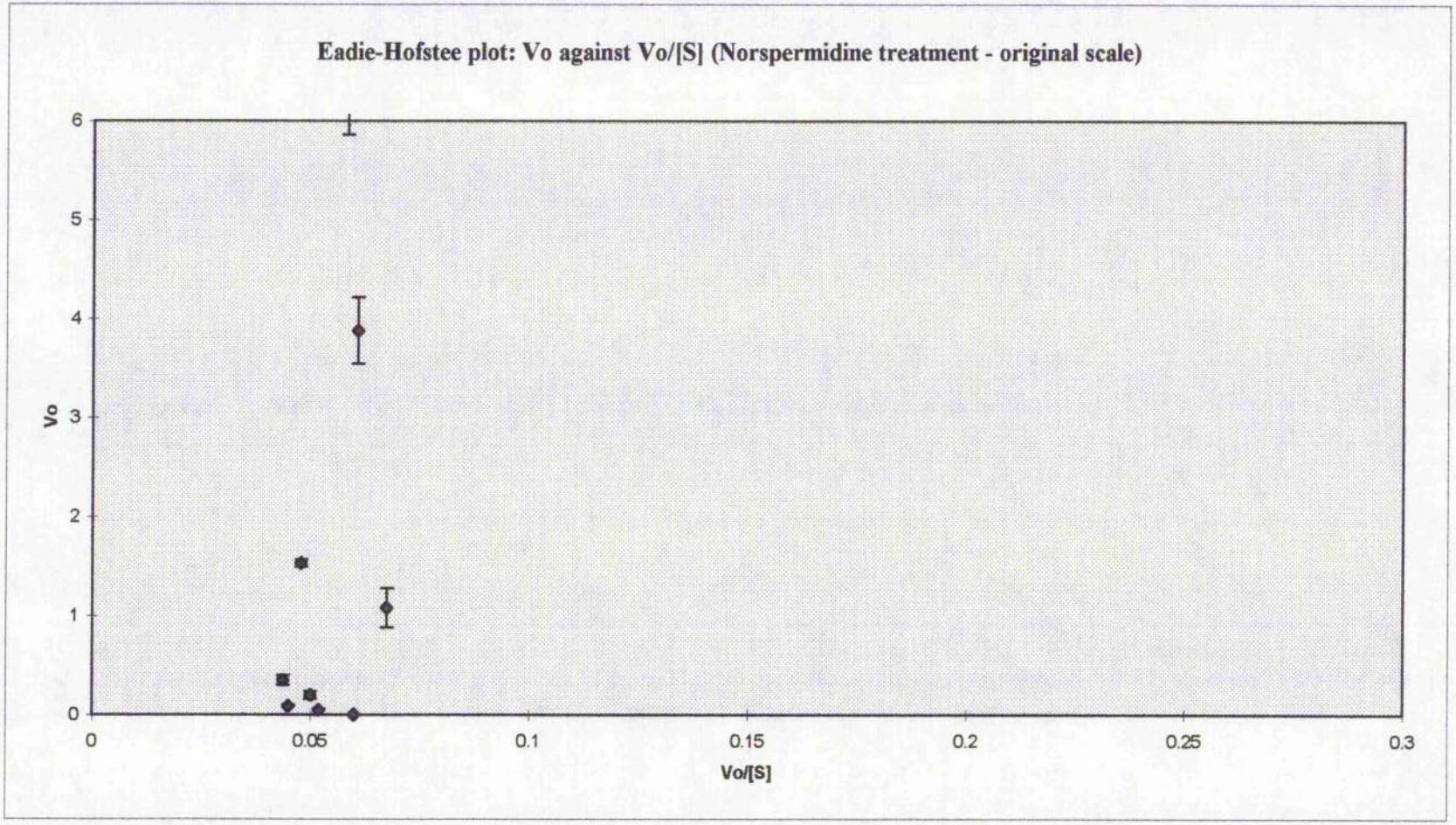


Figure 19. Uptake of varying concentrations of methionine (0.01 - 128 mM) by *Pyrenophora avenae* , exposed to 0.2 mM norspermidine for 4 days. Uptake was allowed to proceed for 15 mins. Results are the means of four replicates and the data were analysed using regression analysis

7.4 DISCUSSION

Exposure of fungus to 1.0 mM CHA or 0.01 mM norspermidine for 2 days had no effect on the uptake of amino acids, polyamines or sugars by *P. avenae*. This is in contrast to the findings of Walters & McPherson (1997), who showed that exposure of fungus to E-BED for two days reduced the uptake of numerous compounds by *P. avenae*. Similarly, longer exposure of *P. avenae* to CHA and norspermidine, for 4 days, had no effect on uptake of those compounds examined.

When the various compounds were taken up by the fungus in the presence of 1.0 mM CHA, only spermine uptake was reduced. Walters & McPherson (1997), however, found that uptake of numerous compounds was reduced when uptake proceeded in the presence of E-BED. CHA and spermine may share a common carrier for fungal uptake, although kinetic studies would be necessary to determine whether the observed inhibition was competitive or non-competitive. Conversely, CHA may not utilise the carrier systems of the other compounds examined, thus explaining why their uptake into *P. avenae* was unaffected.

Competitive studies with 0.01 mM norspermidine only affected uptake of fructose, whose uptake into *P. avenae* was actually increased. This increase was unexpected, but may have been caused by a number of mechanisms. Binding of norspermidine to the carrier protein may have affected binding site affinity for fructose and increased the affinity. Also, since sugar uptake is generally considered to operate via active carrier mediated transport, norspermidine may have affected the disappation of energy and effectively increased the rate of fructose uptake by the fungus, although why this should have only affected fructose is unclear. Kinetic studies would indicate if such effects had occurred: an affinity increase would be reflected by a reduction in K_m while an increase in the rate of substrate transport would lead to an increase in V_{max}.

The comparative lack of effect of CHA and norspermidine is, as discussed, in contrast to the findings of Walters & McPherson (1997). The use of 1.0 mM CHA and 0.01 mM norspermidine as opposed to higher concentrations of the compounds has already been discussed, (section 3.2.2.2, page 85). Although reductions in fresh weight of fungus exposed to such concentrations do not always occur, growth effects are clearly visible. Nevertheless, the effect of higher concentrations of the compounds, 2.75 mM CHA and 0.2 mM norspermidine, on fungal uptake were also examined. Shorter uptake times were investigated in case uptake effects had been masked by the long four hour uptake period.

Thus, exposure of fungus to 2.75 mM CHA for four days resulted in an increase in methionine uptake and no change in spermidine uptake by *P. avenae*. No effect was

observed in the uptake of methionine or spermidine when fungus was exposed to CHA only during the uptake process. This suggests that CHA does not share a common uptake carrier with methionine or spermidine. The effect on methionine uptake differed from previous work in this study and may be the result of the higher CHA concentration or the shorter uptake time. Further work would be necessary to determine which condition led to the uptake effect and indeed, why the effect occurred. Exposure of fungus to CHA may have caused changes to the carrier binding proteins and resulted in an increased affinity for methionine. Alternatively, the energetics of the transport system may have been affected and the rate of uptake thus increased.

Exposure of fungus to 0.2 mM norspermidine for four days resulted in a slight increase in spermidine uptake and reduced methionine uptake. The converse effects were found when the compounds were allowed to be taken up in the presence of norspermidine: i.e. an increase in methionine and a reduction in spermidine uptake was caused. Whether these effects were due to the increased concentration of norspermidine or the reduced uptake time is not known.

Exposure of fungus to norspermidine may have affected the carrier proteins of the fungus or the membrane potential of the cells and therefore affected either the binding affinity of the carrier sites towards the substrates or the energy disappated for active transport. The causes of the differing effects for methionine and spermidine are unclear and future work would be required to clarify the findings.

In the competitive studies, norspermidine may have inhibited the uptake of spermidine competitively or non-competitively. Kinetic studies would be required to establish which mechanism was responsible. Since the two compounds are structurally similar, they may have utilised the same carrier and competed with each other for binding sites. The increase in methionine uptake in the presence of norspermidine may be the result of altered binding affinity of the carrier towards methionine or changes in the energy available for active transport. Further work would be required in this area.

The results so far have been discussed assuming that active carrier mediated transport was operating for fungal uptake of the compounds. Certainly, carrier mediated transport was suggested by saturation kinetics of methionine uptake into *P. avenae* by Walters & McPherson (1997). Further work would be required using, for example, sodium azide, to examine if uptake did indeed require energy.

The kinetics of uptake were examined in fungus exposed to 2.75 mM CHA or 0.2 mM norspermidine for 4 days. For all the concentrations of methionine examined, methionine uptake was reduced in fungus pre exposed to 2.75 CHA. This is, however, in direct contrast to the previous work in this study which showed that fungus exposed to the same concentration of CHA for the same time and allowed to take up methionine for the same period, caused increased amino acid uptake. Griffen (1994) stated that even the age of the culture used could influence the uptake process, therefore, such results, differing from experiment to experiment are not unexpected, even though all cultures were used at approximately the same age.

Exposure of fungus to norspermidine reduced methionine uptake from external concentrations of 0.01 mM - 8 mM, but at higher concentrations (16 mM - 128 mM), no differences in uptake between the norspermidine treated fungus and the control were observed. The reasons for this phenomenon are not clear since methionine uptake into the control fungus had not reached its maximum level at the external concentration of 8 mM. The unaltered methionine uptake into norspermidine treated fungus could not therefore be explained by saturation. The high concentrations of external methionine appeared able to overcome the norspermidine-induced effects causing reduced methionine uptake.

An Eadie-Hofstee plot of the control fungus was best fitted by two straight lines, suggesting the uptake to be biphasic (Figure 15). Such uptake of amino acids is in agreement with previous work on glutamic acid and glutamine into *Pyrenopeziza brassicae* (Walters *et al.* 1997) and methionine into *P. avenae* (Walters & McPherson 1997). K_m values for the high and low affinity systems were 3.84 mM and 520 mM respectively. Such values are in contrast for those reported by Walters & McPherson (1997) for *P. avenae*, (0.02 mM and 1.68 mM). However, as discussed previously, differences can exist between different experiments with respect to the age of the fungal culture used. V_{max} values were 0.87 nmol g^{-1} h^{-1} and 27.3 nmol g^{-1} h^{-1} respectively. This, again, is in contrast to those previous findings of 0.004 mmol g^{-1} h^{-1} and 0.39 mmol g^{-1} h^{-1} by Walters & McPherson (1997).

Eadie-Hofstee plots for fungus exposed to CHA or norspermidine clearly show that inhibition of methionine uptake in these two fungi was not caused by competitive or non-competitive inhibition. The kinetics of biphasic uptake was greatly distorted, more so for norspermidine than CHA. If the hypothesis that such findings are true for uptake of any substance into treated fungus holds, previous attempts to explain variations in uptake in experiments on the basis of classical competitive or non-competitive inhibition were unfounded.

A more likely explanation for the findings lies in the known association between polyamines and cellular membranes. CHA and norspermidine may be binding to those sites associated with the polyamine-induced stabilisation of membrane integrity. Membrane structure may thus be disrupted and cause abberant effects on amino acid, polyamine and sugar transport.

Exactly why the analogues do not act as the putrescine analogue E-BED does with regards to fungal uptake of various compounds, is unknown. E-BED had been shown to reduce uptake of various compounds into fungus and such a reduction in polyamine uptake may be associated with the induced putrescine accumulation found in *P. avenae* by Havis and co-workers (1994a). However, Walters & McPherson (1997) could not conclude whether the E-BED induced reductions in uptake were responsible for the antifungal effects of the compound or indeed, related to the increased putrescine concentration. The present study has indicated that CHA may interfere with membrane structure and/or function but the cause of the CHA induced putrescine accumulation is still unknown.

Porter & Sufrin (1986) suggested that polyamine analogues would utilise the polyamine transport system. Thus, norspermidine was hypothesised to compete with spermidine for uptake into fungal cells. Competitive uptake studies between spermidine and 0.2 mM norspermidine suggested that this was indeed true with spermidine uptake into *P. avenae* inhibited in the presence of norspermidine. However, kinetic studies revealed the apparent destruction of cell uptake systems by the spermidine analogue, although, it should be noted that such studies examined fungus already exposed to the analogue and little is known of norspermidine uptake into fungal cells during growth.

It is interesting that the results failed to demonstrate existence of distinct amino acid, polyamine and sugar uptake systems in *P. avenae*. Norspermidine affected uptake of methionine, fructose and spermidine, three chemically distinct compounds. This is, however, in agreement with work using E-BED which found that uptake of amino acids, polyamines and sugars was inhibited by the putrescine analogue (Walters & McPherson 1997). In mammalian cells, polyamines were reported to utilise a different uptake system from several amino acids (Khan *et al.* 1990), while in the fungus *P. brassicae*, different uptake systems were demonstrated for different amino acids (Walters *et al.* 1997). However, given the destruction of the integrity of the uptake system, the lack of observation of specific uptake systems is not surprising.

Many factors influence uptake into cells including the substrate, organism, extracellular pH, temperature and cellular energy production (Hunter & Segal 1971; Seaston *et al.* 1973; Griffen 1994). Furthermore, fungal uptake can even be affected by the age of the culture used (Griffen 1994). Despite the variability in the results of this study, CHA and norspermidine clearly affected uptake of amino acids, polyamines and sugars into cells of *P. avenae*. Whether these effects were primarily responsible for the antifungal activity of CHA and norspermidine or the result of some other antiproliferative mechanism is unknown.

