CONTROL OF FUNGAL PLANT PATHOGENS

USING NOVEL PUTRESCINE ANALOGUES

NEIL D HAVIS

H.N.D.A., G.I.Biol.

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY AT THE UNIVERSITY OF GLASGOW

OCTOBER 1993

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

CONTENTS

INDEX OF TABLES

INDEX OF FIGURES

ACKNOWLEDGEMENTS

The author wishes to thank Dr D.R. Walters for his supervision, advice, encouragement and patience during this period of study.

I would also like to extend my thanks to all my colleagues who offered advice and encouragement; Mrs Tracy Cowley and Miss Arlene McPherson for their technical assistance; the Library Staff at SAC Auchincruive; Dr S.A. Foster for useful advice at the beginning of this project; the Hannah Research Institute for the use of the Spectrofluorimeter and Mr Alistair Sword for his advice with the statistical analyses of these results.

I would also like to thank the British Technology Group plc for their funding and Dr Richard A'Court of BTG for advice and guidance; and Merrell-Dow Pharmaceutical Co., Cincinnati, U.S.A for the generous gift of the DFMO.

Finally, I would like to thank my parents for the support and help given throughout all my studies.

Parts of the work in this thesis appear in the following papers:

HAVIS, N.D. and WALTERS, D.R. (1992). Field evaluation of DFMO for control of barley powdery mildew. *Tests of Agrochemicals and Cultivars 13,* 16-17.

WALTERS, D.R., HAVIS, N.D., FOSTER, S.A. and ROBINS, D.J. (1992). Control of fungal diseases of arable crops using inhibitors of polyamine biosynthesis. *Brighton Crop Protection Conference, Pests and Diseases 1992,* 645-650.

HAVIS, N.D., WALTERS, D.R., FOSTER, S.A., MARTIN, W., COOK, F.M. and ROBINS, D.J. (1993). Fungicidal activity of the synthetic putrescine analogue, E-1,4-diaminobutane. *Pesticide Science (In Press).*

HAVIS, N.D., WALTERS, D.R., MARTIN, W., COOK, F.M. and ROBINS, D.J. (1992). Fungicidal activity of three putrescine analogues. *Pesticide Science (In Press).*

Parts of this thesis also appear in the following patent applications:

ROBINS, D.J. and WALTERS, D.R. (1992). Antifungal Diamine. UK Patent Application No. GB 2 256 141 A.

ROBINS, D.J. and WALTERS, D.R. (1992). Antifungal 1,4-diaminobut-2-ene derivatives. UK Patent Application No. GB 2 256 142 A.

SUMMARY

The effects of a number of synthetic, novel putrescine analogues were examined on a range of fungal pathogens both *in vivo* and *in vitro*. The three major compounds studied were E-1,4-Diaminobut-2-ene (E-BED), E-N,N,N',N'-Tetraethyl-1,4-diaminobut-2-ene (TED-1) and 1,2-bis(aminomethyl)-4,5 dimethylcyclohexa-1,4-diene (BAD). Each of these compounds gave significant control of *Erysiphe graminis* f.sp. *hordei* Marchal infection of barley seedlings. Post-inoculation treatments with these compounds were more effective than pre-inoculation treatments, which may be related to a perturbation of polyamine biosynthesis in the germinating conidia on the leaf surface. These three compounds all exhibited xylem mobility within the barley plant and E-BED and TED-1 appeared to possess some phloem mobility. The fate of these compounds within plants is still unclear however. The effects of various salts and derivatives of these three compounds on *Erysiphe graminis* infection of barley seedlings were also examined. These novel compounds gave slightly less control of *Erysiphe graminis* infection than active ingredients used in commercially available fungicides, when compared in the glasshouse environment. This may be due to the higher concentrations and superior formulations of the active ingredients used. In a field scale comparison, E-BED gave good early season control of *E. graminis* infection of barley. This control was comparable to that achieved with the commercial standard used and was superior to that produced by the ornithine decarboxylase (ODC) inhibitor, difluoromethylornithine (DFMO). E-BED also produced significant increases in plant height, plant dry weight and grain weight. Mixtures of these novel compounds gave greater control of *E. graminis* infection than sprays of the compounds alone.

These three compounds produced very different effects on other fungal pathogens *in vivo*. Both E-BED and TED-1 gave significant control of *Uromyces viciae-fabae* and *Botrytis fabae* infection of broad bean seedlings. In contrast, BAD gave no control of *U. viciae-fabae* and only poor control of *B. fabae* infection. E-BED and TED-1 gave

significant control of *Phytophthora infestans* infection of potato leaf discs. However,BAD gave poor control of this pathogen. All of these compounds gave significant control of *Podosphaera leucotricha* infection of apple seedlings.

The compounds were also tested against a range of fungal pathogens *in vitro.* This was achieved by supplementing either solid or liquid growth media with the compounds. E-BED reduced growth of *Botrytis cinerea* significantly and was the only compound to reduce growth of the rice blast fungus, *Pyricularia oryzae,* even though this control was poor. E-BED and TED-1 both gave significant reductions in the growth of the oat leaf stripe pathogen, *Pyrenophora avenae.*

The effect of E-BED and TED-1 on polyamine levels and polyamine biosynthetic enzymes in *P. avenae* was examined. E-BED depleted enzyme activities yet produced a very substantial increase in putrescine levels in the fungus. Spermidine levels were reduced while spermine levels increased. The substantial putrescine pool could have reached toxic levels within the fungus, causing the inhibition of growth observed. In contrast, TED-1 decreased putrescine and spermidine levels within *P. avenae,* while leaving spermine levels unchanged. TED-1 also increased soluble ODC activity within the fungus, but reduced soluble S-adenosylmethionine decarboxylase (AdoMetDC) activity. Further experiments indicated that TED-1's growth inhibition may not be related to binding at intracellular binding sites and effects on membrane integrity.

It seems therefore, that different novel putrescine analogues may possess quite different modes of action.

These results indicate that synthetic putrescine analogues possess considerable fungicidal activity.

SECTION ONE

INTRODUCTION

1.1 POLYAMINES AND THEIR BIOSYNTHESIS

Research into polyamines (PAs) in plants, micro-organisms and fungi has been a steadily growing field over the last twenty years. Although the function of polyamines is still not well understood at the molecular level, there is a great deal of information which attests to the importance of polyamines in cellular function. This information has led to the realisation that the design of inhibitors of the polyamine biosynthetic pathway could produce compounds, which will prove useful weapons in the fight against animal and plant diseases.

1.1.1 Polyamines

Antoni van Leeuwenhoek is credited with the discovery of polyamines, when he observed crystals of the insoluble phosphate salt of the polyamine, spermine in human semen. Although this discovery was made in 1678 it was not until 1926 that the structure of this crystal was elucidated, simultaneously in England and Germany. The structure was confirmed by synthesis and given the name spermine.

Since then a number of naturally occurring polyamines have been described. The group name is a misnomer however, as the compounds are actually aliphatic diamines, triamines and tetraamines. These simple organic compounds have low molecular weights, are soluble in water and are cationic at cellular pH. The most widely distributed members of this group are putrescine (1,4-diaminobutane), spermidine (4 azaoctane-1,8-diamine) and spermine (4,9 diazododecane-1,12-diamine). The structures of these compounds are shown in Table 1.

Table 1 : Chemical structures of some naturally occurring polyamines (from Smith, 1972)

Polyamine	Structure	Occurrence
Putrescine	$NH_2(CH_2)_4NH_2$	Widespread
Spermidine	$NH_2(CH_2)_3NH(CH_2)_4NH_2$	Widespread
Spermine	$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	Widespread

These three compounds are the major polyamines found in eukaryotes, including fungi. Synthesis of spermine is non-existent or rare in bacteria (Tabor and Tabor, 1972). In most fungi spermidine appears to be the predominant polyamine, with spermine and putrescine also being present in yeasts and many filamentous fungi (Stevens and Winther, 1979; Nickerson *et al.*, 1977; Bushnell and Bull, 1974). However in lower eukaryotic slime moulds (Mitchell and Rusch, 1973; North and Murray, 1980) and in *Blastocadiella emersonii* (Menucci *et al.*, 1975) putrescine levels were found to be higher than those of spermidine.

Other less widespread polyamines have been described. Examples of such polyamines are 1,3-diaminopropane (Smith, 1970; North and Turner, 1977), 1,5-diaminopentane and 2-hydroxyputrescine (Dion and Cohen, 1972).

Putrescine, spermidine and spermine occur either as free bases or they may be conjugated. The most usual state in micro-organisms is for the polyamines to be free, although Tabor and Dobbs (1970) and Tabor and Tabor (1975) reported

the existence of amines conjugated as acetyl or glutathionyl derivatives in *Escherichia coli* . Hydroxycinnamic acid amines are widely distributed in plants (Martin-Tanguy *et al.*, 1978; Martin-Tanguy, 1985) and, in some plants, have been associated with leaf emergence and flowering (Cabanne *et al.*, 1981; Martin-Tanguy *et al.*, 1987). Slocum and Galston ,(1985a), suggested that conjugated forms of polyamines may be important in regulating levels of free polyamines.

The basic nature of polyamines, which allows them to attach strongly to anions within the cell (Kashiwagi *et al.*, 1986) and their highly soluble properties has made the study of intracellular distribution of polyamines difficult.

1.1.2 Polyamine Biosynthesis

Mammalian cells, lower eukaryotic cells, including fungi and possibly most protozoa, have only one pathway for the synthesis of polyamines. Plant and bacterial cells may utilise two pathways however. These pathways are illustrated in Figure 1. In mammalian cells putrescine is formed by the decarboxylation of ornithine in a reaction which is catalyzed by the enzyme ornithine decarboxylase (ODC; EC 4.1.1.17). In addition to this pathway, higher plants and bacteria are also able to synthesise putrescine from agmatine, which is formed by the decarboxylation of arginine by the enzyme arginine decarboxylase (ADC; EC 4.1.1.19).

Spermidine and spermine are formed by the subsequent addition of aminopropyl groups to putrescine. The aminopropyl donor groups are formed by the decarboxylation of S-adenosylmethionine by the enzyme S-adenosylmethionine decarboxylase (AdoMetDC; EC 4.1.1.50). Spermidine is then formed from putrescine by the addition of an aminopropyl group in a reaction catalyzed by

the enzyme spermidine synthase (EC 2.5.1.16). Spermine is then formed by the addition of another aminopropyl group in a reaction catalyzed by the enzyme spermine synthase (EC 2.5.1.22) (Stevens and Winther, 1979; Pegg and McCann, 1982; Smith, 1985; Walters, 1989; Tabor and Tabor, 1985). S-adenosylmethionine is also required in ethylene biosynthesis, hence an increase in ethylene biosynthesis will often lead to a reduction in polyamine synthesis and *vice versa* (Bakanashvili *et al.*, 1987). The regulation of senescence in plants is governed by this interaction.

The early work on elucidating these biosynthetic pathways was done on *Escherichia coli* by Tabor and Tabor (1964) and Morris and Pardee (1966). Morris and Fillingame (1974) isolated and purified two forms of ornithine decarboxylase and two forms of arginine decarboxylase. These forms have been described as the biosynthetic and biodegradative decarboxylases, respectively. The biodegradative enzymes were found to be induced in an acidic medium, while under normal conditions the biosynthetic forms dominate. Since this early work, the biodegradative enzymes have been found in only about 1 in 10 *E. coli strains* ,while the presence of biosynthetic ornithine decarboxylase has been confirmed in practically all strains of *E. coli* and also in other unicellular organisms, including the lower ones such as *Physarum polycephalum* (Mitchell and Carter, 1977) and *Euglena gracilia* (Lafarge-Frayssinet *et al.*, 1978). Interestingly while the two forms of ornithine decarboxylase from *E. coli* shared a common affinity for pyridoxal phosphate, the two forms from *Physarum polycephalum* were found to have different affinities. Both ornithine decarboxylase and arginine decarboxylase are pyridoxal phosphate dependant enzymes (McCormick, 1977; Smith, 1979).

Stevens and Stevens (1981) examined regulation of ornithine decarboxylase activity during conidial germination of *Aspergillus nidulans* and found no

evidence of multiple forms of this enzyme. Difficulties in enzyme purification rendered them unable to conclude definitely whether ornithine decarboxylase is degraded or inactivated. Several theories exist regarding the regulation of ornithine decarboxylase, including an interconversion between the enzyme forms (Mitchell and Carter, 1977) and the production of a specific inhibitor (Cannellakis *et al.*, 1979). Ornithine decarboxylase activity is probably controlled by it's synthesis and breakdown and indeed, Stevens and Winther (1979) reported the activity of a non-specific proteinase in conidia of *A. nidulans* after germination had occurred. Holm *et al.*,(1989) showed that the synthesis of ornithine decarboxylase was controlled by polyamines in Ehrlich ascites tumour cells, mainly at the level of translational efficiency. Thus, polyamine deprivation resulted in enhanced ornithine decarboxylase synthesis and enzyme activity, while elevation of intracellular polyamine levels leads to a greatly reduced ornithine decarboxylase synthesis and activity.

Arginine decarboxylase activity was reported in one fungus, *Panus tigrinus* (Uhlemann and Reinbothe, 1977). This arginine decarboxylase activity was believed to be attributable to the biodegradative form. However recently, there have been reports of arginine decarboxylase activity in *Ceratocystis minor* and *Verticillium dahliae* (Khan and Minocha, 1989a), as well as in four other phytopathogenic fungi *Helminthosporium maydis*, *H. carbonum*, *Fusarium oxysporum* and *Ceratocystis ulmi* (Khan and Minocha, 1989b). These workers found little or no ornithine decarboxylase activity in *C. ulmi*. They suggested that the arginine decarboxylase activity measured was due to the presence of the biosynthetic form of the enzyme in the fungi.

There are two rate limiting enzymes in polyamine biosynthesis. The first is ornithine decarboxylase, whilst the second is S-adenosylmethionine decarboxylase (AdoMetDC). AdoMetDC has been purified to homogeneity

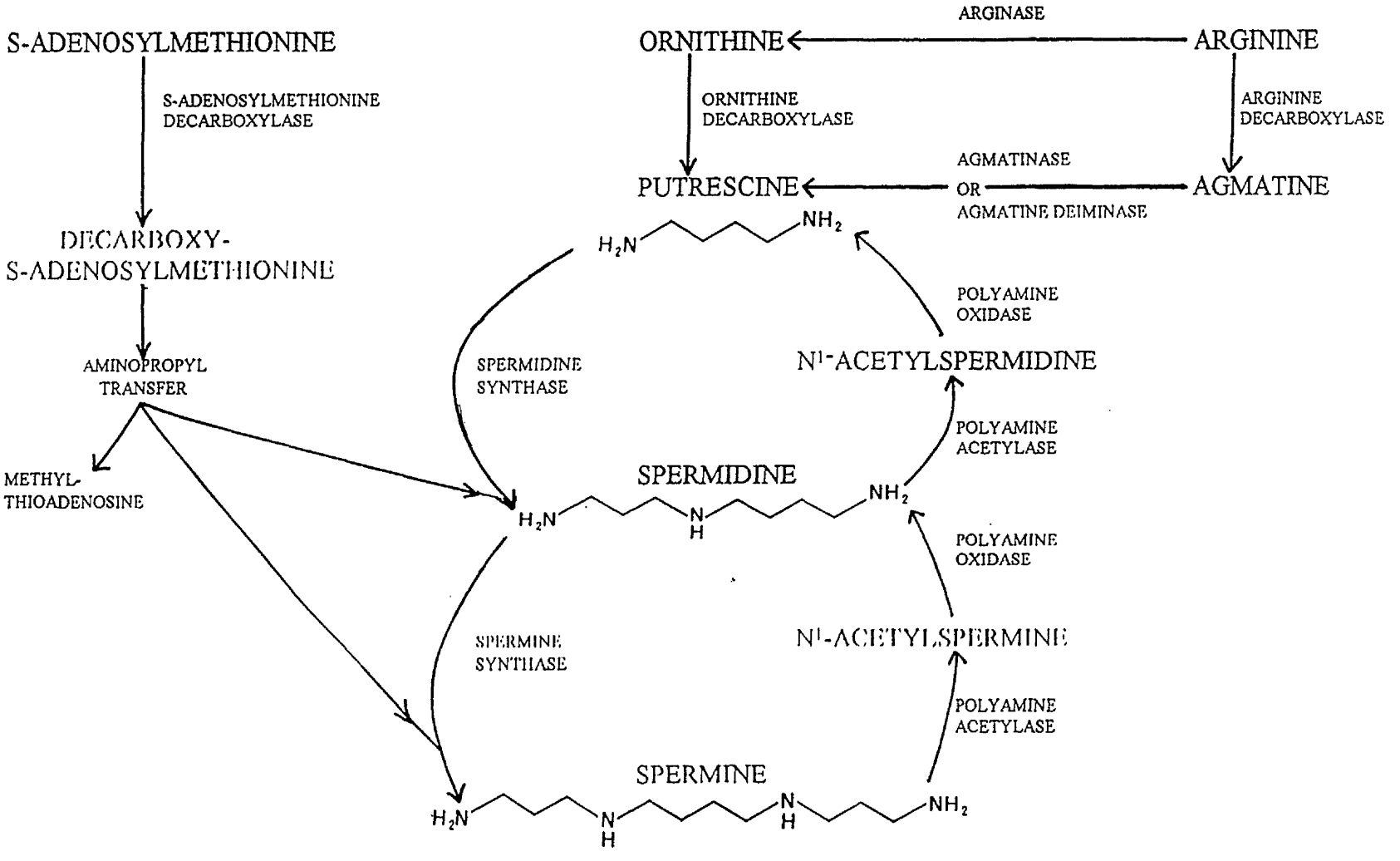


Figure 1. Pathways of Polyamine Metabolism (from McCann, Pegg and Sjoerdsma, 1987).

from *E. coli*, yeast, mammalian tissues and to a lesser extent from a variety of sources, including plants and *Physarum polycephalum* (Williams-Ashman and Pegg, 1981; Pegg, 1984; Tabor and Tabor, 1984; Yamanoha and Cohen, 1985). These enzymes were found to differ in their structure and cation requirement. S-adenosylmethionine decarboxylase from *E. coli* was found to have a requirement for Mg^{2+} for maximum activity and was insensitive to putrescine. Conversely, mammalian AdoMetDC is strongly activated by putrescine and is unaffected by Mg^{2+}. The enzyme purified from *Saccharomyces cerevisiae* (Pösö *et al.*, 1975) was found to have similar properties to mammalian AdoMetDC. The one common feature found in AdoMetDC preparations so far examined has been the presence of pyruvate as a covalently bound prosthetic group. S-adenosylmethionine levels have been shown to be regulated by the intracellular concentration of spermidine (Pegg *et al.*, 1982; Wagner *et al.*, 1982; Mamont *et al.*, 1982). When spermidine content is high, AdoMetDC is repressed and *vice versa*.