Chapter 8

Hypusine biosynthesis

8. HYPUSINE BIOSYNTHESIS

8.1 INTRODUCTION

8.1.1 Review of literature

8.1.1.1 Hypusine: an introduction

The unusual amino acid hypusine was initially discovered in extracts of bovine brain by Shiba and co-workers (Shiba *et al.* 1971). Shortly afterwards, these workers determined the chemical structure of the compound to be [N^{ε}-(4-amino-2-hydroxybutyl)lysine] (Shiba *et al.* 1972). The amino acid was subsequently found both in its free form (Nakajima *et al.* 1971), and bound to protein (Imaoka & Nakajima 1973; Sano *et al.* 1984) in various animal tissues. Hypusine has since been detected in lower eukaryotes such as yeast (Gordon *et al.* 1987), *Drosophila melanogaster* (Gordon *et al.* 1987), *Dictyostelium discoideum* (Sandholzer *et al.* 1989), *Neurospora crassa* (Chen & Yang 1988; Yang *et al.* 1990) and plants (M. H. Park unpublished results). Substantial evidence for the ubiquity of hypusine in all eukaryotic cells would thus now appear to exist. Hypusine has also been detected in many archaebacterial species but not in eubacteria (Schümann & Klink 1989; Bartig *et al.* 1990; Bartig *et al.* 1992).

8.1.1.2 Biosynthesis of hypusine

Hypusine is the product of the post-translational modification of one specific cellular protein which was identified as eukaryotic translation initiation factor 5A (eIF-5A) (Cooper *et al.* 1983). Biosynthesis of the amino acid occurs in two steps. Firstly, free spermidine is cleaved and the aminobutyl moiety transferred to the ε-amino group of a specific lysine residue in the precursor of eIF-5A, forming the intermediate deoxyhypusine. This enzymatic transfer is catalysed by deoxyhypusine synthase. The subsequent reaction step consists of hydroxylation of the second carbon of the added group, catalysed by deoxyhypusine hydroxylase, to complete hypusine synthesis and eIF-5A maturation (Figure 20) (Park *et al.* 1993a).

8.1.1.3 Biological significance of eIF-5A and hypusine

The translation initiation factor eIF-5A was initially identified as a stimulant for two *in vitro* protein synthesis systems (Kemper *et al.* 1976). Indeed, Hershey (1991) stated that the mammalian eIF-5A appeared to act in the initiation phase of protein synthesis by promoting formation of the first peptide bond. However, recent reports of continued protein synthesis in yeast after the apparent complete depletion of eIF-5A suggest that the *in vivo* activity of eIF-5A may lie outwith the realm of protein synthesis (Park *et al.* 1993b).

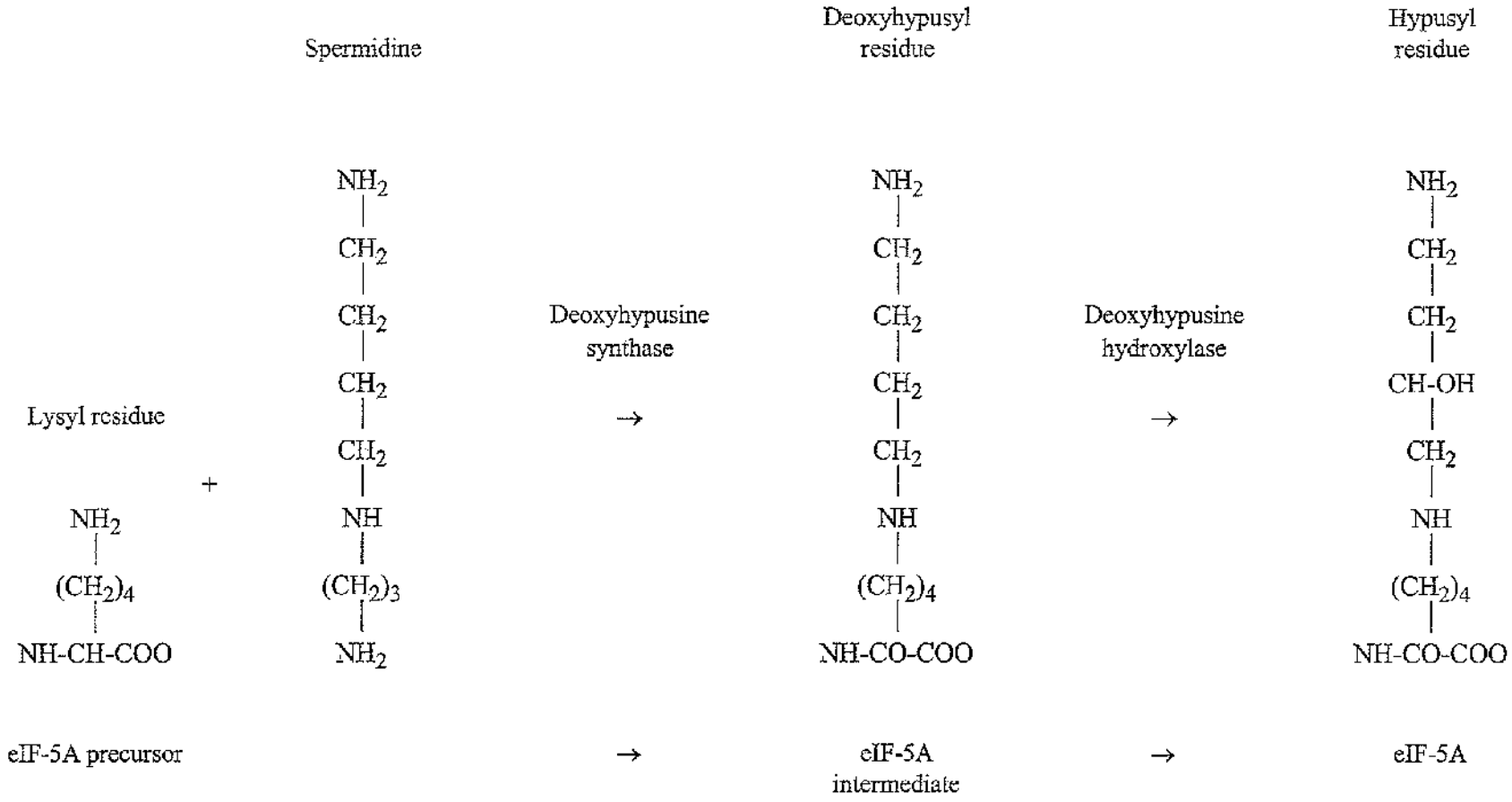


Figure 20: Biosynthesis of hypusine (Reproduced from Park *et al.* 1993b)

Hershey and co-workers studied the function of eIF-5A by genetically modifying yeast cell lines, having identified two genes responsible for eIF-5A in yeast. Expression of either of the two genes alone promoted growth of the cells while disruption of both genes was lethal (Schnier *et al.* 1991; Schwelberger *et al.* 1993). Similarly, incorporation of a plasmid containing either one of the genes into a double null mutant caused cells to grow at almost the normal rate (Schnier *et al.* 1991; Schwelberger *et al.* 1993). The necessity of eIF-5A for yeast cellular growth was demonstrated when cells depleted of eIF-5A ceased to divide (Park *et al.* 1993b).

Ambiguity thus exists with respect to the exact role of eIF-5A in cellular biology. It may participate in protein synthesis itself or some other function relating to cellular proliferation. However, studies such as those described above, as well as the highly conserved nature of the protein, do suggest that role to be critical (Park *et al.* 1993a). The activity of eIF-5A is undoubtedly conferred by the hypusine residue (Park *et al.* 1991), and further studies have thus attempted to elucidate the exact cellular role of hypusine.

An increase in hypusine synthesis coincided with an increase in protein synthesis when human lymphocyte cells were activated to grow (Cooper *et al.* 1982). Various groups of researchers have demonstrated that modification of the hypusine residue of eIF-5A results in the failure of eIF-5A to stimulate protein synthesis *in vitro* (Park 1989; Smit-McBride *et al.* 1989; Park *et al.* 1991).

Studies have also demonstrated an association between hypusine synthesis and cellular proliferation with general correlations between the two processes having been reported for various mammalian cell lines (Chen 1983; Torrelio *et al.* 1984; Gerner *et al.* 1986). Interestingly, the post-translational events leading to hypusine synthesis occur only after initiation of cell growth (Cooper *et al.* 1982; Chen 1983; Torrelio *et al.* 1984).

Thus, as with eIF-5A, the precise cellular role of hypusine would appear to be unknown at present. Yet, whether it be associated with protein synthesis or some other function relating to cellular proliferation, synthesis of the amino acid is critical for cell viability. The necessity of hypusine has been substantiated further through studies involving depletion of the spermidine substrate and inhibition of the two biosynthetic enzymes involved in the synthesis of hypusine.

8.1.1.4 Polyamine biosynthesis and hypusine

Although the significance of polyamines in cellular replication and growth has been known for almost 50 years (section 1.1.2.1, page 4), it was not until relatively recently that a

specific physiological role for spermidine was discovered: that of hypusine formation (Park *et al.* 1981). Early work, as reviewed in Chapter 1, found that cellular growth inhibition by the ODC inhibitor DFMO was accompanied by changes in intracellular polyamine levels, including a reduction in spermidine (Tabor & Tabor 1984). However, the cessation of cellular growth was observed to be delayed with respect to the spermidine depletion (Hölttä *et al.* 1979). Hölttä and co-workers (1979) thus speculated that the delay in growth inhibition was not due to specific spermidine loss but rather due to depletion of some spermidine derived component that was required for cellular proliferation and/or growth. Subsequent studies revealed that DFMO treatment of cells not only caused depletion of spermidine but resulted in a reduced rate of hypusine synthesis (Gerner *et al.* 1986; Park 1987).

In further studies, the effects of the ODC inhibitor, APA, were examined. As with DFMO, inhibitor treatment of HTC cells resulted in the arrest of cellular proliferation accompanied by reductions in both the spermidine level and hypusine content (A. Shirahata unpublished results). Similarly, treatment of HTC cells with APA resulted in reduced hypusine content and spermidine levels, however, treated cells appeared to grow as well as control cells in this instance (Beppu *et al.* 1996).

Thus, evidence relating the inhibition of polyamine biosynthesis and resultant effects on cellular proliferation and growth to hypusine synthesis was apparent. Further work with polyamine biosynthesis inhibitors revealed further the necessity of spermidine for hypusine formation and cell growth. Byers and co-workers exposed L1210 cells to AbeAdo, an inhibitor of AdoMetDC. As expected, spermidine and spermine levels were depleted while putrescine levels increased. The cellular growth rate declined and was followed by the onset of cytostasis whereby cells cease to grow in order to conserve intracellular levels of polyamines (Byers *et al.* 1992). The onset of cytostasis was, however, delayed relative to the depletion of spermidine and thus, AbeAdo-induced cytostasis was proposed to be associated with hypusine synthesis (Byers *et al.* 1992).

eIF-5A precursor levels did indeed become measurable upon the onset of AbeAdo-induced cytostasis, indicating a reduction in hypusine biosynthesis. Cells could be rescued from cytostasis by the addition of exogenous spermidine upon which the level of accumulated precursor decreased (Byers *et al.* 1992). Spermine also could rescue cells from cytostasis but was thought to undergo intracellular conversion to spermidine and the spermidine, not spermine rescue the cells (Byers *et al.* 1992). Many polyamine analogues were tested for their ability to reverse AbeAdo-induced cytostasis but only one of those was successful; the substrate for deoxyhypusine synthase, N-(3-aminopropyl)-1,4-diamino-cis-but-2-ene (Byers

et al. 1992). Other analogues tested failed to rescue cells and were themselves unable to act as substrates for deoxyhypusine synthase. Interestingly, the trans-isomer of N-(3-aminopropyl)-1,4-diamino-cis-but-2-ene did act as a substrate for deoxyhypusine synthase yet failed to rescue AbeAdo-induced cytostatic cells (Byers *et al.* 1992). In later work, two synthetic analogues, 1-methylspermidine and 1,12-dimethylspermine were tested for their ability to rescue cells. 1-methylspermidine was successful but 1,12-dimethylspermine failed to reverse AbeAdo-induced cytostasis. Treatment by 1-methylspermidine, a substrate for deoxyhypusine synthase, caused a reduction in the levels of unmodified eIF-5A and this correlation between hypusine synthesis and AbeAdo-induced cytostasis was further suggested by the failure of 1,12-dimethylspermine, which was not a substrate of deoxyhypusine synthase, to affect levels of eIF-5A (Byers *et al.* 1994).

8.1.1.5 Inhibition of hypusine biosynthetic enzymes

8.1.1.5.1 Deoxyhypusine synthase

Inhibition of deoxyhypusine synthase, the first enzyme of hypusine biosynthesis, was achieved using several mono-, di- and polyamines (Park & Wolff 1988). These initial studies indicated some structural features that were possibly important with regards to enzyme inhibition and thus, led to more detailed work by Jakus and co-workers (1993). These researchers demonstrated effective inhibition of deoxyhypusine synthase by several diamines and polyamines. 1,8-diaminooctane and 1,7-diaminoheptane were strong inhibitors of the enzyme while caldine (norspermidine) and N-(3-aminopropyl)cadaverine provided moderate inhibition. Inhibition was observed to be greatest with compounds that resembled spermidine in carbon chain length. The exception to this was the effective inhibition afforded by 1,3-diaminopropane (Jakus *et al.* 1993). This inhibition was, however, explained by product inhibition with 1,3-diaminopropane being produced when spermidine is cleaved prior to its addition to the lysine residue of eIF-5A (Park & Wolff 1988).

Since the fungicide guazatine, a guanylated polyamine derivative, had been reported to successfully inhibit deoxyhypusine synthase (Murphey & Gerner 1987), guanyl derivatives of these inhibitory diamines and polyamines were also synthesised. Monoguanyl derviatives were found to be more effective than the parent compounds, and indeed, the bisguanyl derivatives in inhibiting the enzyme. N^1-guanyl-1,7-diaminoheptane was the most effective inhibitor examined, having an IC_{50} of 0.017 μM (Jakus *et al.* 1993).

Compounds were tested for their ability to inhibit hypusine formation *in vivo* in CHO cells. All of the compounds tested: mono-guanyl derivatives of 1,7-diaminoheptane and 1,8-diaminooctane and bis-guanyl derivatives of 1,6-diaminohexane, 1,7-diaminoheptane and 1,8-diaminooctane successfully reduced hypusine formation. N^1-guanyl-1,7-

diaminoheptane caused almost complete cessation of hypusine production at a concentration of 10 μM (Jakus *et al.* 1993). Similar recent studies using HTC cells exposed to the parent diamines revealed interesting results. None of the four diamines examined affected cell growth and, indeed, 1,3-diaminopropane failed to produce an effect on hypusine content (Beppu *et al.* 1996). 1,6-diaminohexane and 1,7-diaminoheptane did, however, reduce hypusine synthesis, as did 1,8 diaminooctane although to a lesser degree. The lack of effect on cellular growth aside, the reduction in hypusine formation *in vivo* by the diamines was unlikely to wholly be caused by inhibition of deoxyhypusine synthase. Spermidine content was reduced in cells exposed to the inhibitors and this may have contributed to the observed reduction in hypusine content (Beppu *et al.* 1996).

Guanylated diamine studies concluded that N^1-guanyl-1,7-diaminoheptane and N^1-guanyl-1,8-diaminooctane inhibit deoxyhypusine synthase *in vitro*, hypusine formation *in vivo* and reduce cellular growth in a correlated manner. Thus, the growth inhibition would appear to be due to enzyme inhibition and not the result of non-specific toxic effects. Compounds showed little effect on cellular polyamine levels, implying that the inhibitors do not interfere with other enzymes for which spermidine is a substrate (Jakus *et al.* 1993, Park *et al.* 1994). However, the growth inhibition afforded by, for example, the mono-guanyl derivative of 1,6-diaminohexane and the bis-guanyl derivatives of 1,6-diaminohexane and 1,8-diaminooctane may have been due to causes other than inhibition of hypusine synthesis. *In vivo* studies revealed that these compounds reduced growth of CHO cells but failed to produce comparative reductions in hypusine content. Intervention in cellular processes other than hypusine synthesis is likely to account for the arrest of growth by these compounds (Park *et al.* 1994).

Further studies revealed that N^1-guanyl-1,7-diaminoheptane was actively taken up into CHO cells utilising the polyamine transport system. Guanylated diamines generally inhibited growth of other cell lines although the degree of inhibition varied and appeared to depend on the efficiency of inhibitor transport in different cell lines (Park *et al.* 1994). N^1-guanyl-1,7-diaminoheptane further demonstrated its antiproliferative ability with the supression of growth of mouse neuroblastoma and murine erythroleukemia cells at micromolar concentrations (Chen *et al.* 1996), as well as several other tumorigenic cell lines (Shi *et al.* 1996).

Work progressed when diamine and triamine analogues and derivatives were synthesised and tested for their ability to inhibit deoxyhypusine synthase. Few of these compounds were comparable with N^1-guanyl-1,7-diaminoheptane *in vitro* and none were as efficient in

reducing hypusine synthesis *in vivo*. The arrest of cellular growth by these compounds could not be wholly attributed to inhibition of deoxyhypusine synthase (Lee *et al.* 1995).

8.1.1.5.2 Deoxyhypusine hydroxylase

Other studies have focussed on inhibition of deoxyhypusine hydroxylase, the second enzyme of hypusine biosynthesis, although this work is somewhat limited. The enzyme was successfully inhibited by metal chelators such as α,α-dipyridyl (Park *et al.* 1982), and hydralazine (Paz *et al.* 1984) *in vitro*. Polyamines are also known to inhibit the enzyme (Abbruzzese *et al.* 1989). Inhibition of deoxyhypusine hydroxylase by L-mimosine *in vivo* resulted in the arrest of cell cycle progression (Watson *et al.* 1991). *In vivo* inhibition was also observed in CHO cells cultured in the presence α,α-dipyridyl. Deoxyhypusine was observed to accumulate in cellular protein (Park *et al.* 1982; Beppu *et al.* 1996).

8.1.2 Aim of studies

Knowledge of the critical nature of hypusine for cellular proliferation and/or growth led to the supposition that control of amino acid production could provide a means of regulating cell growth. Control of growth of cancerous and normal cells has been achieved through the inhibition of hypusine biosynthetic enzymes (Park *et al.* 1994; Hanauske-Abel *et al.* 1994). In addition, eIF-5A has been implicated in human immuno-deficiency virus type 1 (HIV-1) replication and disruption of eIF-5A function may provide a means of combatting the virus (Ruhl *et al.* 1993).

Spermidine analogues were originally designed to interfere with the synthesis of spermidine via disruption of the enzyme spermidine synthase. They were also anticipated to act through disruption of cellular function(s) of spermidine. In hypusine synthesis, the provision of spermidine is critical, although in specific work related to AbeAdo-induced cytostasis various spermidine analogues have been observed to act as suitable substitutes for spermidine. Importantly, various analogues have been unable to act as substrates for deoxyhypusine synthase. Indeed, in related work, N^1-acetylspermidine was found to be unable to compete with spermidine *in vivo* (Gerner *et al.* 1986). Perhaps the most interesting aspect of work carried out so far lies in the observation that N-(3-aminopropyl)-1,4-diamino-trans-but-2-ene acted as a substrate for deoxyhypusine synthase yet failed to support hypusine synthesis (Byers *et al.* 1992). Presumably, analogues such as this compound could 'disable' biologically active eIF-5A, prevent hypusine formation and thus prove invaluable in preventing cellular proliferation and/or growth.

However, as observed in the studies relating to inhibition of the hypusine biosynthetic enzymes, many compounds differ in their effect on hypusine synthesis *in vitro* and *in vivo*.

Hypusine synthesis may also be affected without biosynthetic enzyme inhibition. Much work in this area is required. Nevertheless, the proposed use of spermidine analogues to affect the enzymes responsible for the synthesis of hypusine presents a specific antiproliferative strategy. Clearly, a specific target in an antiproliferative approach is undoubtedly superior to a wide ranging attempt to affect any cellular function(s) for which spermidine is important.

8.2 EFFECT OF VARIOUS DEOXYHYPUSINE SYNTHASE INHIBITORS ON MYCELIAL GROWTH OF *PYRENOPHORA AVENAE* ON SOLID MEDIA

8.2.1 Introduction and Objectives

Although evidence of the presence of hypusine in *Neurospora crassa* does exist (Chen & Yang 1988; Yang *et al.* 1990), perturbation of hypusine formation as an antifungal or fungicidal strategy has not yet been explored. Given that CHA and spermidine analogues have demonstrated antifungal and fungicidal activity but no mode(s) of action have as yet been determined, investigation of fungal hypusine biosynthesis was deemed appropriate. Before attempting to study the effect of CHA and norspermidine on hypusine biosynthesis, the effects of known inhibitors of deoxyhypusine synthase on mycelial growth of *Pyrenophora avenae* on solid media was studied. This would give a clear indicator if perturbation of hypusine biosynthesis did indeed affect growth of *P. avenae*. Many of the known deoxyhypusine synthase inhibitors are related to guazatine (Panoctine, Rhone-Poulenc), a cereal seed dressing, thus antifungal activity was expected. Nevertheless, various inhibitors of deoxyhypusine synthase were generously supplied by Dr. J. E. Folk of the Enzyme Chemistry Section, National Institutes of Health, USA (Table 37). Although effects on fungal growth on solid media are not always reliable indicators of *in vivo* fungal growth, such a study was appropriate given the limited amounts of compounds available.

8.2.2 Materials and Methods

P. avenae was maintained as described in section 3.1.2.1 (page 76). Fungus was grown on solid media amended with the various deoxyhypusine synthase inhibitors using the procedure described in section 3.1.2.2 (page 76), but modified to allow for the very limited amounts of inhibitors available.

Filter-sterilised inhibitor solutions (10 ml) were added to 30 ml of sterile potato dextrose agar (PDA) at 45-47°C to obtain the final desired concentrations of each compound. Concentrations varied from 1.0 mM depending on the amount of material available. Although 1.0 mM and lower concentrations of CHA and novel spermidine analogues did not produce inhibition of mycelial growth on solid media, norspermidine had inhibited growth at that concentration. The limited amounts of deoxyhypusine synthase inhibitors did not permit higher concentrations to be used for these studies. Sterile medium (10 ml) was added aseptically to a 60 mm plastic Petri dish. A 6 mm cork borer was used to remove plugs of mycelium from stock plates which were inverted and placed in the centre of each Petri dish. Inoculated plates were incubated in the dark at 24°C. Radial mycelial growth, excluding the mycelial plug, was measured 6 days after inoculation with *P. avenae*. Three measurements were taken from each plate. Control plates contained culture medium only.

Four replicates were used for each treatment with significance assessed using Student's *t*-test. All experiments were repeated and similar results obtained.

8.2.3 Results

All of the inhibitors examined reduced radial mycelial growth of *P. avenae* at the concentrations tested (Table 38, Plates 5-6). Four of the compounds completely inhibited fungal growth, with 4a doing so at a concentration as low as 0.61 mM. Compounds 48, 49 and 4e affected growth more profoundly than is reflected in the radial measurement data: growth was less dense in the presence of these compounds (Plate 6).

Table 37. Structures of deoxyhypusine synthase inhibitors

Compound	Structure
3a [1]	$H_2NC(=NH)NH(CH_2)_8NH(CH_2)_8NHC(=NH)NH_2$
3b [1]	$H_2NC(=NH)NH(CH_2)_7NH(CH_2)_7NHC(=NH)NH_2$
4a [1]	$H_2N(CH_2)_8NH(CH_2)_8NHC(=NH)NH_2$
4c [1]	$H_2N(CH_2)_4NH(CH_2)_4NHC(=NH)NH_2$
11 [1]	$H_2N(CH_2)_3O(CH_2)_3NHC(=NH)NH_2$
12 [1]	$H_2N(CH_2)_3S(CH_2)_3NHC(=NH)NH_2$
21 [1]	$H_2NC(=NH)NH(CH_2)_7NHCH_2CH_3$
42 [2]	$H_2NC(=NH)NH(CH_2)_7NHC(=NH)NH_2$
43 [2]	$H_2NC(=NH)NH(CH_2)_8NHC(=NH)NH_2$
48 [2]	$H_2NC(=NH)NH(CH_2)_7NH_2$
49 [2]	$H_2NC(=NH)NH(CH_2)_8NH_2$

Note. Compounds are referred to as numbers as detailed in [1] Lee *et al.* 1995 and [2] Jakus *et al.* 1993.

Table 38: Effect of deoxyhypusine synthase inhibitors on mycelial growth of *Pyrenophora avenae*

Treatment	Concentration (mM)	Mycelial growth, measured as mean colony radius (mm)	Percentage reduction in radial mycelial growth
Control		25.7 ± 0.37	
4a	0.61	NG	100
3b	0.97	NG	100
4e	0.84	20.7 ± 0.62	19.5
21	0.90	11.7 ± 0.21	54.4
42	1.0	1.2 ± 0.13	95.3
43	0.83	NG	100
48	1.0	20.1 ± 0.43	21.8
49	1.0	17.8 ± 0.41	30.7
Control		24.3 ± 0.37	
3a	1.0	NG	100
11	1.0	12.2 ± 0.31	49.8
12	1.0	7.4 ± 0.18	69.5

Note: Values are shown as the means ± SE of three measurements from each of four replicates.

For all treatments, significant differences were observed from the controls ($p \leq 0.001$).