Spermidine synthase is the enzyme which converts putrescine to spermidine. This aminopropyltransferase has been purified from *E. coli* (Bowman *et al.*, 1973), bovine brain and rat prostrate (Raina *et al.*, 1984) and from pig liver (Yamanoha *et al.*, 1984). Spermidine synthase is not thought to be a rate limiting enzyme. Spermine synthase has also been purified from bovine brain (Pajula *et al.*, 1979; Pajula, 1983). Unlike spermidine synthase it is not inhibited by an excess of the substrate, decarboxylated S-adenosylmethionine (Pajula, 1983), but is very strongly inhibited by the product, methyl-thioadenosine (Pajula and Raina, 1979; Pajula *et al.*, 1979). Spermidine synthase has also been purified in extracts from *Lathyrus sativus* (Suresh and Adiga, 1977). Although the reactions of these two enzymes are not reversible putrescine can be formed from spermine and spermidine via the enzymes spermidine-N^1-acetyltransferase and polyamine oxidase (PAO; EC 1.4.3.4)

(Pegg *et al.*, 1981). Polyamine oxidase has been reported in the Gramineae (Smith, 1985), in *Medicago sativa* L. (alfalfa) (Bagga *et al.*, 1991) and in a range of fungal species (Yamada *et al.*, 1980). In plants, polyamine oxidases appear to be localised in cell walls e.g. of oat leaves (Kaur-Sawhney *et al.*, 1981) and maize (Torrigiani *et al.*, 1988).

Putrescine can be oxidised by the enzyme diamine oxidase (DAO, EC 1.4.3.6). Diamine oxidases are found in most organisms but occur only in selected tissues (Shaff and Beaven, 1976). In plants a copper containing diamine oxidase is particularly active in the *Leguminosae* (Smith, 1980). Torrigiani *et al.* (1989) demonstrated diamine oxidase activity in *Helianthus tuberosus* and suggested that DAO has a role in regulating the cellular level of putrescine and thus of polyamines.

1.2 FUNCTIONS OF POLYAMINES

Owing to their ubiquitous nature, polyamines have an important role to play in the functioning of all cells. These roles merit closer examination.

1.2.1 Associations with Nucleic Acids

Polyamines have an important association with nucleic acids, largely due to the protonated nature of polyamines and the negatively charged phosphate groups of nucleic acids. Polyamines have been found in nucleic acids isolated from viral, bacterial and mammalian cells. In cell free systems, polyamines are capable of precipitating nucleic acids, increasing their melting point, protecting against enzymatic degradation, x-irradiation and mechanical shearing. Polyamines have been shown to promote ribosomal subunit aggregation and protect against dissociation (Bachrach, 1973; Cohen, 1971; Stevens, 1970;

Tabor and Tabor, 1972; Tabor and Tabor, 1976). Liquori *et al.* (1967) used X-ray techniques to suggest possible site-specific models in which the polyamines might stabilise the DNA double helix (Figure 2). Bloomfield and Wilson (1981) put forward the theory of counterion condensation. In their model, DNA has a linear distribution of negative charge and polyamines act as points of positive charge without a specific site of interaction. Marton and Morris (1987) argued that both non-specific and specific interactions between polyamines and DNA are of importance.

1.2.1.1 DNA

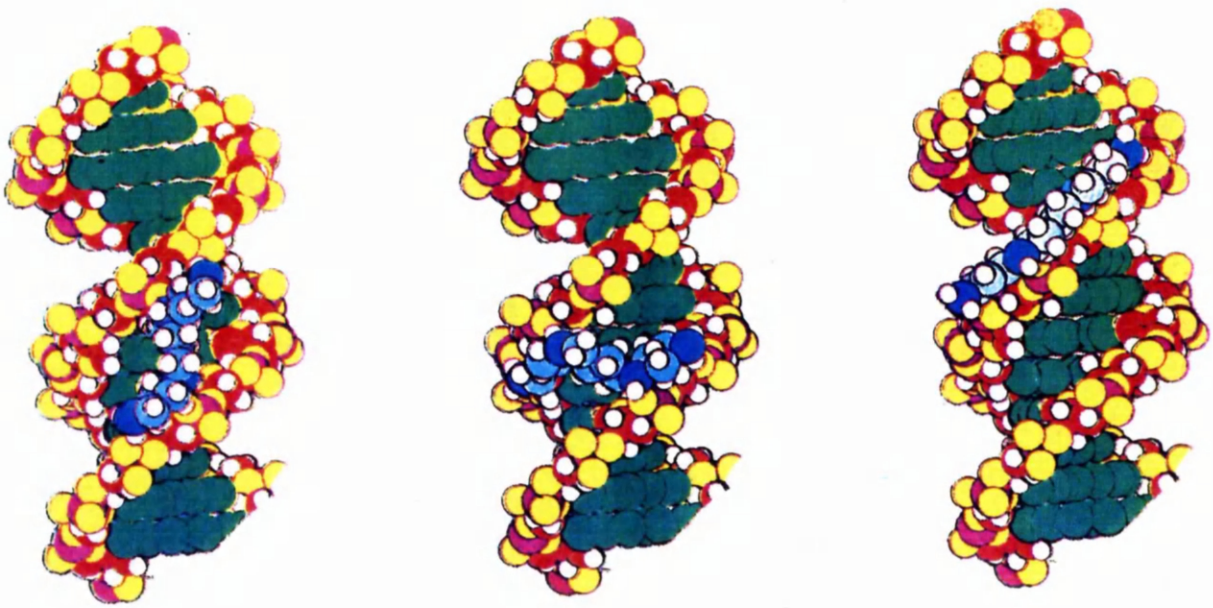
Polyamines have been shown to affect rates of DNA synthesis in the bacterium, *E. coli* (Dion and Cohen, 1972). Work on polyamine starved cells of *E. coli* by Geiger and Morris, (1978; 1980) showed a slowing of the rate of replication fork movement. No evidence was found for an effect of polyamines on initiation of chromosome replication. These observations led to them to suggest that in bacteria polyamines may act as cofactors for DNA replication rather than as regulatory molecules.

In mammalian cells it was observed that appropriate polyamine levels were necessary for DNA replication but not for entry into S phase (Morris *et al.*, 1977; Seyfried and Morris, 1979). Spermidine has been described as requirement for DNA synthesis in eukaryotes (Porter, 1983).

1.2.1.2 RNA

Marton and Morris (1987) showed that specific transfer RNAs (tRNAs) exhibit different stoichiometry and nature of binding to polyamines. Spermidine stabilises the secondary and tertiary structures of tRNA (Tropp and Redfield,

Figure 2. Probable configurations of the spermine-DNA complex (Liquori *et al.*, 1967). The coloured molecules are as follows; Dark blue, spermine nitrogen; light blue, spermine carbon; purple, DNA phosphorous; yellow, DNA oxygen; red, DNA carbon; green, DNA bases; white, hydrogen.



1983) and spermine enhances the binding of manganese to tRNA (Nöthig-Laslo *et al.*, 1985). Cohen and Greenberg (1981) observed that spermidine is a component of plant viral RNA cores.

1.2.1.3 Ribosomes

Cohen and Lichtenstein (1960) were the first workers to show that ribosomes extracted from whole cells contain polyamines. Polyamines and Mg^{2+} are involved in a complex relationship based on competition for binding sites on ribosomes (Choi and Carr, 1967 ; cited by Marton and Morris, 1987). Replacement of Mg^{2+} with polyamines at critical sites can lead to an unfolding of the ribosomal particles with subsequent inactivation. This inactivation is possibly due to the role of a steric factor for cations in maintenance of ribosomal tertiary structure (Weiss *et al.*, 1973). Coccuci and Bagni (1968) (cited by Smith, 1972) showed putrescine and spermidine can stimulate ribosomal activity in *Helianthus tuberosos* L.

1.2.2 Regulation of Cell Membrane Properties

The cationic nature of polyamines which leads them to associate with nucleic acids, also allows them to interact with the phospholipid groups of membranes. Early work showed that spermine could stabilise osmotically fragile bacteria and bacterial protoplasts, but it was Mager (1962) who first showed that membrane stabilisation was due to an interaction involving the cell membrane and polyamines.

Tabor and Tabor (1964) compared the concentrations of sucrose and spermine needed to protect protoplasts from lysis. A concentration of 0.5M sucrose was used compared with 1.0 mM of spermine. They concluded that spermine does

not act through the osmotic mechanism proposed for sucrose but rather it forms complexes with the acidic groups in the cell membrane. These complexes reduce repulsive forces without diminishing cohesive forces thereby stabilising the membrane.

Mammalian membranes are also stabilised by polyamines. Little (1962) showed that the delayed haemolysis of reticulocytes, which occurs when they are suspended in a saline solution, can be partially inhibited by spermidine. Chun *et al.* (1976) found that humans with sickle cell anaemia showed elevated blood polyamine levels and that polyamines affected the electrokinetic properties of the membrane glycoproteins. Ballas *et al.* (1983) showed that polyamines decreased erythrocyte deformability and stabilised the membrane skeleton, reducing the risk of fragmentation.

Polyamines have also been shown to interact with membranes in plants. Spermine was shown to reduce the leakage of ions from plant suspension cultures (Srivastava and Smith, 1982) and betacyanin from wounded beet storage tissues (Naik and Srivastava, 1978 ; Altman, 1982). Altman *et al.* (1977) showed that spermidine and spermine have a stabilising influence on oat protoplasts in relation to lysis. It has been suggested that polyamines may interfere with plant hormone responses at the membrane either by competing for binding sites or by counteracting the changes in permeability induced by hormones (Naik *et al.*, 1980 ; Kyriakidis, 1983).

1.2.3 Effect on Protein Synthesis

Polyamine deficient mutants of *Escherichia coli* were used in the work that showed polyamines influence protein synthesis. Morris and Jorstad (1973) showed that growth of these mutants in the absence of polyamines depressed the

rates of protein synthesis and the rate of growth to the same extent. In addition to this, Young and Srinivasan (1972) found that the addition of putrescine resulted in an almost immediate increase of protein synthesis. This increase preceded changes in the rate of both DNA and RNA synthesis.