NG no growth

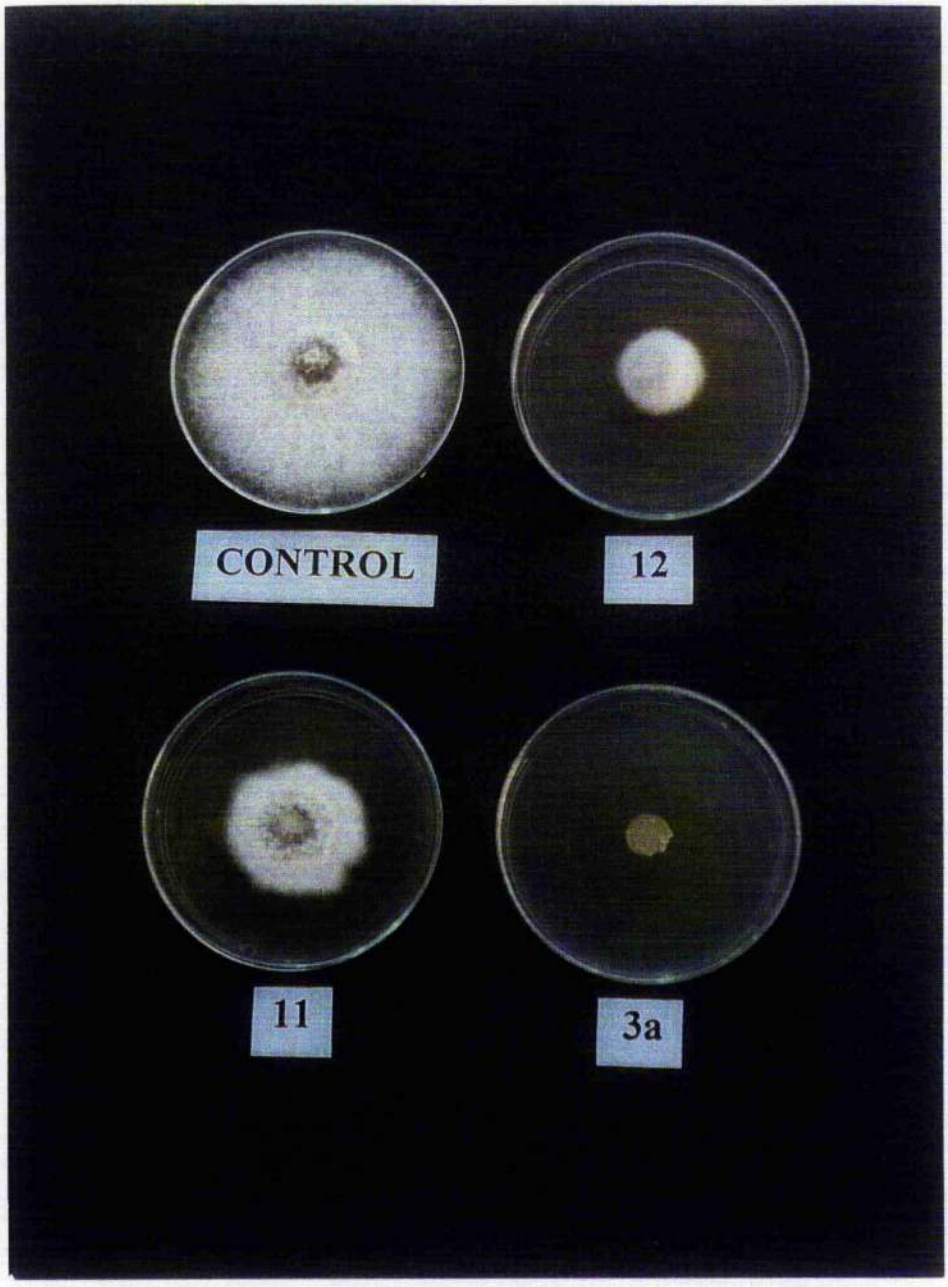


Plate 5. Effect of 1.0 mM concentrations of deoxyhypusine synthase inhibitors on mycelial growth of *Pyrenophora avenae*, grown on solid media for six days.

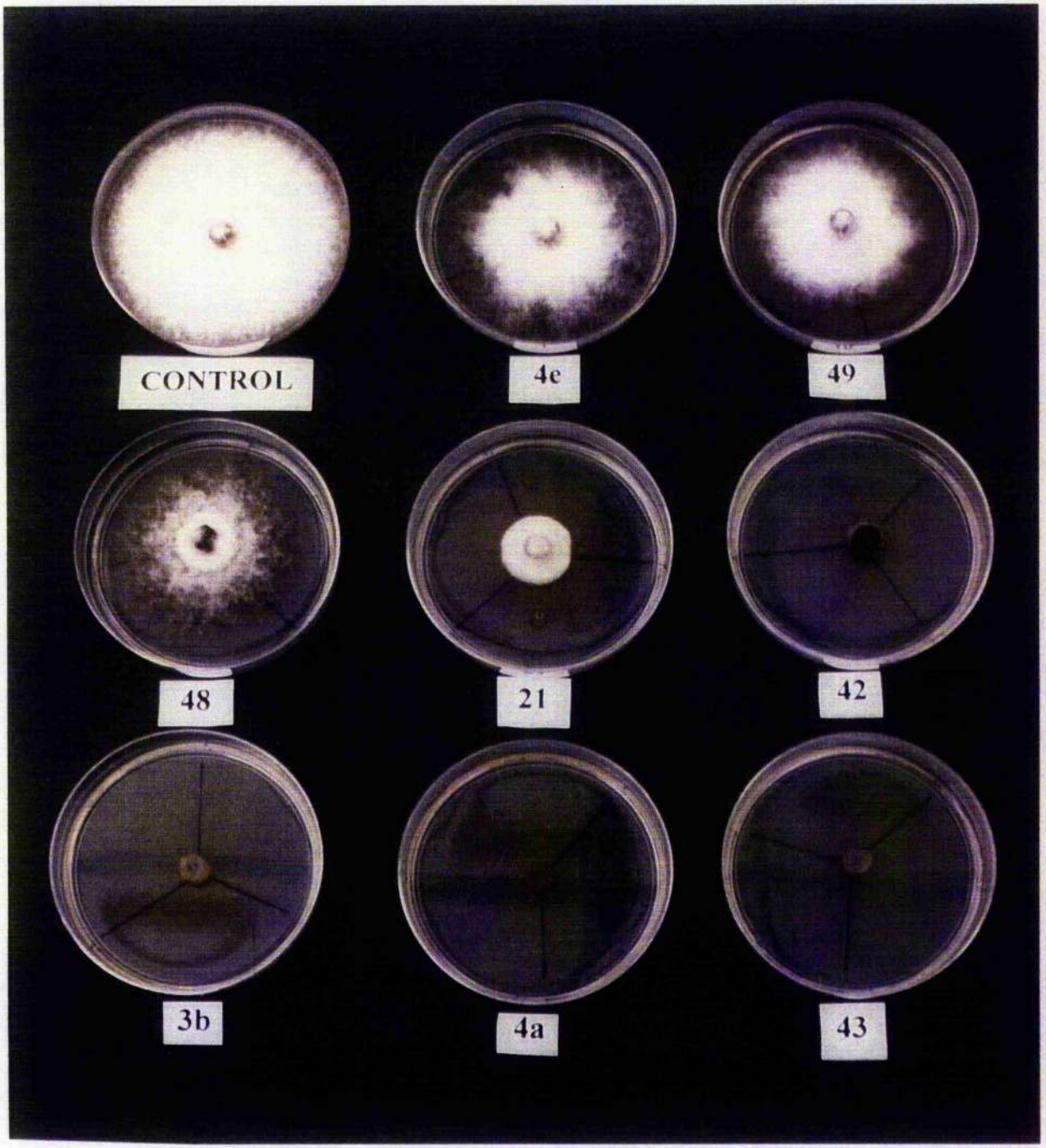


Plate 6. Effect of varying concentrations * of various deoxyhypusine synthase inhibitors on mycelial growth of *Pyrenophora avenae*, grown on solid media for six days.

* For concentrations used, see Table 38, page 190

8.3 EFFECT OF VARIOUS COMPOUNDS ON THE GROWTH OF *PYRENOPHORA AVENAE* IN LIQUID CULTURE

8.3.1 Introduction and Objectives

Given that known inhibitors of deoxyhypusine synthase do indeed inhibit mycelial fungal growth of *P. avenae* on solid media, it was decided to determine the amount of deoxyhypusine and hypusine in fungus treated with CHA and norspermidine and thus investigate the effect of these compounds on hypusine biosynthesis. Various other compounds have demonstrated an effect on hypusine and deoxyhypusine formation *in vivo*, albeit in animal cells. Exposure of fungus to such compounds was thought to be worthwhile as an aid to developing the deoxyhypusine/hypusine determination procedure. Thus, if compounds acted in fungus as they do in animal cells, a series of known effects would be available for which comparison of CHA and norspermidine effects could be made.

DFMO (Gerner *et al.* 1986; Park 1987) and APA (A. Shirahata unpublished results; Beppu *et al.* 1996) are known to reduce synthesis of hypusine in cultured cells. 1,3-diaminopropane and 1,6-diaminohexane have been reported to inhibit deoxyhypusine synthase (Park & Wolff 1988; Jakus *et al.* 1993) while 2,2'-dipyridyl, (α,α-dipyridyl), is known to inhibit the hydroxylation process and thus allow deoxyhypusine to accumulate (Park *et al.* 1982). Deoxyhypusine is normally present in cells at very low levels and can only be detected through disruption of deoxyhypusine hydroxylase.

8.3.2 Materials and Methods

P. avenae was grown in liquid culture in the presence of the various compounds associated with the inhibition of hypusine synthesis as detailed in section 3.2.2.2 (page 85). DFMO and APA[7] were both used at 0.1 mM, limited amounts of these compounds not permitting higher concentrations to be used. 2,2'-dipyridyl was used at a concentration of 0.01 mM. A preliminary experiment had shown that fungal growth was completely prevented by 1.0 mM 2,2'-dipyridyl, whereby the normally yellow coloured PDB became pink in colour, indicating extreme toxicity. 1,3-diaminopropane and 1,6-diaminohexane were used at concentrations of 1.0 mM, such a concentration chosen on the basis of previous work which had shown CHA to affect *P. avenae* growth when used at 1.0 mM. Fungus was grown for 4 days in order to amass the material required for further analysis. Four replicates were used for each experiment and experiments were repeated with similar results. Statistical significance was assessed using Student's *t*-test.

[7] APA was kindly provided by Professor A. Khomutov of the Russian Academy of Science, Moscow.

8.3.3 Results

1,3-diaminopropane failed to reduce the fresh weight of *P. avenae* at the concentration examined, although, it did appear to affect fungal growth in liquid culture. Fungus exposed to the compound was darker than the control fungus. DFMO, APA, 1,6-diaminohexane and 2,2'-dipyridyl all reduced the fresh weights of fungal mycelium grown in liquid culture in their presence for 4 days (Table 39), albeit, not always significantly.

Table 39: Effect of various compounds on the growth of *Pyrenophora avenae* in liquid culture for 4 days

Treatment	Concentration (mM)	Growth of fungus, measured as mean fresh weight (g)
Control		3.7 ± 0.31
DFMO	0.1	3.2 ± 0.20
APA	0.1	2.8 ± 0.18
Control		3.9 ± 0.28
1,3 diaminopropane	1.0	4.0 ± 0.19
1,6 diaminohexane	1.0	2.7 ± 0.19 *
Control		3.3 ± 0.12
2,2'-dipyridyl	0.01	2.8 ± 0.19

Note: Values are shown as the means ± SE of four replicates.

Significant difference from control is shown as: * $p \leq 0.05$

8.4 Effect of various compounds on fungal hypusine and deoxyhypusine content

8.4.1 Introduction and Objectives

Numerous methods have been reported for determination of hypusine and deoxyhypusine. The majority of these procedures involve, firstly, the collection of cellular protein and then acid hydrolysis of that protein to release the bound amino acids. Ion-exchange chromatography is used to 'clean up' the sample by removing the bulk of the amino acids from hypusine and deoxyhypusine. Methods then vary in their preference for pre or post HPLC column derivatisation before separation and detection of the derivatives fluorometrically. Such a method using o-phthalaldehyde (OPA) pre column derivatisation was used by Beninati and co-workers (1990) while Bartig & Klink (1992) used 4-dimethylaminoazobenzene-4'-sulphonyl chloride derivatisation. Post column OPA derivatisation was employed by Sano and co-workers (1984) and Beppu *et al.* (1996).

Alternatively, various groups of workers have used deoxyhypusine synthase enzyme assays to study hypusine biosynthesis. One such *in vitro* enzyme assay procedure involves examination of the formation of radio-labelled products from labelled spermidine or lysine. Ion exchange chromatography is necessary to separate the products before radioactive counting to assess the flux of starting materials through to products (for example, Park *et al.* 1991). Another assay procedure involves measurement of the radioactivity found formed in protein bound hypusine and deoxyhypusine from labelled spermidine. Bound products are separated by gel electrophoresis before radioactivity counting of each protein (Chen & Dou 1988; Dou & Chen 1990). Assays have also been reported for deoxyhypusine hydroxylase, (for example, Csonga *et al.* 1996), however, all enzyme assays require the use of purified enzyme and precursor eIF-5A protein and were thus deemed unsuitable for the purposes of the present study.

It was decided to determine hypusine and deoxyhypusine content in fungal tissue by adapting the method reported by Beppu *et al.* (1996). These authors argued that previously reported methods using such a procedure of protein collection, acid hydrolysis, ion exchange chromatography and HPLC analysis, were unsatisfactory with regards to separation and sensitivity. However, the procedure reported by Beninati *et al.* (1990), using pre column OPA derivatisation was deemed satisfactory. Therefore, both of these previously reported studies were consulted during the 'design' of the present method. These procedures were deemed the most suitable in view of the resources and equipment at the disposal of the present study.

8.4.2 Materials and Methods

Authentic hypusine was kindly provided for the study by Dr. T. Shiba of the Protein Research Foundation, Osaka, Japan. Deoxyhypusine was generously donated by Dr. J. E. Folk.

The method reported by Beppu *et al.* (1996) initially involved collection of cellular protein. Briefly, HTC cells were washed twice and suspended in phosphate buffered saline before being sonicated and the lysate centrifuged. Protein in the supernatant was then determined.

Thus, fungus (7 g) was ground in a pre chilled mortar and pestle with 7 mls of a phosphate buffer, pH 7.6. This pH was chosen since it was the pH of the grinding buffer used for ODC and AdoMetDC enzyme assays. Portions of the crude extract were sonicated on ice using a Soniprep 150 for 10 cycles of 10 seconds on/20 seconds off before being pooled and centrifuged at 16000 *g* for 25 min at 0°C, as for free polyamine analysis. Protein assays were carried out using the method of Lowry *et al.* (1951) with BSA as the standard.