In studies of cell free systems from mammalian and plant sources, polyamines were found to stimulate the synthesis of high molecular weight proteins rather than low molecular weight proteins (Atkins *et al.,* 1975 ; Takemoto *et al.,* 1983). It has been suggested that these results indicate a premature termination of polypeptide elongation in the absence of polyamines. Konecki *et al.* (1975) looked at protein synthesis in isolated, ionic buffer washed, ribosomes. They found that there was a requirement for polyamines for maximum protein synthesis. Spermine was found to give the maximum stimulation but it was also inhibitory at higher concentrations well within the normal physiological range. Spermidine and putrescine on the other hand were only inhibitory at concentrations outwith the normal physiological range.

1.2.4 Differentiation, Growth and Development

The discovery that polyamines stimulate growth in micro-organisms and in animal and plant tissues followed the pioneering work of Herbst and Snell (1948) who showed that putrescine was essential for the growth of *Haemophilus parainfluenzae*. Their initial finding led to a great number of experiments to determine the effects polyamines have on growth and development.

An absolute requirement of polyamines for growth has been shown in *Neurospora crassa* (Deters *et al.*, 1974) and *Aspergillus nidulans* (Sneath, 1955). Trinci (1969) described a high growth rate in *A. nidulans* during

germination and this prompted Stevens *et al.* (1978) to examine enzyme activities in this fungi during this period. They found that both ornithine decarboxylase and S-adenosylmethionine decarboxylase activities were increased over a hundred-fold during germ tube emergence. Increased ODC activities have also been observed during germination of *Neurospora crassa* (Sikora and McDougall, 1978), *Physarum polycephalum* (Mitchell and Rusch, 1973) and *Saccharomyces cerevisiae* (Brawley and Ferro, 1979).

In initial studies in animal tissues a clear correlation was demonstrated between polyamine concentrations, protein and RNA content in developing chick embryos (Raina, 1963 ; Caldarera *et al.*, 1965). ODC and AdoMetDC activities were also shown to peak during foetal development in mammals (Snyder *et al.*, 1970 ; Russell, 1970). Fozard *et al.* (1980) reported that inhibition of ODC by DFMO during mammalian embryogenesis resulted in the arrest of embryonic development. There is also ample evidence to suggest an important role for polyamines in mammalian cell differentiation. Oka *et al.* (1981) demonstrated that spermidine is an essential requirement for milk protein synthesis, a differentiated function in mammary glands. They also demonstrated the requirement for increased putrescine synthesis for mammary cell growth. On the other hand, polyamines have also been reported to have an inhibitory effect on mammalian cell differentiation (Sunkara and Prakash, 1984).

In plants, polyamine biosynthesis and concentration have usually been found to peak in the more active areas of cell division (Bagni and Serafini-Fracassini, 1979), although some workers have found this not always to be the case (Palavan *et al.*, 1984 ; Felix and Harr, 1987). Feirer *et al.* (1984) showed that in carrot cell cultures there is a putrescine requirement for the transition from disorganised growth into somatic embryogenesis. However, work done by Meijer and Simmonds (1988) on somatic embryogenesis in two alfalfa lines

indicated that the requirement for polyamines in initiation of somatic embryogenesis may not be universal.

In whole plant systems, Jarvis *et al.* (1985) found evidence for the requirement of polyamines for root initiation and early growth in *Phaseolus.* Kakkar and Rai (1987) later showed that spermine is associated with enhanced rooting and that spermidine in combination with indoleacetic acid increased carbohydrate content. Similar findings have been made in mung beans (Chatterjee *et al.*, 1983 ; Shyr and Kao, 1985) and apple (Wang and Faust, 1986). Kaur-Sawhney *et al.* (1988) found dramatic differences between vegetative and floral-determined tissues cultured *in vitro.* Cytokinin levels were 10 times higher in the vegetative tissue, whilst spermidine levels were 4.5-fold higher in explants on floral bud medium. The application of exogenous spermidine decreased vegetative bud growth and induced the appearance of floral buds in vegetative medium. However polyamine levels were much higher than those of cytokinin and auxin in the medium and the authors suggested that polyamines may possibly be masking cytokinin effects.

High ornithine decarboxylase levels have been reported in tomato ovaries (Heimer and Mizrahi, 1982 ; Heimer *et al.*, 1979) and in tobacco ovaries (Slocum and Galston, 1985b). High free and bound polyamine levels have also been discovered in early periods of fruit growth of apple (Biasi *et al.*, 1988), mandarin (Nathan *et al.*, 1984), avocado (Applebaum, 1986) and tobacco (Slocum and Galston, 1985b). Application of putrescine to flowering crops has been shown to increase fruit set in apples (Costa and Bagni, 1983) and olives (Rugini and Mencuccini, 1985). Prakash *et al.* (1988) demonstrated that pollen tube growth in *Catharanthus* is sensitive to spermidine levels *in vitro.* Promotive effects were shown at 0.01 mM but inhibitory effects appeared at 0.1 mM.

Polyamines also play less well known roles within cells, such as metabolic buffering of pH in plants exposed to aerial pollution (Priebe *et al.*, 1978) or to acid stress (Young and Galston, 1983). Polyamines have also demonstrated a role in plant responses to osmotic stress (Slocum *et al.*, 1984), chilling stress (Guye *et al.*, 1986) and in fungal-infected plants (Walters, 1989). Kaur-Sawhney and Galston (1979) also proposed a role for polyamines in plant senescence regulation, since it is well known that elevated polyamine levels normally accompany tissue juvenility and the addition of exogenous polyamines to excised tissue delays senescence. These workers suggested that the senescence-delaying effects of polyamines may be due to inhibition of protease and RNase activity and to decreased ethylene biosynthesis. Indeed, polyamines have been shown to inhibit the production of ethylene in various plant senescing systems (Kushad and Dumbroff, 1991).

1.3 INHIBITION OF POLYAMINE BIOSYNTHESIS

Inhibition of polyamine biosynthesis by specific inhibitors has allowed the elucidation of the roles polyamines play in living cells. The vast majority of research has centred on mammalian systems This is a result of the intensive research into the possible anti-cancer value of polyamine biosynthesis inhibitors. In comparison, research into the mechanisms of these inhibitors in plants and fungi is still in it's early stages. However this field of research is rapidly expanding as the antifungal properties of these compounds are tested against a range of agriculturally important pathogens.

In general, these inhibitors have been classed into three categories :

a) Competitive inhibitors, which are usually analogues of the substrate or product of an enzyme catalyzed reaction

b) Antagonists of pyridoxal phosphate - molecules which interact with the cofactor

c) Specific pathway enzyme inhibitors e.g. compounds which act against amino acid decarboxylases, S-adenosylmethionine decarboxylase and spermidine and spermine synthases.

Only the inhibitors in class c) are irreversible

1.3.1 Inhibitors of Ornithine Decarboxylase

Since ornithine decarboxylase is one of the rate limiting enzymes in polyamine biosynthesis, it is one of the most obvious points of attack, in terms of inhibitor design.

1.3.1.1 Substrate Based Inhibitors

One of the earliest competitive inhibitors of ODC synthesised was α-methylornithine (Russell, 1973). Since then a number of competitive ornithine analogues have been synthesised and their activity tested *in vitro*. None of these compounds was found to be more potent than α-methylornithine (Bey *et al.*, 1987).

L-canaline is an ornithine analogue which inhibits ODC by forming a Schiff base with the cofactor of ODC, pyridoxal phosphate, leading to an inhibitory effect (Rahiala *et al.*, 1971). The α-hydrazino analogue of ornithine has also been shown to be a potent inhibitor of *E. coli* and rat prostrate ODC (Johanssen *et al.*, 1973; Harik and Snyder, 1974). However, like α-methylornithine, α-hydrazinoornithine has proved disappointing in *in vivo* experiments. Neither proved potent enough to significantly decrease spermidine concentration or selective enough to be free of undesirable biological effects (Hölttä *et al.*,

1981). In addition both increased the half life of ODC, leading to an accumulation of ODC (Harik *et al.*, 1974; McCann *et al.*, 1977).

In a bid to overcome these disadvantages irreversible inhibitors of ODC were designed. This effort was closely associated with the development of the concept of substrate-induced irreversible inhibition of enzymes (Abeles and Maycock, 1976). The α-difluoromethyl analogue of ornithine (DFMO) was one of the earliest synthesised irreversible inhibitors (Bey, 1978; Metcalf *et al.*, 1978) and with over 500 published reports on it's biochemical effects on a range of systems, from cultured cells to human diseases, easily the most studied. Originally it was synthesised by Merrell-Dow Pharmaceuticals (Cincinnati) as an anticancer drug. DFMO acts by binding to ornithine decarboxylase forming an aldimine between the aldehyde of the pyridoxal phosphate and the α-amino group of DFMO. DFMO then undergoes decarboxylation and the enzyme is subsequently inactivated due to the alkylation of a nucleophilic residue at the active site. No enzyme activity was found even after extensive dialysis and hence DFMO was classed an irreversible inhibitor.

DFMO has been shown to deplete cellular polyamine levels, but this process is incomplete as spermine is not eliminated. As a result, DFMO produces cytostatic rather than cytotoxic effects in mammalian systems. However in systems where it can eliminate spermine DFMO can exhibit cytotoxic effects (Sunkara *et al.*, 1983). This has been observed in typanosomes, where ODC activity, DNA and RNA synthesis and cell proliferation are inhibited (Bacchi *et al.*, 1987). The passive system utilised by DFMO to enter animal cells means that high intracellular concentrations have to be used in order to build up reasonable intracellular doses (Erwin and Pegg, 1982).

Other ornithine analogues have been synthesised as irreversible inhibitors. Both α-monofluoromethylornithine (Bey, 1978; Kollonitsch *et al.*, 1978) and α-ethylornithine (Danzin *et al.*, 1981) have been shown to decrease ODC activity in rat tissues. (E)-2-(fluoromethyl)dehydroornithine has been found to be an enzyme activated inhibitor of ODC 10 times more potent than DFMO *in vitro* (Bey *et al.*, 1983). However, this compound did not prove more efficient than DFMO at decreasing ODC activity in rat tissues (Mamont *et al.*, 1986).