To precipitate the protein, Beppu *et al.* (1996) added 10 % trichloroacetic acid (3 ml) to HTC cell supernatant (1 ml) which contained 0.5 - 10 mg of protein. The resulting precipitate was washed once with 10 % trichloroacetic acid and then powdered using acetone and ether, aiming to remove low molecular weight compounds, including trichloroacetic acid.

Thus, 10 % trichloroacetic acid (30 ml) was added to supernatant (10 ml) and the precipitate allowed to settle. Fungus generally contained 0.2 - 0.3 mg of protein per ml, thus approximately 2 - 3 mg of protein was collected. The supernatant was collected after filtering the mixture under gravity, allowing complete recovery of protein. Filtration under vacuum resulted in impregnation of the filter paper with the protein sample. The precipitate was washed once using 10 % trichloroacetic acid. Acetone (20 ml) was added followed by diethyl ether (30 mls) and the solvents removed *in vacuo*.

To the HTC cell residue, Beppu and co-workers (1996) added 500 pmol of an internal standard, a synthesised analogue of deoxyhypusine synthase, before hydrolysing the mixture in 6 M hydrochloric acid for 13 hours at 120°C.

These authors had tested many compounds for use as an internal standard and thus, 1,6-diaminohexane (500 pmol) was added to the fungal residue. Under the HPLC conditions described by the authors, 1,6-diaminohexane was retained for 12.4 mins with hypusine and deoxyhypusine retained for 14.4 and 17.2 mins respectively. Thus, adequate separation

between the internal standard and the desired compounds was envisaged. 6 M hydrochloric acid (20 ml) was added and the mixture hydrolysed for 13 hours, using an oil bath.

Upon completion of hydrolysis, the fungal sample was treated as the authors had done for hydrolysed HTC cell protein. Solvents were removed *in vacuo* and the residue dissolved in distilled water (10 ml). The solution was passed through a 0.45 µm syringe filter and 1 M pyridine (100 µl) added.

Using the method originally described by Samejima and co-workers (1976), the filtrate was applied to a small column (10 x 130 mm) of CM23 carboxymethyl cellulose, a fibrous cation exchanger (Whatman), equilibrated with 0.025 M pyridine-acetic acid buffer, pH 5. The column was eluted sequentially with 0.025 M (10 ml), 0.05 M (5 ml), 0.075 M (5 ml) and 0.1 M (5 ml) pyridine-acetic acid buffers, pH 5. Hypusine, deoxyhypusine and the internal standard were predicted to be recovered in the last fraction and this was evaporated to dryness *in vacuo* before dissolving the residue in distilled water (1 ml) and freezing at -20°C until required for HPLC analysis. Beppu *et al.* (1996) dissolved residues in HPLC elution buffer before directly injecting aliquots into the HPLC apparatus. In the present study, only one sample could be generated at any one time and intensive HPLC apparatus use meant that samples had to be frozen until their generation was complete. Nothing was known of the stability of the samples in the HPLC elution buffer, thus water was chosen as the medium for freezing the samples. Indeed, Beninati *et al.* (1990) froze stock solutions of hypusine and deoxyhypusine at -20°C in distilled water.

Thus, fungus grown as a control was prepared for HPLC analysis along with *P. avenae* exposed for 4 days to those compounds described in section 8.3: DFMO, APA, 1,6-diaminohexane, 1,3-diaminopropane, 2,2'-dipyridyl, 1.0 mM CHA and 0.01 mM norspermidine. Such CHA and norspermidine concentrations were chosen on the basis of previous work. The lower concentrations, as opposed to 2.75 mM CHA and 0.2 mM norspermidine, were used since large amounts of fungal material were necessary to collect sufficient protein for analysis.

An additional sample was prepared which would act as a test for hypusine presence, as described by Beppu *et al.* (1996). Fungus grown as a control was prepared in the manner as described previously. After ion-exchange chromatography, solvents were removed *in vacuo* from the final fraction recovered and the residue dissolved in 0.01 M hydrochloric acid (200 µl). 100 µl of the sample was frozen to await HPLC analysis. To the other 100 µl, 100 µl of 40 mM sodium periodate solution was added and the mixture left to stand at room temperature for 12 hours before being frozen to await HPLC analysis. Oxidative reaction

with sodium periodate is envisaged to cleave the β-aminoethanol portion of hypusine. Thus, HPLC analysis of the treated residue (50 µl) should reveal the absence of a peak when compared to the untreated sample (25 µl), indicating therefore that the peak should be hypusine. Twice the amount of the sodium periodate treated sample has to be injected compared to the untreated sample to allow for the 50 % dilution by sodium periodate.

HPLC apparatus consisted of an eluant pump (Model LC-10AD, Shimadzu, Japan) and a reagent pump (Model constaMetric 3500, LDC Analytical, Florida) connected to an auto-injector (Model SIL-9A, Shimadzu, Japan), fluorometric detector (Model 821-FP, Jasco, Japan) and chromato recorder (Model C-R6A, Shimadzu, Japan).

HPLC conditions were as follows: a C18 column (Spherclone ODS-2, 150 x 4.6 mm i.d., Phenomenex, UK) connected with a guard cartridge (ODS-2, 30 x 4.6 mm i.d., Phenomenex, UK), was kept at ambient temperature (22°C). Eluant buffer contained 0.1 M $NaH_2PO_4.H_2O$, adjusted to pH 2 using phosphoric acid, with 10 mM sodium *n*-hexylsulfate and 3.5 % acetonitrile added. Buffer was eluted at a flow rate of 1 ml/min and fresh eluant was prepared daily.

Amines were detected fluorometrically by the OPA post column method as described by Seiler & Knödgen (1985). Reagent was prepared by dissolving boric acid (25 g) and potassium hydroxide (22 g) in 500 ml distilled water. 1.5 ml Brij-35 solution, 1.5 ml of β-mercaptoethanol and a solution of 200 mg of o-phthalaldehyde, dissolved in 2.5 ml methanol, were added to the solution. The reagent was stored in a dark bottle and prepared fresh daily. The derivatising reagent was delivered in the HPLC system at a rate of 1 ml/min.

The column eluate was thus mixed with the derivatising reagent in a 1:1 ratio and passed through a mixing column (50 x 4.6 mm i.d., filled with glass beads, 75µm, Supelco, UK), before fluorescence was detected using an excitation wavelength of 334 nm and emission wavelength of 440 nm.

8.4.3 Results

Due to the extremely limited amount of hypusine available, initial workings of the HPLC apparatus were undertaken using samples of 1,6-diaminohexane, the internal standard. Problems were immediately apparent with respect to the reproducibility of peaks. Indeed, a number of repeated injections of the same 1,6-diaminohexane solution resulted in ever decreasing peak areas although retention times remained steady. A similar phenomenon was observed with authentic deoxyhypusine, a small amount of which was used in case the

problems lay with 1,6-diaminohexane. Several means of fine tuning were employed in an attempt to solve the problem. Fresh derivatising reagent was prepared during a single day, although it was not thought that the reagent would deteriorate so quickly. Indeed, this made little difference to the results obtained. Injection volumes were increased from 50 µl to 150 µl, to ensure that excess sample was present and the full 50 µl was loaded onto the column. Gain and response settings on the detector were also adjusted.

A mechanical problem did develop in that a loop was blocked, preventing sample reaching the column. Partial blocking may have contributed to the problems detected prior to discovery and clearance of the blockage.

Subsequently, an attempt to detect vitamins A and E for which the HPLC apparatus was normally used, was successful, indicating that mechanical problems had ceased. Such problems did, however, account for some considerable time, during which attempts were being made to develop the HPLC methodology.

The occurrence of multiple peaks (so called 'split peaks') indicated a void in the system. Attempts were made to eliminate the void and were apparently successful. Collection of a chromatogram of authentic hypusine was attempted. At this point, it was difficult to determine whether the appearance of multiple peaks was a characteristic of the compound i.e. contaminants, or that a void was still present in the system. Elimination of any void was attempted and consultation with Dr. Shiba revealed that the hypusine was indeed likely to consist of two diastereoisomers.

Aliquots of the control sample were injected and revealed many peaks early on in collection of the chromatogram yet little in the region where hypusine and the internal standard were expected. Aliquots of one of the treatment samples (1.0 mM CHA) were also injected. Still variability occurred with respect to the peak areas.

The possibility of carry over of sample, a possible cause of peak area irregularity was investigated. Thus, three injections of a sample were made before running 3 aliquots of distilled water through the system. No peaks were observed in the water samples and thus, carry over of sample was excluded.

Ultimately, comparison of the control sample with authentic hypusine revealed that indeed, hypusine may be present in the fungus (Figure 21). However, given the inherent variability of the repeated injections, the work could not be pursued further within the time permitted. A matter of weeks of HPLC apparatus use were negotiated originally when a much longer

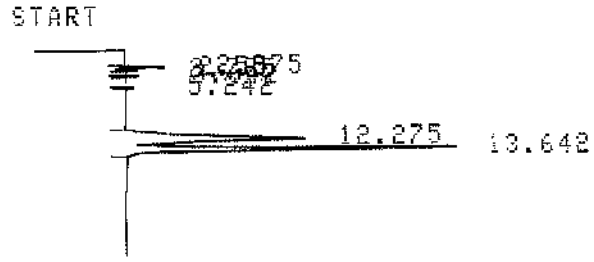
time frame is generally accepted to be required for fine-tuning of HPLC methods. Further consultation with Dr. Shiba also revealed that the two peaks in the hypusine sample were not likely to correspond to two diastereoisomers, but that a contaminant was present.

Nevertheless, the work did indicate areas which may improve the workings of the HPLC system. The second (reagent) pump may have been operating variably. Pressure fluctuations may have been the cause of the erratic peak areas observed. Little resistance is offered against the pump and the inclusion of an inert column between the pump and the T-piece where the reagent meets the eluate may provide such an obstacle for the pump to operate against, and thus improve efficiency of the pump.

Indeed, the use of two pumps was an unusual means of operating for those who frequently used the HPLC apparatus. Pre column derivatisation would exclude the need for the second pump and may increase efficiency of the system.

Peaks corresponding to 1,6-diaminohexane were undetected in the fungal samples examined by HPLC. The inclusion of a compound to be used as an internal standard which acted more like hypusine and deoxyhypusine in the preparative procedure is desirable. 1,6-diaminohexane may have been lost from the samples in the ion-exchange chromatography step.

Clearly, continuation of this work, developing a satisfactory method for hypusine and deoxyhypusine determination would be prudent. Methods reported until now have been for amino acid determination in cultured animal cells and mammalian organs. Fungal samples may require alternative or additional 'clean up' steps to remove compounds which hinder HPLC detection of hypusine and deoxyhypusine.

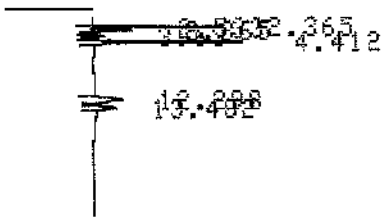


1.

CHROMATOPAC C-R6A
SAMPLE NO 0
REPORT NO 1103

FILE 7
METHOD 0061

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.375	7734			1.3055	
2	2.55	4127	V		0.6965	
3	2.785	3578	V		0.604	
4	3.642	3154			0.5324	
5	5.242	1691			0.2854	
6	12.275	282239			47.6385	
7	13.642	289936	V		48.9377	
	TOTAL	592460			100	



2.

CHROMATOPAC C-R6A
SAMPLE NO 0
REPORT NO 1104

FILE 7
METHOD 0061

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.365	26518			19.0823	
2	2.533	6948	V		4.994	
3	2.865	17817	V		12.8213	
4	3.63	3054			2.1979	
5	4.412	46908			33.7551	
6	12.298	25006			17.9941	
7	13.482	12723			9.1553	
	TOTAL	139967			100	

Figure 21. HPLC Chromatograms of authentic hypusine and extract from *Pyrenophora avenae*

Chromatogram 1 (authentic hypusine) produced peaks at 12.28 and 13.64 minutes.

Chromatogram 2 (fungus grown as control) produced peaks at 12.30 and 13.48 minutes.

8.5 Discussion

The antifungal activity demonstrated against *P. avenae* grown on solid media by the inhibitors of deoxyhypusine synthase is in agreement with those findings which show that the compounds affect *in vitro* enzyme activity and *in vivo* growth of CHO cells (Jakus *et al.* 1993; Lee *et al.* 1995). Compound 3a, guazatine, was extremely effective, affording complete control of fungal growth at a concentration of 1.0 mM. Given that guazatine is a commercial fungicide, this control is not surprising. Similarly, the closely related compounds 3b and 4a completely prevented fungal growth when used at concentrations which were in fact less than 1.0 mM.

Compound 4a is the mono-guanylated derivative of bis-guanylated 3a and had demonstrated reduced inhibitory activity against deoxyhypusine synthase *in vitro* compared to 3a in previous work (Lee *et al.* 1995). However, growth inhibition of CHO cells *in vivo* by 4a was greater than that provided by 3a, leading the authors to conclude that growth inhibition was not wholly caused by an effect on deoxyhypusine synthase (Lee *et al.* 1995). In the present study, 4a reduced fungal growth at a lower concentration (0.61 mM) compared to 3a (1.0 mM). However, since both compounds completely prevented fungal growth, further study would be necessary to conclude which of the compounds was more effective. Nevertheless, the antifungal activity demonstrated by the compounds agrees with previous work which showed the compounds to possess antiproliferative activity.