1.3.1.2 Product Based Inhibitors

Putrescine analogues have been used to inhibit ornithine decarboxylase, since the discovery that putrescine and other diamines suppress ODC activity (McCann, 1980; Heby and Jänne, 1981). This suppression is believed to be the result of a polyamine-elicited induction of a protein called 'antizyme', which forms an inactive enzyme-antizyme complex with ODC protein (Heller *et al.*, 1976; Cannellakis *et al.*, 1979; Seely and Pegg, 1983). Other analogues have been used to reduce ODC activity and polyamine levels in animal tissues. Examples of these analogues include, 1,3-diaminopropane, 1,5-diaminopentane and 1-6 diaminohexane (Danzin and Mamont, 1987). However doubts have been expressed about the value of these effects as these compounds are subject to rapid metabolism and can fulfil the requirement for endogenous polyamines in polyamine deprived systems (Hölttä *et al.*, 1981). As well as lacking a specific point of action these diamines exhibit significant toxicity.

Competitive inhibitors of ODC have been synthesised. Both DL-α-hydrazino-∂-aminovaleric acid (DL-HAVA) and diaminobutene were found to be potent competitive inhibitors of ODC, block putrescine formation for several hours and produce significant reductions in putrescine and spermidine concentrations in animal tissues (Inoue *et al.*, 1975; Kato *et al.*, 1978). However both compounds

have drawbacks. Diaminobutene was shown to be metabolised to the unsaturated spermidine analogue and may fulfil the role of the natural polyamine (Kameji *et al.*, 1979). Despite this it was shown to be a potent inhibitor of diamine oxidase (Relyea and Rando, 1975). DL-HAVA exhibited inhibitory activity against other pyridoxal-phosphate dependant enzymes (Inoue *et al.*, 1975), as does 1-aminoxy-3-propane, a new competitive inhibitor of ODC *in vitro* (Khomutov *et al.*, 1985). As with ornithine based competitive inhibitors, these compounds have the major disadvantages of stabilising ODC (McCann *et al.*, 1977; Jänne *et al.*, 1981) and increasing enzyme protein (Persson *et al.*, 1985).

Enzyme activated irreversible inhibitors based on putrescine have also been synthesised. 5-Hexyne-1,4-diamine, or acetylenic putrescine was discovered at the same time as DFMO (Metcalf *et al.*, 1978; Danzin *et al.*, 1979a) and proved to be a potent inhibitor of mammalian ODC. However this compound was found to inhibit 4-aminobutyrate aminotransferase *in vivo* and caused secondary pharmacological effects in brain tissue (Danzin *et al.*, 1979b). Casara *et al.* (1985) synthesised 6 heptyne-2,5-diamine and found that *in vitro* most of the ODC inhibitory activity lay in the (2R, 5R) stereoisomer. This compound, also known as MAP (for methyl acetylenic putrescine), was found to be 10 times more potent than DFMO *in vivo*. It was also shown to be as organ specific as ornithine analogues and chemically stable. Two other putrescine analogues that irreversibly inhibit ODC *in vivo* are α-monofluoromethylputrescine and α-difluoromethylputrescine. Neither of these were more potent than MAP and unfortunately both were oxidised to their acids *in vivo* and inhibited GABA-T (Danzin *et al.*, 1982). Two dehydroputrescine analogues, *trans*-hex-2-ene-5-yne-1,4diamine (Metcalf *et al.*, 1978) and (E)-2-(fluoromethyl) dehydroputrescine (Bey *et al.*, 1983) were shown to be efficient inhibitors of

ODC *in vivo*, although toxicity problems with these compounds in animals has limited further investigations.

1.3.1.3 Other Inhibitors

As well as the diamine, putrescine, the triamine spermidine has been shown to regulate the activity of ornithine decarboxylase in mouse fibroblasts through translation control (Clark and Fuller, 1975). Holm *et al.* (1989) showed that spermidine alone had a regulatory effect on ODC synthesis in Ehrlich ascites-tumour cells. They found a marked inhibition even by exposure to micromolar spermidine concentrations. Porter *et al.* (1987) discovered that the spermidine analogue N^1N^8-bis(ethyl)spermine can also suppress ODC activity in L1210 cells. This compound has also been shown to suppress ODC activity in mouse cell lines, as did the analogue N^1,N^{14}-bis(ethyl)homospermine (Ghoda *et al.*, 1992). A bisbenzyl polyamine analogue MDL 27695 repressed ODC activity in rat hepatoma cells (Bitonti *et al.*, 1989). At present, a combination of mechanisms are believed to be responsible for the activity of spermidine and it's analogues. It is thought that a post translational modification, via antizyme binding to ODC, contributes to an enhanced degradation of the enzyme protein

1.3.2 Inhibitors of S-adenosylmethionine Decarboxylase

S-adenosylmethionine decarboxylase (AdoMetDC) is a key enzyme in the biosynthesis of polyamines since it is involved in the rate limiting step in the formation of spermidine and spermine. The activity of AdoMetDC is known to be negatively regulated by these two polyamines and positively regulated by their precursor putrescine (Pegg *et al.*, 1988). After ornithine decarboxylase, this enzyme is the next most obvious point of attack for polyamine biosynthesis inhibitors.

1.3.2.1 Substrate Based Inhibitors

A number of analogues of S-adenosylmethionine have been synthesised to date and their activity tested. Only two were found to be potent inhibitors of AdoMetDC *in vitro*. These are S-adenosyl-3-methylthiopropylamine and 5'-(di-methylsufonio)-5'-deoxyadenosine. The K_i values for these compounds against AdoMetDC isolated from *Saccharomyces cerevisae* and *Escherichia coli* range from 7.0-13.0μM. So far, no work has been carried out on these compounds *in vivo* (Pegg and Jacobs, 1983). Bey *et al.* (1978) reported the synthesis of the α-difluoromethyl derivative of AdoMet but, unfortunately, this compound appeared to have no inhibitory activity against AdoMetDC (Kolb *et al.*, 1982).

1.3.2.2 Product Based Inhibitors

It is well known that AdoMetDC is quite strongly inhibited by it's product, decarboxylated AdoMet (Pegg and Williams-Ashman, 1987). In the last few years, much work has been carried out in the search for an irreversible inhibitor of S-adenosylmethionine decarboxylase. Kramer *et al.* (1989) described the effect of an irreversible inhibitor, S-(5'-Deoxy-5'-adenosyl)-methylthioethylhydroxylamine (AMA), an analogue of decarboxylated AdoMet. Against L1210 leukaemia cells, a 100μM concentration of AMA reduced growth by 50%. This inhibitor is believed to act by the reaction of it's hydroxylamine moiety with the pyruvate residue of AdoMetDC.

1.3.2.3 Other Inhibitors

The most studied inhibitors of S-adenosylmethionine belong to a group of compounds called the diguanidines. The best studied members of this group are

methylgloxal bis (guanylhydrazone) (MGBG), ethylglyoxal bis (guanylhydrazone) (EGBG) and ethylmethylglyoxal bis (guanylhydrazone)sulphate (EMGBG). MGBG was synthesised in the early 1960's but it's reversible inhibition of AdoMetDC was not described until 10 years later (Corti *et al.*, 1974; Hölttä *et al.*, 1973). MGBG was shown to be most effective against putrescine activated mammalian AdoMetDC with a K_i of less than 1μM (Williams-Ashman and Pegg, 1981; Williams-Ashman and Seidenfield, 1986). It was also shown to affect Mg^{2+} activated AdoMetDC and AdoMetDC which did not require putrescine or metal cations (Pegg, 1984; Pegg and Jacobs, 1983). MGBG reduces cellular contents of spermidine and spermine, although it is still considered non specific, as it's antiproliferative effects may be due to inhibition of other processes. There is evidence that MGBG can damage mitochondria and inhibit DNA synthesis within the organelle (Feverstein *et al.*, 1979) and affect lipid metabolism by inhibiting fatty acid oxidation (Nikula *et al.*, 1984).

MGBG can also inhibit diamine oxidase activity, interfere with polyamine transport, and induce spermidine/spermine-N^1-acetyltransferase (Porter *et al.*, 1981; Williams-Ashman and Seidenfield, 1986; Pegg, 1986). These effects can cause toxicological problems, such as an accumulation of putrescine to toxic levels in cancer patients treated with the drug (Hölttä *et al.*, 1973). Pretreatment of cells with DFMO can overcome this problem. One advantage that MGBG possesses is that it is actively transported into mammalian cells (Porter *et al.*, 1981) and can reach very high intracellular concentrations.

EGBG, the ethyl analogue of MGBG, was found to be a superior inhibitor of S-adenosylmethionine decarboxylase than MGBG, although it's effect on mitochondrial activity has not been fully elucidated. EMGBG is another analogue of MGBG which was found to be an extremely powerful competitive

inhibitor of eukaryotic AdoMetDC with an apparent K_i value of 12ηM. Like EGBG it did not inhibit the proliferation of mouse L1210 leukaemia cells *in vitro* (Elo *et al.*, 1986).

Two derivatives of MGBG, 4,4'-diaminidinodiazoaminobenzene; diaminazine aceturate (Berenil) and p,p'-(pentamethylenedioxy) dibenzamidine; pentamidine methionate (pentamidine) have been shown to inhibit AdoMetDC activity from rat liver, yeast and *E. coli* (Karvonen *et al.*, 1985). In *Trypanosoma brucei brucei*, only Berenil inhibited AdoMetDC irreversibly. The inhibition produced by pentamidine was reversible. Both inhibitors have been shown to inhibit human spermidine/spermine acetyltransferase (SSAT) and pentamidine is also a strong inhibitor of polyamine oxidase, when N-acetylspermine is the substrate (Libby and Porter, 1992).

Other irreversible inhibitors of S-adenosylmethionine decarboxylase have been described. These include 5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxy-adenosine (MDL 73811) (Byers *et al.*, 1991) and 5'-deoxy-5'-[N-methyl-N-(3-hydrazinopropyl)] aminoadenosine (Madhubala *et al.*, 1988).

1.3.3 Inhibitors of Spermidine Synthase

A variety of inhibitors of spermidine synthase have been identified. Of the early inhibitors examined S-adenosyl-L-homocysteine sulfone (Hibasami *et al.*, 1980a) and S-5'-deoxyadenosyl-(5')-2-methylthioethylamine (Samejima and Nakazawa, 1980) were the most effective.

Cyclohexylamine was shown to be a strong inhibitor of mammalian spermidine synthase (Hibasami *et al.*, 1980b). Cyclohexylamine, which is sold as dicyclohexylammonium sulfate by Sigma Chemical Company and has been

incorrectly described as dicyclohexylamine, has been shown to inhibit spermidine synthases from trypanosomes, plants and some bacteria (Pegg and Williams-Ashman, 1987). It has also been reported that cyclohexylamine is readily taken up in cells and depletes spermidine in mammalian tissues and cells, plants and susceptible bacteria (Pegg and Williams-Ashman, 1987).

S-adenosyl-1,8-diamino-3-thiooctane (AdoDato) is a mechanism-based inhibitor which has an IC_{50} (concentration at which fifty per cent inhibition of growth occurs) of less than 20ηM for mammalian spermidine synthase in assay conditions comparable to those *in vivo* (Pegg *et al.*, 1983). As well as being active against a range of spermidine synthases, AdoDato applied to cultured cells leads to a striking depletion of spermidine but also to an increase in putrescine, spermine and decarboxylated AdoMet (Pegg *et al.*, 1982). There was little change in cellular polyamine concentration and as a result it has been suggested that AdoDato produces a compensatory increase in the amount of AdoMet, which is then utilised by the spermine synthase, which is not inhibited by AdoDato.

Recently Shirahata *et al.* (1988) reported the discovery of a more potent inhibitor of pig spermidine synthase than AdoDato. *Trans*-4-methylcyclo-hexylamine is a putrescine competitive inhibitor. Interestingly the activity of this compound was found to rest in the *trans*-isomer, which was found to be over 200 times more potent than the *cis*-isomer.

1.3.4 Inhibitors of Spermine Synthase

A variety of synthetic analogues of the product of spermine synthase, methylthioadenosine(MTA), have been found to inhibit spermine synthase. One of these 5'-Methylthiotubericidin is somewhat less active than MTA *in*

vitro but is more stable in the cell as it is not a substrate for MTA phosphorylase (Coward, 1983). Pegg *et al.* (1986) described three new potent inhibitors of spermine synthase. These were 5'-[(3-aminopropyl)amino]-5'-deoxyadenosine, 9-[6(R,S),8-diamino-5,6,7,8-tetradeoxyβ-D-*ribo*-octofuranosyl]-9H-purin-6-amine (AdoDap) and dimethyl(5'-adenosyl)sulfonium perchlorate [$AdoS^+(CH_3)_2$]. $AdoS^+(CH_3)_2$ and AdoDap were specific in their action against spermine synthase in SV-3T3 cells and blocked spermine synthesis but there was a compensatory increase in the production of decarboxylated AdoMet and in the concentration of spermine and there was no effect on cell growth (Pegg and Coward, 1985; Pegg *et al.*, 1986). Neither of these two compounds could deplete residual spermine pools formed by DFMO.

More recently, Baillon *et al.* (1989) found that N-alkylated-1,3-diaminopropane derivatives inhibited mammalian spermine synthase *in vitro* and in cultured rat hepatoma cells. However although these derivatives depleted spermine in growing cells, spermidine levels increased and growth continued unabated.

1.3.5 Cellular Responses to Polyamine Depletion

Polyamines are known to be an absolute requirement for normal cell division to occur and as a result polyamine biosynthesis is very tightly regulated (Jänne *et al.*, 1981). Most examinations of cellular compensatory mechanisms to polyamine depletion have been undertaken on cultured animal cells. However it is very probable that similar responses occur in plant and fungal cells. The responses observed to polyamine depletion include enhanced uptake of extracellular polyamines and diminished excretion of intracellular polyamines, increased production of the target enzyme resulting from gene amplification, the manufacture of secondary enzymes and the formation of analogues not normally found within the cell.

Points at which inhibtors act

A = α-MFMO

α-DFMO

α-hydrazinoornithine

1,5-diaminopentane

1-6-diaminohexane

MAP

N^1N^8 bis(ethyl)spermine

B = MGBG

EMGBG

EGBG

Berenil

Pentamidine

C = CHA

AdoDato

S-adenosyl-L-homocysteine sulfone

D = AdoDap

$AdoS^+(CH_3)_2$

E = Berenil

Pentamidine

F = Berenil

Pentamidine

G = Pentamidine

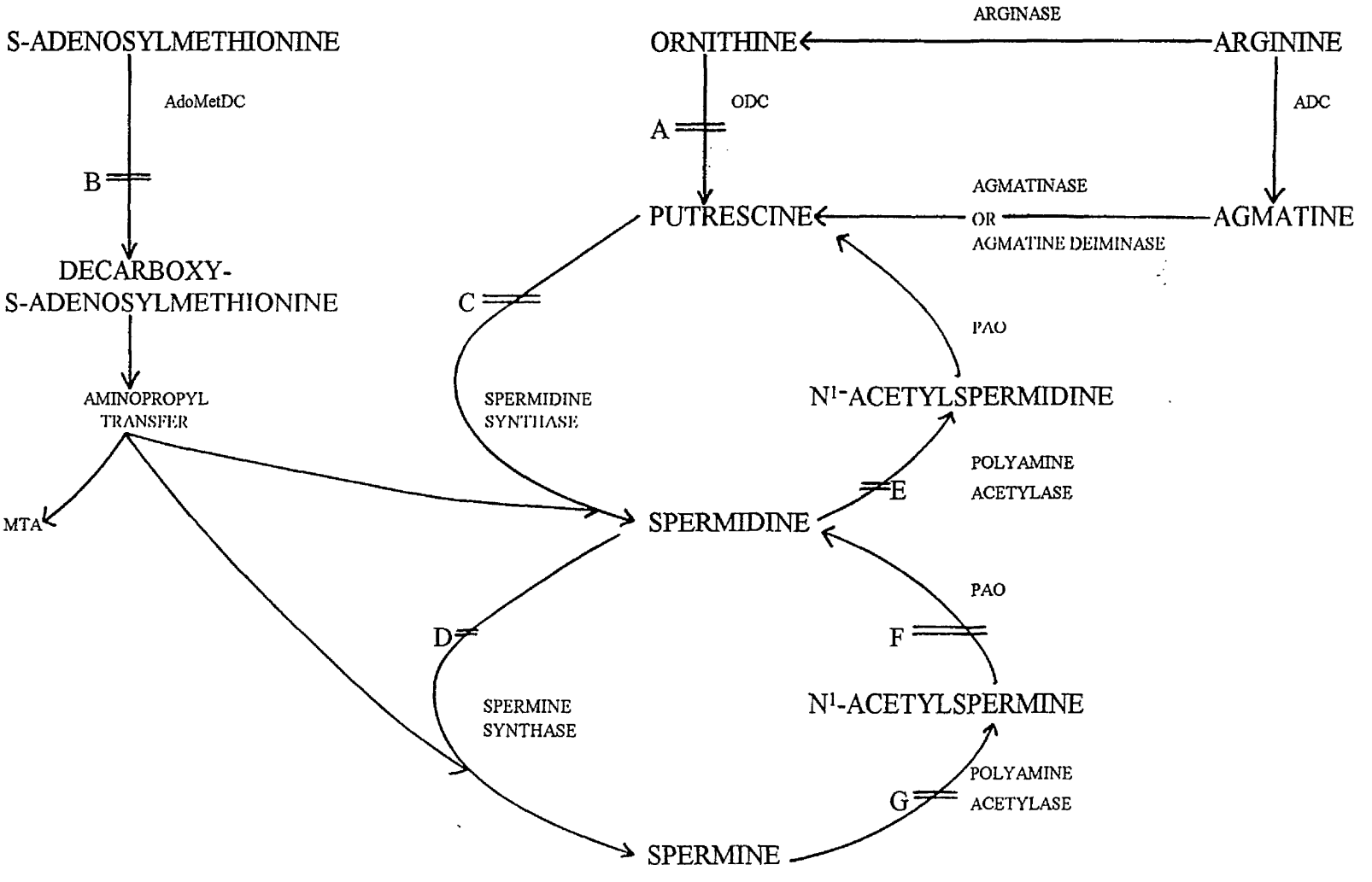


Figure 3 Pathways of Polyamine Metabolism and Points of Action of Inhibitors

Deprivation of polyamines leads to the cells retaining polyamines. BHK-21 cells exposed to α-methylornithine or MGBG excreted considerably less polyamines into the medium than did untreated cells (Melvin and Keir, 1978). Interestingly, putrescine analogues acting as inhibitors of ODC caused increased excretion from the cell. This phenomenon is probably related to the diamines replacing the polyamines at intracellular binding sites, such as from ribosomes, thus allowing their excretion. The mechanisms which enable enhanced uptake of extracellular polyamines into deprived cells are as yet unknown. Cultured carcinoma cells, which were pre-treated with DFMO for 48 hours to reduce putrescine and spermidine pools, were shown to take up extracellular polyamines very efficiently (Jänne *et al.*, 1981). As polyamines share the same uptake pathways as certain inhibitors e.g. MGBG, then this 'priming' of cells may be of use in cancer treatments. Moreover, lower MGBG concentrations could then be used and toxicity risks reduced.