Lee and co-workers (1995) made comparisons of the effects of 3a and 3b with the guanylated derivatives of 1,8-diaminooctane and 1,7-diaminoheptane. Firstly, 3b was found to be more effective at inhibiting deoxyhypusine synthase *in vitro* than 3a and also more effective at inhibiting growth of CHO cells. In the present study, both compounds completely prevented fungal growth, thus confirming the antiproliferative activity already reported. Further study would be necessary to distinguish between efficacy of the compounds.

3a provided greater inhibition of deoxyhypusine synthase *in vitro* compared to bis-guanylated 1,8-diaminooctane (compound 43) but less inhibition compared to the mono-guanylated derivative, compound 49. A similar relationship was observed between 3b and the bis-guanylated derivative of 1,7-diaminoheptane (compound 42) and its mono-guanylated counterpart, compound 48. Thus, mono-guanylated derivatives were shown to provide increased inhibitory activity of deoxyhypusine synthase *in vitro* over bis-guanylated derivatives (Lee *et al.* 1995). However, the present study demonstrated that compounds 42 and 43, the bis-guanylated derivatives, afforded greater control of fungal growth than their mono-guanylated counterparts, compounds 48 and 49, disagreeing with the previous work.

Jakus and co-workers (1993) had also found the mono-guanylated derivatives inhibited deoxyhypusine synthase *in vitro* more effectively than the bis-guanylated derivatives. This disagreement with the present study was further substantiated since Jakus *et al.* (1993) and Park *et al.* (1994) had indicated that the mono-guanylated compounds 48 and 49 provided better control than the bis-guanylated compounds 42 and 43 in reducing hypusine formation and cellular proliferation of CHO cells.

As with 3b and 3a, the 1,7-diaminoheptane derivatives were more effective than 1,8-diaminooctane derivatives both *in vitro*, (Jakus *et al.* 1993; Lee *et al.* 1995) and *in vivo* (Jakus *et al.* 1993). However, the present study showed little differences between the 1,7-diaminoheptane derivatives (3b, 42 and 48) compared to their 1,8-diaminooctane counterparts (3a, 43 and 49). Further study, involving a range of inhibitor concentrations would be necessary before any firm conclusions could be made about the respective efficacies of these compounds.

Previous work indicated that N^1-guanyl-1,7-diaminoheptane (compound 48) is the strongest known inhibitor of deoxyhypusine synthase both *in vitro* and *in vivo* (Jakus *et al.* 1993; Park *et al.* 1994). However, the present study found the inhibitor to only reduce mycelial growth of *P. avenae* by 21.8 %, when used at a concentration of 1.0 mM. Workers noted that the inhibitory activity exerted by N^1-guanyl-1,7-diaminoheptane in various cell lines depended on uptake of the compound into cells (Park *et al.* 1994). Transport into fungal cells may thus have affected the degree of efficacy afforded by the compound. Compounds 48 and 49 did affect fungal growth more so than is reflected in the radial mycelial growth measurements (Plate 6). Indeed, Mandels (1965) stated that no necessary correlation between the spread of mycelial growth on a solid surface and the total amount of fungus produced existed and that the use of linear measurements for quantifying growth was misleading. Clearly, compounds 48 and 49 were less effective than 42 and 43. Further work using fungus grown in liquid culture would be prudent.

The effects of the remaining compounds, 4e, 11, 12 and 21 agree with the work of Lee *et al.* (1995) who showed that each compound effectively reduced deoxyhypusine synthase activity *in vitro* and reduced growth of CHO cells *in vivo*. Perhaps the control afforded by 4e is less than would have been expected given the low IC_{50} value of 0.16 μM and good inhibitory activity shown against CHO cells, however, Plate 6 does indicate that growth was more markedly affected than suggested by the mycelial growth measurements.

Interestingly, compounds 42, 43, 48, 49 and perhaps 4e are the only compounds for which evidence exists that their *in vivo* inhibitory activity is indeed related to deoxyhypusine

synthase inhibition (Jakus *et al.* 1993; Lee *et al.* 1995), although Park *et al.* (1994) suggested that the growth inhibition of bis-guanylated 1,8-diaminooctane may not be wholly due to inhibition of hypusine biosynthesis. The remaining compounds are likely to affect hypusine formation by some means other than deoxyhypusine synthase inhibition. Thus, their antifungal activity may be the result of more than one mode of action.

The inhibitory effects of the ODC inhibitors DFMO and APA against *P. avenae* grown in liquid culture are in agreement with previous work carried out in such areas. The growth inhibitory activity of DFMO is well documented, with such effects indeed being observed against fungal growth (for example, West & Walters 1989). APA has been shown to affect HTC cellular growth (A. Shirahata unpublished results) although the compound had little effect on the growth of CHO cells (Beppu *et al.* 1996). Such DFMO and APA treatment of cultured cells has been associated with a reduction in spermidine content as well as a reduction in the rate of hypusine synthesis (Gerner *et al.* 1986; Park 1987; A. Shirahata, unpublished results; Beppu *et al.* 1996). It should thus be noted that DFMO and APA perhaps cause a reduction in hypusine formation as a result of the lack of spermidine in the cells and not in fact due to an effect on the hypusine biosynthetic enzymes (Park 1987; Beppu *et al.* 1996). Nevertheless, perturbation of hypusine formation is envisaged to occur in fungus exposed to the compounds, and such fungus will thus be important in aiding the measurement of fungal hypusine and deoxyhypusine.

Fungal growth inhibition by 2,2'-dipyridyl agrees with the previous work carried out by Beppu *et al.* (1996), who showed that CHO cellular growth was affected by this compound. Hypusine content of CHO cells decreased while deoxyhypusine content increased. As spermidine levels did not differ from the control cells, inhibition of deoxyhypusine hydroxylase was suggested to have occurred (Beppu *et al.* 1996). Deoxyhypusine hydroxylase inhibition in fungus would allow detection of deoxyhypusine, and thus fungus exposed to 2,2'-dipyridyl should aid the development of a method to determine deoxyhypusine content of fungus.

The diamines 1,3-diaminopropane and 1,6-diaminohexane had no effect on CHO cellular growth (Beppu *et al.* 1996), thereby disagreeing with the findings of the present study where the compounds inhibited fungal growth. In such previous work, 1,3-diaminopropane had little effect on hypusine content of CHO cells (Beppu *et al.* 1996), even though it is well documented to inhibit deoxyhypusine synthase *in vitro* (Park & Wolff 1988; Wolff *et al.* 1992; Jakus *et al.* 1993). 1,6-diaminohexane reduced hypusine content of CHO cells, however, since spermidine content was also reduced, the reduction in hypusine may have

been due to a lack of spermidine rather than inhibition of deoxyhypusine synthase (Beppu *et al.* 1996). 1,6-diaminohexane has also demonstrated inhibitory activity towards deoxyhypusine synthase *in vitro* (Jakus *et al.* 1993). Whatever effects these compounds have on fungal hypusine biosynthesis, hypusine content is likely to be altered, and thus, the use of these compounds could prove useful in determining a method for the study of hypusine formation in fungal cells.

In spite of this preliminary work, hypusine and deoxyhypusine levels in control and treated fungus are still undetermined as yet. The study has shown that known inhibitors of deoxyhypusine synthase can successfully inhibit mycelial growth of *P. avenae* on solid media. Whether these compounds do in fact act by inhibition of the enzyme remains to be seen. Some compounds may act by inhibition of deoxyhypusine synthase and others by disruption of hypusine formation via alternative mechanisms.

Spermidine analogues have demonstrated inhibitory activity towards deoxyhypusine synthase *in vitro*. For example, caldine, otherwise known as norspermidine, affected the enzyme with an IC_{50} value of 41.2 μm (Jakus *et al.* 1993). *In vitro* inhibition of the enzyme by the compound was also observed by Wolff and co-workers (1992) and Park & Wolff (1988). However, N^1-acetylspermidine did not possess inhibitory properties against deoxyhypusine synthase *in vitro* (Jakus *et al.* 1993), nor did it compete with spermidine in hypusine formation (Gerner *et al.* 1986). Clearly, further work in this area would be prudent to determine whether an effect on hypusine formation and/or its biosynthetic enzymes is caused by CHA and norspermidine in *P. avenae*.

Chapter 9

General discussion

9. GENERAL DISCUSSION

This study has shown that CHA and norspermidine possess fungicidal and antifungal activity against a number of plant pathogens grown on host plants and on artificial media. Furthermore, the compounds controlled powdery mildew on barley in the field. Such findings for CHA are in agreement with reported inhibition of *Gaeumannomyces graminis* (West & Walters 1989) and powdery mildew on barley (West & Walters 1988). In addition, growth inhibitory activity has been demonstrated against other organisms such as tumours (Ito *et al.* 1982) and carrot cell cultures (Feirer *et al.* 1985). Norspermidine has been shown to inhibit growth of cultured cells (Sunkara *et al.* 1988), tumours (Prakash *et al.* 1988) and potatoes (Masse *et al.* 1988).

N^1- and N^8-acetylspermidine possessed limited antifungal activity although fungicidal activity was observed against powdery mildew in previous work (S. A. Foster unpublished results). Such findings are in agreement with reports of inhibition of cultured cell growth by the spermidine analogues, N^1,N^8-bis(ethyl)spermidine (BES) and N^1,N^8-bis(propyl)spermidine (BPS) (Porter *et al.* 1985).

In addition, novel spermidine analogues possessed antifungal activity and although some analogues did not affect growth of *P. avenae*, previous work had shown that two of the analogues possessed fungicidal activity against a number of plant pathogens grown on host plants (N. D. Havis unpublished results). Thus, CHA, norspermidine, N^1- and N^8-acetylspermidine and novel spermidine analogues have been shown to provide control of a wide range of plant pathogenic fungi on plants and when grown on artificial media.

Interestingly, there was generally little difference in the relative efficacies of CHA and norspermidine, at comparable concentrations, against plant pathogens on host plants. Norspermidine did appear to affect apple powdery mildew and potato late blight more effectively than CHA, but the differences were small. Against plant pathogenic fungi grown on artificial media however, norspermidine was much more effective than CHA, with lower concentrations required for growth inhibition. The reasons for such differences are not known, but it should be noted that differences do exist between fungi growing on plants and fungi growing on artificial media. On plants, the inhibitors act upon early development of the fungus while on solid media or in liquid culture, fungal mycelium is exposed to the inhibitors. *In vitro* growth may not therefore reflect *in vivo* sensitivity. Also, since biotrophic fungi cannot be grown easily on artificial media, other species of fungi must be used for *in vitro* analyses. Differences may therefore exist in the effects of the inhibitors on the biotrophic fungal species and those fungal species grown in culture.

Although the compounds demonstrated antifungal and fungicidal activity, they did not affect spermidine biosynthesis of *P. avenae* grown in liquid culture. CHA is a known inhibitor of spermidine synthase from a variety of sources including mammalian cells (Hibasami *et al.* 1980), trypanosomes, plants and bacteria (Pegg *et al.* 1983; Pösö *et al.* 1983; Paulin *et al.* 1983; Mattila *et al.* 1984; Sindhu & Cohen 1984). *In vivo* inhibition of spermidine synthase has also been demonstrated in protoplasts from Chinese cabbage leaves (Greenberg & Cohen 1985), while growth inhibition of pathogenic yeasts was associated with polyamine biosynthesis inhibition (Pfaller *et al.* 1988, 1990). The present study is therefore in disagreement with the widely regarded view that CHA is an inhibitor of spermidine synthase.

However, there are a number of reports which indicate that such effects of CHA are not true for every organism. The enzyme from some bacteria was unaffected by the compound (Pösö *et al.* 1983; Mattila *et al.* 1984). CHA growth inhibitory action has also been demonstrated against potatoes on inhibitor amended medium, and organogenesis in tobacco callus culture systems without an effect on spermidine biosynthesis (Tiburcio *et al.* 1987; Masse *et al.* 1988). Similarly, growth of *Agrobacterium tumefaciens* was reduced by CHA, yet was not associated with a reduction in polyamine biosynthesis (Ponappa *et al.* 1992). Pegg & Williams-Ashman (1987) noted how information on the specificity of CHA was lacking. AdoDato is known to be a more specific inhibitor of spermidine synthase (Pegg 1988), although, for the purposes of this study, CHA was used as it is commercially available and inexpensive.

Little is known of the effects on spermidine synthase of norspermidine, N^1- and N^8-acetylspermidine. A norspermidine-induced effect on potato and maize root growth on inhibitor amended media was associated with spermidine biosynthesis inhibition (Masse *et al.* 1985, 1988). However, Sunkara *et al.* (1988) concluded that an effect by the compound on HeLa cell growth in culture was related to mitosis in cells. N^1,N^8-bis(ethyl)spermidine and N^1,N^8-bis(propyl)spermidine were associated with an inhibition of spermidine biosynthesis in cultured L1210 cells (Porter *et al.* 1985). However, one other analogue possessed inhibitory activity yet its mode of action was unclear (Porter *et al.* 1985). Thus it can be seen that while a lack of effect on spermidine synthase by CHA, norspermidine, N^1- and N^8-acetylspermidine and the novel spermidine analogues in this study was somewhat unexpected, such effects are clearly not unusual in other organisms.

Consideration should be given at this point to the difficulties in comparing work in fungal systems to work in related areas. Many examples are available of different effects of the same inhibitor with regards to different organisms, testament to the diversity of organisms.

Examples include the ODC inhibitor RR-MAP which was extremely effective against growth of rat HTC cells (Mamont *et al.* 1984), yet only partially effective against growth of powdery mildew on barley (West & Walters 1988). Also the AdoMetDC inhibitor, EMGBG, failed to affect mouse leukemia cells in culture (Elo *et al.* 1986), yet was effective in reducing mycelial growth of *P. avenae* on solid media (Foster & Walters 1990).

The lack of effect on spermidine synthase, in spite of demonstrating antifungal and fungicidal activity should not be unexpected in view of previous work using putrescine analogues. Such analogues were indeed effective in reducing plant pathogens grown on host plants and in culture. However, varying effects were observed on polyamine biosynthetic enzyme activities and intracellular polyamines in *P. avenae*. The authors concluded that the effects could not wholly account for the observed antifungal and fungicidal activity (Havis *et al.* 1994a, c; Havis *et al.* 1997). Interestingly, some cyclic analogues demonstrated fungicidal activity yet failed to even affect growth of *P. avenae* in culture or polyamine biosynthesis in the fungus (Havis *et al.* 1996a).

Despite not causing an effect on spermidine synthase activity in *P. avenae*, CHA caused an accumulation of putrescine, the cause of which could not be determined. Such an increase in putrescine has also been found in *P. avenae* exposed to E-BED and has been implicated in the antifungal activity of the putrescine analogue (Havis *et al.* 1994a). Although an accumulation of putrescine has been associated with maintenance of membrane integrity (Srivastava & Smith 1982), it is likely that this increase may have lead to destruction of the membrane, as suggested by DiTomaso *et al.* (1989) for maize roots.

Although Walters & McPherson (1997) found that E-BED caused reductions in uptake of amino acids, polyamines and sugars in *P. avenae*, the findings of the present study indicated over-whelmingly that destruction of the biphasic uptake system was caused by exposure of the fungus to CHA or norspermidine. This was thought to be caused by the compounds destabilising the membranes and thus destroying the transport mechanism. Further evidence of the association of polyamine analogues with cell membranes is found in the reported displacement of spermine from intracellular binding sites in *Phytophthora infestans* by E-TED (Havis *et al.* 1996b).

Thus, a prudent area for future work would be the examination of the effect of the compounds on fungal cellular membranes. Ion leakage is a known symptom of membrane damage and conductivity measurements would be a useful aid to determining whether or not membranes were damaged by exposure to the inhibitors. A complicating factor would arise

from efflux of amines from the fungus as a compensatory measure to any fluctuations in the polyamine intracellular pool.

Inhibition of spermidine synthase did not occur in fungus exposed to any of the compounds: CHA, norspermidine, N^1- and N^8-acetylspermidine and the novel spermidine analogues. Only CHA caused an increase in putrescine in *P. avenae*. It should be noted that other perturbations in polyamine metabolism may have occurred following treatment with CHA or the other compounds and yet may have been masked by the many compensatory mechanisms available to cells to respond to fluctuations in the free polyamine pool. However, any undetected changes clearly have little effect on the antifungal or fungicidal activity of the compounds. Thus, alternative modes of action should be sought.

Perturbation of hypusine biosynthesis was shown to be possible in fungal tissue, providing the deoxyhypusine synthase inhibitors tested act against fungi as they do in cultured animal cells. Such inhibition of hypusine synthesis clearly had antifungal effects. The possibility that CHA and the spermidine analogues act by inhibiting hypusine biosynthesis warrants further work.

Many more antiproliferative mechanisms have been suggested as possible functions of polyamine biosynthetic inhibitors and polyamine analogues in various organisms and are thus worthy of consideration for future work in fungal systems. A number of bis(benzyl)polyamine analogues were reported to be potent inhibitors of growth of malaria causing parasites (Bitonti *et al.* 1989). Such analogues were thought to act by binding to DNA and causing disruption of macromolecular synthesis (Bitonti *et al.* 1989). Such a mechanism was originally hypothesised by Porter & Sufrin (1986) when they suggested analogues to be more effective than irreversible enzyme inhibitors.

Depletion of spermidine has been suggested to be associated with the reduction of the trypanosome spermidine-containing enzyme co-factor, trypanothione, which is obligatory for glutathione reductase activity (Fairlamb *et al.* 1985). Porter & Sufrin (1986) hypothesised that analogues may act by inhibiting or regulating polyamine biosynthetic enzyme activity. It is feasible that other enzymatic processes may involve polyamines and that CHA or spermidine analogues may exert their effects on such alternative cellular functions. Indeed, hypusine biosynthesis is one such example of another cellular process involving spermidine. Zarb & Walters (1994a, c) suggested that polyamine biosynthesis inhibitors may be involved in amino acid synthesis in *Paxillus involutus* and *Laccaria proxima*.

Inhibitors of polyamine biosynthesis were implicated in aberrant methylation in trypanosomes (Byers *et al.* 1991). Indeed, exposure of fungus to E-BED inhibited DNA methylation in *P. avenae* (Walters 1997). Many authors have implicated polyamines and methylation with differentiation of fungi, suggesting that polyamines are able to control expression of genes by interfering with the methylation process (Cano *et al.* 1988; Ruiz-Herrera 1994). An inhibitor of polyamine biosynthesis has also been reported to inhibit DNA methylation (Cano *et al.* 1988; Cano-Canchola *et al.* 1992), thus, clearly, this may be an area worthy of future consideration with regards to determining the mode of action of CHA and the spermidine analogues.

Another cellular process in which polyamines are implicated is protein phosphorylation. Phosphorylation is necessary for the activation of some enzymes and polyamines have been implicated in such regulation of enzyme activity. Differing effects are apparent with polyamines stimulating some enzymes (Velutthambi & Poovaiah 1984), yet depressing activity of others (Bothma & Dubery 1991). Little work has examined fungal protein phosphorylation with regards to the effects of polyamine biosynthetic enzyme inhibitors or polyamine analogues, thus, further work in this area may be prudent.

In conclusion, this study has shown that CHA and norspermidine possess the ability to control plant pathogenic infections on host plants under glasshouse conditions and in the field. Furthermore, pathogens grown on and in artificial media were controlled by CHA, norspermidine, N^1- and N^8-acetylspermidine and a number of novel spermidine analogues. Spermidine synthase activity in fungal tissue did not appear to be affected by exposure of the fungus to the compounds. An increase in putrescine was caused by exposure of *P. avenae* to CHA, the cause of which is unknown. It is unlikely that this increase accounted for the observed antifungal activity of CHA. Membrane integrity was drastically affected in fungus exposed to CHA or norspermidine, disrupting the methionine transport system. However, it is not clear whether this effect is primarily involved in the antifungal activity of the compounds or if it is a symptom of another mechanism. Spermidine is involved in another cellular process, hypusine synthesis. *P. avenae* growth has been shown likely to be inhibited by the prevention of hypusine synthesis. The biosynthesis of hypusine is clearly worthy of further investigation in fungal tissue treated with CHA or norspermidine.

Although fungicidal effects of the compounds may differ from antifungal effects, clearly, study of the antifungal mode(s) of action of CHA and the spermidine analogues may be useful in the search for new fungicides with novel modes of action. In spite of plentiful food production in some areas of the world, agricultural productivity must increase to meet rising populations. Crop diseases will continue to reduce potential productivity and

fungicides will remain essential as tools to control such disease. However, fungicide resistance and environmental and toxicological considerations mean that new active ingredients with novel modes of action are required. The antifungal and fungicidal activity exhibited by CHA and spermidine analogues may be useful in the continuing search for novel fungicidal agents.

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Appendices

11. APPENDICES

Appendix 1. Effect of CHA and norspermidine against rust on broad bean

Treatment	Mean percentage of leaf area affected by disease
Control	11.5 ± 1.16
0.5 mM pre CHA	4.5 ± 0.80 ***
1.0 mM pre CHA	3.3 ± 0.77 ***
2.0 mM pre CHA	4.8 ± 0.70 ***
0.5 mM pre norspermidine	4.5 ± 1.20 ***
1.0 mM pre norspermidine	5.5 ± 1.14 ***
2.0 mM pre norspermidine	4.1 ± 0.44 ***
0.5 mM post CHA	7.5 ± 1.25 *
1.0 mM post CHA	8.1 ± 1.54 ns
2.0 mM post CHA	6.7 ± 1.64 *
0.5 mM post norspermidine	4.7 ± 1.06 ***
1.0 mM post norspermidine	6.1 ± 0.76 ***
2.0 mM post norspermidine	4.9 ± 0.66 ***

Note: Significant differences from control are shown as: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

ns no significant difference

Appendix 2. Effect of CHA and norspermidine against chocolate spot on broad bean

Treatment	Mean percentage of leaf area affected by disease
Control	22.9 ± 1.49
0.5 mM pre CHA	17.2 ± 2.37 ns
1.0 mM pre CHA	13.7 ± 1.56 ***
2.0 mM pre CHA	15.4 ± 0.95 ***
0.5 mM pre norspermidine	14.8 ± 2.27 **
1.0 mM pre norspermidine	16.1 ± 0.93 ***
2.0 mM pre norspermidine	16.6 ± 1.71 **
0.5 mM post CHA	20.4 ± 2.06 ns
1.0 mM post CHA	21.4 ± 1.85 ns
2.0 mM post CHA	16.9 ± 0.77 ***
0.5 mM post norspermidine	21.8 ± 1.24 ns
1.0 mM post norspermidine	18.9 ± 1.61 ns
2.0 mM post norspermidine	18.6 ± 1.33 *

Note: Significant differences from control are shown as: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

ns no significant difference

Appendix 3. Effect of CHA and norspermidine against powdery mildew on barley

Treatment	Mean percentage of leaf area affected by disease
Control	58 ± 10.2
0.5 mM pre CHA	11 ± 1.9 *
1.0 mM pre CHA	6 ± 1.9 **
2.0 mM pre CHA	7 ± 0.8 **
0.5 mM pre norspermidine	8 ± 2.0 **
1.0 mM pre norspermidine	9 ± 3.3 **
2.0 mM pre norspermidine	7 ± 2.6 **
0.5 mM post CHA	22 ± 9.4 *
1.0 mM post CHA	15 ± 2.2 *
2.0 mM post CHA	9 ± 3.1 **
0.5 mM post norspermidine	13 ± 3.8 **
1.0 mM post norspermidine	12 ± 4.5 **
2.0 mM post norspermidine	7 ± 1.4 **

Note: Significant differences from control are shown as: * $p \leq 0.05$; ** $p \leq 0.01$

Appendix 4. Effect of varying concentrations of CHA and norspermidine on mycelial growth of *Pyrenophora avenae* on solid media

Treatment	Mean radial mycelial growth (mm) Days of growth		
	3 days	6 days	8 days
Control	12.0 ± 0.26	28.7 ± 0.42	36.2 ± 0.79
0.05 mM CHA	12.2 ± 0.30	29.5 ± 0.44	37.5 ± 0.31
0.1 mM CHA	12.0 ± 0.59	28.8 ± 0.32	37.9 ± 0.26
0.5 mM CHA	11.8 ± 0.64	26.4 ± 0.99	37.0 ± 0.47
1 mM CHA	12.2 ± 0.59	26.1 ± 0.93 *	35.4 ± 1.00
2 mM CHA	3.7 ± 0.22 ***	12.2 ± 1.49 ***	16.2 ± 2.04 ***
0.05 mM norspermidine	12.6 ± 0.31	28.6 ± 0.47	37.6 ± 0.36
0.1 mM norspermidine	2.8 ± 0.31	28.3 ± 0.62	38.0 ± 0.36
0.5 mM norspermidine	10.2 ± 0.45 **	25.3 ± 0.68 ***	3.8 ± 0.68 *
1 mM norspermidine	7.5 ± 0.68 ***	15.8 ± 1.59 ***	21.6 ± 2.39 ***
2 mM norspermidine	4.4 ± 0.15 ***	9.8 ± 0.27 ***	13.3 ± 0.70 ***