One of the major difficulties encountered in attempting to inhibit ornithine decarboxylase and S-adenosylmethionine decarboxylase has been the rapid turnover of these enzymes due to their short half life. Holm *et al.* (1989) showed that the ornithine decarboxylase inhibitor 2-hydrazinoornithine reduces ODC activity *in vitro* but increased the activity of the enzyme *in vivo.* This increase was believed to be the result of reduced enzyme degradation and increased ornithine decarboxylase synthesis. MGBG produced a similar effect on S-adenosylmethionine decarboxylase, inhibiting the enzyme *in vitro* yet causing an accumulation *in vivo.* Indeed it has been observed that inhibitors of ornithine decarboxylase can increase S-adenosylmethionine decarboxylase activity in cells (Foster and Walters, 1990; Halline *et al.*, 1989). The reasons for this are as yet unknown.

Alhonen-Hongisto (1980) found that depletion of spermidine and spermine resulted in an increase in S-adenosylmethionine decarboxylase. However, no comparable reaction was found when diamine concentrations were reduced, suggesting that the quantity of the enzyme present is controlled at the transcription level by spermidine and/or spermine. In a more recent study using Ehrlich ascites tumour cells, polyamines were shown to exert a feedback control on S-adenosylmethionine decarboxylase synthesis (Persson *et al.*, 1989). However the authors could not pinpoint the enzymes involved.

Ornithine decarboxylase inhibitors produce high levels of spermine in cells. This is probably due to increased amounts of S-adenosylmethionine becoming available for the production of decarboxylated S-adenosylmethionine, which in turn is available for spermine synthesis. Putrescine levels are reduced by ornithine decarboxylase inhibitors and this will encourage spermine production as the diamine acts as a natural inhibitor of spermine synthase (Hannonen *et al.*, 1972).

As mentioned earlier, polyamine deprivation can lead to the formation of analogues not usually found in cells. Alhonen-Hongisto and Jänne (1980) reported the formation of cadaverine in DFMO treated tumour cells. They also found that cadaverine had been converted to analogues of spermidine and spermine by decarboxylated adenosylmethionine. These higher derivatives appeared to take over some of the roles of the natural polyamines. These reactions were readily suppressed by micromolar concentrations of spermidine and spermine.

In cancer therapy these compensatory mechanisms can influence the effectiveness of treatments, especially if the inhibitors are applied individually. DFMO resistant tumour cells have already been reported. This resistance is

believed to be the result of over production of the target enzyme, which maintains polyamine levels (Alhonen-Hongisto *et al.*, 1982). Alhonen-Hongisto *et al.* (1985) concluded that this reduced antiproliferative effect of DFMO was due to an amplification of the ornithine decarboxylase gene.

1.4 CANCER RESEARCH

A great deal of research into polyamine biosynthesis inhibitors has been undertaken with the express aim of discovering valuable new anti-cancer drugs. The extensive nature of these studies merits a brief review.

DFMO, a potent, specific and irreversible inhibitor of ornithine decarboxylase, decreased intracellular putrescine and spermidine concentrations, and inhibited cell growth in a number of culture systems and in animal tumours *in vivo* (Sunkara *et al.*, 1987). Long term application of DFMO to animals and humans produced some side effects e.g. diarrhoea, weight loss and debilitation in rats, and diarrhoea and hearing loss in man. However all the side effects noted were reversible (Luk *et al.*, 1983; Abeloff *et al.*, 1984, 1986).

Initial studies on DFMO were very promising. Mamont *et al.* (1978) showed that DFMO exhibited antiproliferative effects against HTC cells, L1210 leukaemia cells and MA-160 human prostatic adenoma cells. In addition, antiproliferative effects were confirmed against human embryo fibroblasts (Hölttä *et al.*, 1979), HeLa cells (Sunkara *et al.*, 1980) and Ehrich ascites carcinoma cells (Oredsson *et al.*, 1980). Initially, it was believed that DFMO was a cytostatic agent but experiments carried out by Sunkara *et al.* (1985) indicated that DFMO is a cytostatic or cytotoxic agent depending on the cell line employed. It has been suggested that these differences may reflect differences among cell types in their capacity for polyamine biosynthesis

(Sunkara *et al.*, 1987). The passive uptake of DFMO into cells means that high dose rates are required in order to build up intracellular concentrations. δ-methylacetylenic putrescine (MAP) has been shown to be a more potent inhibitor of ODC *in vitro* and *in vivo*. It produces similar anti-tumour effects, albeit at lower doses (Bartholeyns *et al.*, 1984; Mamont *et al.*, 1984).

MGBG, the potent inhibitor of S-adenosylmethionine decarboxylase, is known to be an antileukemic agent and is reported to have some activity against solid tumours, but its clinical use has been limited due to its severe toxicity (Pegg, 1988). A great deal of interest has been shown in the use of MGBG in combination with DFMO. Two different systems were used in the evaluation of this combination. The first regime used a sequential application of DFMO followed by MGBG, since it has been shown that DFMO pretreatment can enhance cellular uptake of MGBG. Difficulties in this regime were encountered as reports began to be produced of MGBG restoring the intracellular polyamine levels reduced by DFMO (Kallio and Jänne, 1983). However, despite this, a promising result was found in children with two different leukemias (Siimes *et al.*, 1981), with all the patients achieving a remission. However experiments with adult patients yielded less promising results (Porter *et al.*, 1983; cited by Porter and Jänne, 1987). The other regime used a concomitant application of DFMO and MGBG. It yielded less promising results, but led to a direct advancement in the concept of increasing drug transport (Porter and Jänne, 1987). Various workers concluded that MGBG's antiproliferative effects were due to mechanisms other than the inhibition of S-adenosylmethionine decarboxylase (Pleshkewych *et al.*, 1980; Porter *et al.*, 1981).

DFMO has been shown to be of maximum chemotherapeutic value when it is combined with an appropriate cytotoxic agent (Fozard and Koch-Weser, 1982; Sjoerdsma and Schechter, 1984). DFMO has demonstrated a synergistic effect

when used in combination with adriamycin or vindesine against L1210 leukaemia, hepatoma solid tumour in rats and EMT6 solid tumour in mice (Bartholeyns and Koch-Weser, 1981). Promising results have been produced by a combination of DFMO and interferon against Lewis lung (3LL) carcinoma (Sunkara *et al.*, 1987). More recently, Khan *et al.* (1992) showed that a protein kinase C inhibitor, H-7 potentiated the anti-proliferative effects of DFMO on NIH 3T3 and 3T3/SV40 cells in culture.

Porter and Sufrin (1986) discussed the possibility of using analogues of polyamines and methionine as anticancer agents. They suggested that once inside the cell polyamine analogues could exert intracellular antiproliferative effects in one of several ways. Among the mechanisms proposed were : inhibiting polyamine biosynthetic enzymes, competing for binding sites with polyamines at binding sites associated with cell proliferation, binding at polyamine sites and disrupting critical macromolecular structure, and function and serving as vectors for biologically active moieties. They examined analogues of spermidine and found antiproliferative activity. They also suggested that this approach may have advantages over specific enzyme inhibition. These advantages included : utilisation of polyamine transport and cell penetration at low concentrations, inhibition of more than one enzyme at a time, no compensatory increases in secondary enzymes and the chance to decrease the pools of all polyamines, including spermine.

1.5 PROTOZOAL RESEARCH

Perhaps the most successful applications of polyamine biosynthesis inhibition have come in the field of protozoal research. The discovery of ornithine decarboxylase in *Trypanosoma brucei brucei* by Bacchi *et al.* (1979) raised the

possibility of using DFMO against African trypanosomiasis. Present drug treatments in use for trypanosomal infections have serious side effects and in the case of one drug, melarsoprol, a 5% mortality rate.

DFMO proved to be a fairly weak inhibitor of ornithine decarboxylase from *T. b. brucei in vitro* (Garofalo *et al.*, 1982). However, in studies of infected mice DFMO proved highly effective and complete cures were obtained by administering 2% DFMO for 3 days in drinking water. However, these studies were carried out on an acute *T. b. brucei*, which did not infect the Central Nervous System (CNS) and as such was unrepresentative of the true clinical situation. Using more advanced CNS model infections, Clarkson *et al.* (1983) showed that DFMO treatment, in combination with bleomycin, could control a *T. b. brucei* infection which neither could control singly. This remarkable synergistic effect was also found with other veterinary trypanocides, such as Berenil, in acute models.

DFMO is believed to act in a cytostatic rather than cytotoxic way. Bacchi *et al.* (1983) observed that DFMO induces a striking morphological change in *T. b. brucei* found in the bloodstream. Long slender types became stumpy after prolonged exposure. DFMO eliminated putrescine pools, reduced spermidine pools by up to 75% and decreased the respiration rate of these bloodstream forms.

In experiments on human *Trypanosma* infections the most dramatic results have been observed against *Trypanosoma brucei gambiense* sleeping sickness, where DFMO successfully cured late and early stage infections of the disease (Taelman *et al.*, 1987). Other polyamine biosynthesis inhibitors are now being tried in model situations. Δ-MFMO.CH_3 has given promising results and has the added advantage of accumulating in the parasite at eight times the rate of

DFMO (Bacchi *et al.*, 1987). One trypanosome, *Trypanosoma cruzi* (Chagas' disease) has given conflicting experimental results. DFMO gave no control of the trypanosome even at high concentration. However DFMA applications successfully inhibited this tryapanosome. (Yakubu *et al.*, 1992). Hunter *et al.* (1991) suggested that this inhibition was due to a conversion of DFMA to DFMO and a subsequent inhibition of ODC. It may be that DFMA is acting on arginase decarboxylase activity after all, since Majumder *et al.* (1992) have recently discovered ADC activity in *Trypanosoma cruzi*. *Trypanosoma brucei brucei* has also been shown to be cured in mice by the irreversible inhibitor of S-adenosylmethionine decarboxylase MDL 73811 (Byers *et al.*, 1991). These workers suggested that the increase on S-adenosyl-L-methionine produced by this inhibitor may be responsible for its antitrypanosomal effect.