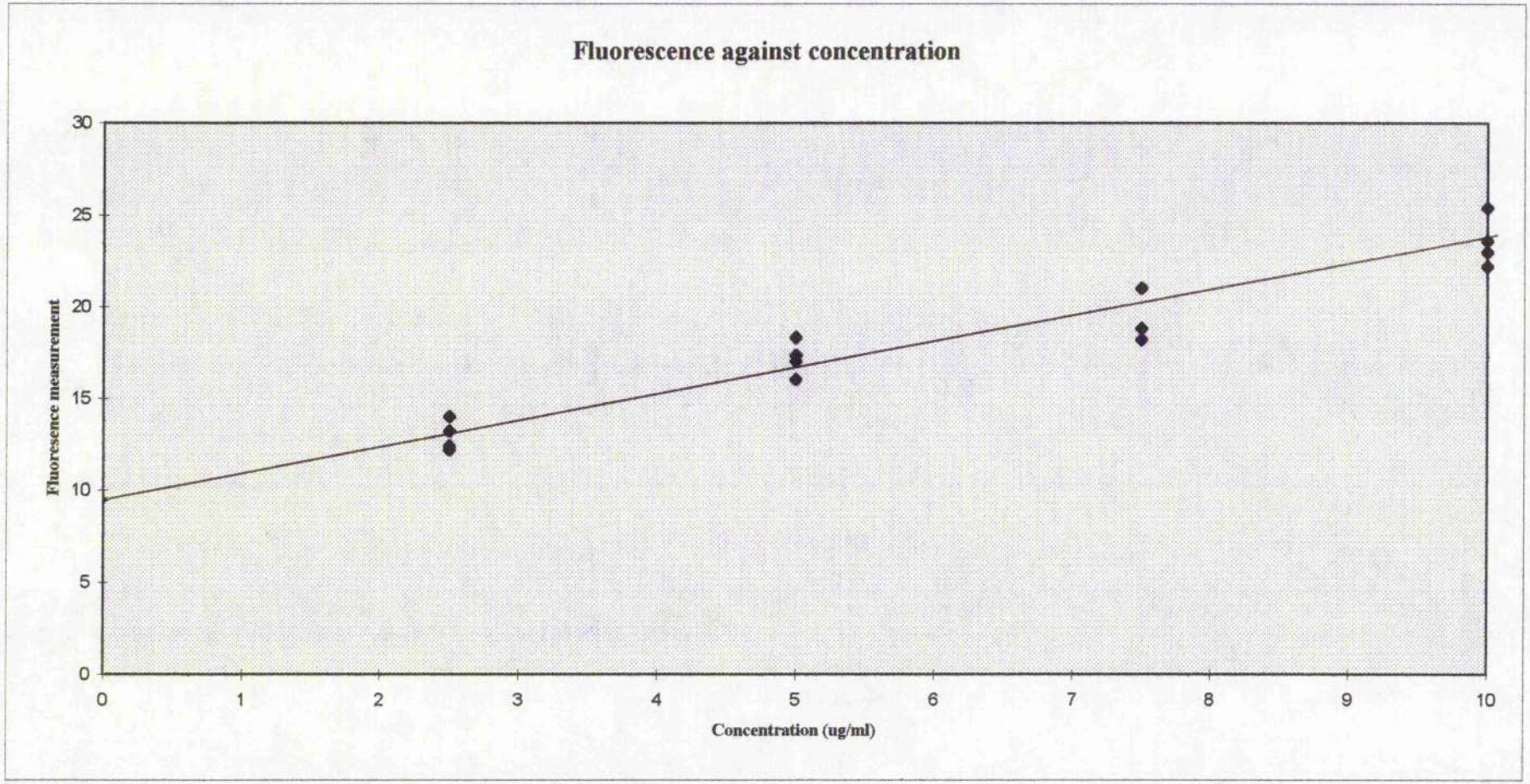
Note: Significant differences from control are shown as: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Appendix 5. Effect of varying concentrations of CHA and norspermidine on mycelial growth of *Pyricularia oryzae* on solid media

Treatment	Mean radial mycelial growth (mm) Days of growth		
	7 days	14 days	21 days
Control	12.7 ± 0.14	27.2 ± 0.37	35.2 ± 0.63
1 mM CHA	11.8 ± 0.11 ***	25.9 ± 0.19 **	35.1 ± 0.40
2 mM CHA	10.7 ± 0.14 ***	22.8 ± 0.30 ***	30.9 ± 0.47 ***
3 mM CHA	7.8 ± 0.34 ***	18.4 ± 0.58 ***	27.4 ± 0.64 ***
4 mM CHA	7.2 ± 0.18 ***	19.4 ± 0.15 ***	28.9 ± 0.36 ***
5 mM CHA	3.6 ± 0.23 ***	14.4 ± 0.36 ***	22.6 ± 0.66 ***
1 mM norspermidine	10.2 ± 0.11 ***	23.8 ± 0.18 ***	33.2 ± 0.37 *
2 mM norspermidine	8.0 ± 0.21 ***	20.4 ± 0.26 ***	29.1 ± 0.42 ***
3 mM norspermidine	4.6 ± 0.15 ***	15.8 ± 0.24***	22.9 ± 0.60 ***
4 mM norspermidine	3.2 ± 0.27 ***	14.1 ± 0.95 ***	23.9 ± 1.12 ***
5 mM norspermidine	0.6 ± 0.15 ***	8.8 ± 1.10 ***	17.4 ± 1.39 ***

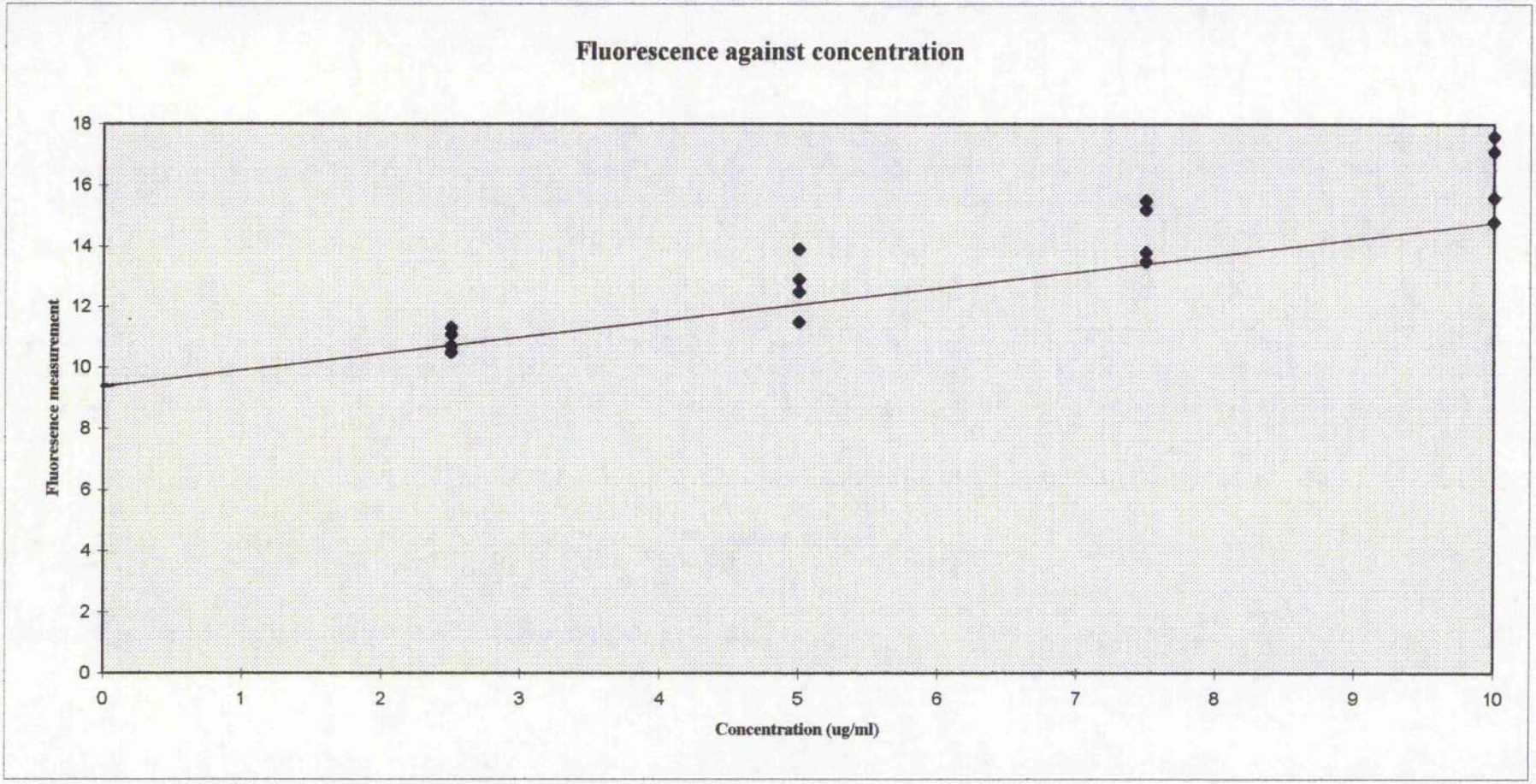
Note: Significant differences from control are shown as: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Appendix 6. N-1- /N-8-acetylspermidine calibration curve



Regression equation is: Fluorescence = 9.75 + 1.35(Concentration), $p < 0.001$

Appendix 7. N-acetylputrescine calibration curve



Regression equation is: Fluorescence = 9.11 + 0.55(Concentration), $p < 0.001$

Appendix 8a. Effect of varying concentrations of novel spermidine analogues of mycelial growth of *Pyrenophora avenae* on solid media.

Treatment	Mean radial mycelial growth (mm) Days of growth		
	3 days	6 days	8 days
Control	11.8 ± 0.68	30.9 ± 0.58	37.2 ± 0.34
0.05 mM C59	10.9 ± 0.31	29.6 ± 0.96	35.4 ± 0.75
0.1 mM C59	10.8 ± 0.32	30.7 ± 0.54	36.9 ± 0.15
0.5 mM C59	12.3 ± 0.38	30.8 ± 0.37	36.9 ± 0.19
1.0 mM C59	12.4 ± 0.23	31.6 ± 0.26	36.8 ± 0.18
2.0 mM C59	12.6 ± 0.19	30.8 ± 0.21	36.8 ± 0.18
0.05 mM C73	11.8 ± 0.24	30.8 ± 0.39	36.7 ± 0.22
0.1 mM C73	11.5 ± 0.26	31.0 ± 0.52	35.8 ± 0.18 **
0.5 mM C73	11.8 ± 0.18	30.9 ± 0.23	37.4 ± 0.19
1.0 mM C73	10.9 ± 0.31	29.2 ± 0.66	34.8 ± 0.27 ***
2.0 mM C73	9.9 ± 0.42 *	28.3 ± 0.80 *	34.1 ± 0.57 ***
0.05 mM LAS 2/11	12.4 ± 0.19	30.8 ± 0.59	35.9 ± 0.31 ***
0.1 mM LAS 2/11	11.0 ± 0.30	30.8 ± 0.47	36.5 ± 0.29
0.5 mM LAS 2/11	11.2 ± 0.60	29.5 ± 0.93	35.4 ± 0.48 **
1.0 mM LAS 2/11	10.4 ± 0.38	28.7 ± 0.29 **	36.0 ± 0.24 *
2.0 mM LAS 2/11	10.1 ± 0.54	28.6 ± 0.38 **	33.9 ± 0.31 ***
0.05 mM LAS 2/12	11.5 ± 0.34	30.1 ± 0.38	36.0 ± 0.21 *
0.1 mM LAS 2/12	11.2 ± 0.22	30.1 ± 0.40	37.1 ± 0.23
0.5 mM LAS 2/12	9.8 ± 0.41 *	29.8 ± 0.34	36.8 ± 0.17
1.0 mM LAS 2/12	10.1 ± 0.15 *	28.6 ± 0.31 **	34.8 ± 0.58 **
2.0 mM LAS 2/12	5.3 ± 0.14 ***	13.9 ± 0.60 ***	19.6 ± 1.46 ***

Note: Significant differences from control are shown as: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Appendix 8b. Effect of varying concentrations of novel spermidine analogues of mycelial growth of *Pyrenophora avenae* on solid media.

Treatment	Mean radial mycelial growth (mm) Days of growth		
	3 days	6 days	8 days
Control	11.7 ± 0.38	31.0 ± 0.78	38.4 ± 0.51
0.05 mM LAS 2/26	12.2 ± 0.28	32.0 ± 0.35	37.8 ± 0.35
0.1 mM LAS 2/26	9.9 ± 0.38 **	28.2 ± 0.98 *	37.7 ± 0.26
0.5 mM LAS 2/26	9.2 ± 0.60 **	25.9 ± 1.12 **	36.2 ± 0.22 **
1.0 mM LAS 2/26	10.7 ± 0.36	29.0 ± 1.08	37.1 ± 0.26 *
2.0 mM LAS 2/26	10.7 ± 0.37	28.8 ± 0.22 *	37.6 ± 0.29
0.05 mM LAS 2/27	12.2 ± 0.28	31.6 ± 0.74	39.1 ± 0.34
0.1 mM LAS 2/27	8.7 ± 0.70 **	26.0 ± 1.22 **	34.9 ± 0.69 ***
0.5 mM LAS 2/27	11.9 ± 0.31	31.5 ± 0.31	37.9 ± 0.15
1.0 mM LAS 2/27	9.2 ± 0.85 *	27.7 ± 1.37	36.9 ± 0.82
2.0 mM LAS 2/27	10.6 ± 0.31 *	30.9 ± 0.36	38.0 ± 0.21
0.05 mM LAS 2/28	10.7 ± 0.56 *	30.2 ± 0.25	37.6 ± 0.23
0.1 mM LAS 2/28	12.2 ± 0.15	31.7 ± 0.41	38.4 ± 0.18
0.5 mM LAS 2/28	11.0 ± 0.37	30.0 ± 0.35	38.2 ± 0.35
1.0 mM LAS 2/28	10.8 ± 0.22	30.0 ± 0.29	37.3 ± 0.17
2.0 mM LAS 2/28	5.6 ± 0.38 ***	17.1 ± 1.20 ***	25.2 ± 1.73 ***
0.05 mM LAS 3/8	10.8 ± 0.28	31.8 ± 0.32	38.5 ± 0.61
0.1 mM LAS 3/8	11.7 ± 0.33	31.8 ± 0.43	38.7 ± 0.41
0.5 mM LAS 3/8	11.6 ± 0.38	31.5 ± 0.44	39.2 ± 0.25
1.0 mM LAS 3/8	11.2 ± 0.21	30.2 ± 0.24	38.8 ± 0.39
2.0 mM LAS 3/8	9.6 ± 0.40 ***	27.8 ± 0.74 **	36.8 ± 0.59

Note: Significant differences from control are shown as: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$