DFMO has also been administered successfully against the parasitic protozoan *Pneumocystis carinii* (Sjoerdsma *et al.*, 1984; Golden *et al.*, 1984). *P. carinii* pneumonia (PCP) is now the most lethal infection in patients with acquired immune deficiency syndrome (Kovacs and Masur, 1985; Fauci *et al.*, 1984). Previous drug treatments with either cotrimaxole or pentamidine had produced a mortality rate of 60% per episode (Kovacs and Masur, 1985). In a clinical study DFMO produced a favourable response in 74% of patients who underwent a treatment period of at least 14 days (Schechter *et al.*, 1987).

In addition to these promising results, DFMO in combination with bis (benzyl) polyamine analogues cured murine malaria (Bitonti *et al.*, 1989). At the same time these bis (benzyl) polyamine analogues were also found to be potent inhibitors of two chlorquine-resistant human malaria parasites, *Plasmodium falciparum, in vitro.* The authors suggested that the cytotoxic effects could be caused by binding of the analogue to DNA with subsequent disruption of

macromolecular biosynthesis and cell death. These new compounds could offer an exciting lead in the search for new agents for chemotherapy of malaria.

1.6 HIGHER PLANT RESEARCH

Polyamines are known to be involved in an overwhelming array of plant growth and developmental processes. While it is known that polyamines play important roles in plant physiological processes, such as stress responses, emphasis in this section will be given to growth and in addition, the effects of polyamine biosynthesis inhibition or perturbation in plants will be considered. This is important, since inhibition or perturbation of polyamines biosynthesis in phytopathogenic fungi will be examined later in this thesis and any effects on the host plant must be taken into account.

1.6.1 Polyamines in Plant Growth and Development

Heimer *et al.* (1982) were the first workers to demonstrate the significant level of ornithine decarboxylase was correlated with cell division frequency in tobacco suspension cultures and tomato ovaries. Application of DFMO to tobacco cells blocked cell division whilst leaving cell enlargement unaltered (Berlin and Forche, 1981), suggesting that DFMO blocks only one stage in the cell cycle. Walker *et al.* (1985) observed that the application of D-arginine, D-arginine plus DFMO, MGBG or cyclohexylamine (CHA), reduced polyamine titres and inhibited cell division in *Acer saccharum* seedlings. Cell elongation was unaffected. Gallardo *et al.* (1992) examined alterations in free polyamine levels in *Cicer arietinum* seeds during the onset of germination. Highest polyamine concentrations were found in the embryonic axes attached to cotyledons. In these axes RNA synthesis coincides with an accumulation of spermine and that of DNA with an increase in spermidine and putrescine.

Schwartz *et al.* (1986) found high spermine content in maize root apices and a localisation of ornithine decarboxylase activity in meristematic zones. In a series of studies on oat protoplasts, the application of exogenous polyamines stimulated both DNA synthesis and a limited amount of mitotic activity (Galston *et al.*, 1978; Kaur-Sawhney *et al,* 1980).

Felix and Harr (1989) reported on the influence of a series of effective inhibitors on endogenous polyamine levels on plant growth in germinated tomato seeds and callus cultures of *Abutilon theophrasti* Medic. Only two of the compounds examined, MGBG and Berenil, inhibited germination of the tomato seeds. 1,4-diaminobutane and the ornithine analogue, canaline, slightly inhibited growth of *A. theophrasti* at 1.0 mM concentrations. The other compounds had no effect. These results led the authors to suggest that inhibition of germination could be the result of reduced spermidine levels. 1-Aminooxy-3-aminopropane has been shown to inhibit all the decarboxylases involved in polyamine biosynthesis yet it produced an accumulation of putrescine in the tomato hypocotyl. This may have been produced by the transport of the polyamines from cotyledons into hypocotyls and roots. Finally, the authors suggested that their results indicated a possibility of building up putrescine levels to toxic levels, and the possibility of using the enhanced spermidine uptake apparatus, found after DFMO treatment, to deliver cytotoxic homologues of spermidine into cells. The effect of exogenous polyamines and difluoromethylornithine on seed germination and root growth of *Arabidopsis thaliana* has been investigated. Root growth was stimulated by low putrescine concentrations but inhibited by higher concentrations. DFMO produced an inhibition of root growth, which was reversed by putrescine. Spermidine and spermine had no effect on root growth but inhibited seed germination (Mirza and Bagni, 1991).

Many experiments have been carried out on polyamines in carrot somatic embryogenesis systems. Montague *et al.* (1978) demonstrated that arginine decarboxylase activity and putrescine concentration increased when carrot cell cultures were shifted from callus medium to embryogenesis medium. Feirer *et al.* (1984) showed that DFMA could block the transition from disorganised growth into somatic embryogenesis, without affecting cell growth. Addition of putrescine restored the embryogenic potential. Khan and Minocha (1991) examined the effect of cyclohexylammonium sulphate (CHAP), an inhibitor of spermidine synthase, on somatic embryogenesis systems and cellular polyamine content. Somatic embryogenesis was only slightly delayed by 0.5 mM CHAP. This concentration substantially reduced spermidine levels but increased putrescine levels. In combination with MGBG, CHAP caused an increase of up to four fold in putrescine levels, whilst decreasing spermidine and spermine. Minocha *et al.* (1991) showed that AdoMetDC activity peaked after 2 days in carrot cell cultures. This activity could be inhibited by 2,4 D, DFMO, MGBG and Cyclohexylammonium sulphate. Polyamine biosynthesis inhibitors have also been studied in *Hevea brasiliensis* (El Hadrami and D'auzac, 1992) and *Nicotiana tabacum* cv Xanthi (Burtin *et al.*, 1989).

There have been several investigations into the roles polyamines play in root formation and growth. Palavan-Unsal (1987) observed that the arginine decarboxylase pathway was responsible for meeting the putrescine requirement for root growth in *Phaseolus*. Rugini *et al.* (1993) examined the effect of putrescine on *in vitro* rooting of difficult-to-root woody species. Putrescine increased the rooting of apple and basal explants of olive, decreased it in walnut, but did not affect chestnut, almond, jojoba and apricot rooting. Total free polyamine content was inversely correlated with the response to exogenous putrescine treatment. In contrast to the findings of Palavan-Unsal, Burtin *et al.* (1990) examined root formation in leaf explants of tobacco cultivated *in vitro*

and found that ornithine decarboxylase was responsible for the putrescine biosynthesis during early and later stages of tobacco root development. These workers found that the greatest change observed during root formation was in the level of hydroxycinnamoylputrescines, a conjugated form of the diamine. These authors also found that during callus formation arginine decarboxylase appears active in the biosynthesis of large levels of free amines, while ornithine decarboxylase appears active in the biosynthesis of putrescine conjugates (Burtin *et al.* 1989). A specific role for these hydroxycinnamoylputrescines has not yet been elucidated. Chriqui *et al.* (1986) found a synergistic effect of auxins and ornithine in rhizogenesis in *Datura innoxia* leaf explants, when exogenous putrescine was applied. These workers then suggested that ODC and ADC may each have important, but separate, roles to play in root formation of cell cultures.

As well as root formation, floral initiation has been examined in intact plants and in *in vitro* systems with a view to gaining a better understanding of polyamines and polyamine conjugate metabolism. Cabanne *et al.* (1981) found that polyamine conjugates, primarily mono- and di-caffeoylputrescine, accumulated in apical shoots and leaves of *Nicotiana tabacum* cv. Xanthi plants during late development. The authors proposed that these conjugates may be related to the appearance of ripening to flower. Lower conjugate levels were found in the shoot apices of a non flowering hybrid of the *Nicotiana* stock RMB7. However, direct comparisons for this hybrid are difficult and changes in polyamine-conjugate levels may be the result of a genetic factor independent of the flowering effect (Dumas *et al.* 1981; 1982). Torrigiani *et al.* (1987) compared free and bound polyamines in cultures of thin layer tobacco cells taken from floral determined tissues and from vegetative stem tissues. They found a slight difference in the rate of appearance of the major polyamines between the vegetative and floral-bud forming tissues, but both showed 10-20

fold increases first of spermidine, then of putrescine levels. Polyamine conjugate levels increased significantly during the course of explant development and they were found at approximately 10-fold higher levels than free polyamines. The results obtained showed a correlation between polyamines and conjugates and bud formation, but showed no differences between tissue types, implying that polyamines may influence bud formation in general, rather than floral bud formation specifically.

In comparison, the roles of polyamines in pollen development have been poorly studied. *In vitro* pollen tube growth in *Catharanthus* was stimulated by spermidine at 0.01 mM and inhibited at 0.1 mM. MGBG reduced germination at 0.5 mM and totally inhibited germination at 1.5 mM (Prakash *et al.*, 1988). Rajam (1988) examined germination and tube growth of pollen grains of lily (*Lilium longiflorum* Thunb). DFMO and DFMA both inhibited germination and tube growth, especially when used in combination. Their effects were reversed by putrescine and spermidine indicating the participation of polyamines in normal growth and development of pollen.

Floral development has also been studied in some detail. High ornithine decarboxylase activities have been reported in the developing ovaries of the tomato flower (Heimer *et al.*, 1979; Heimer and Mizrahi, 1982). These authors showed that DFMO could block development and that this inhibition was reversible by the addition of putrescine. Slocum and Galston (1985a) carried out a detailed study of enzyme activity and free and bound polyamine levels in developing tobacco ovaries. They found an increase in ornithine decarboxylase activity of up to 3-fold during ovary development and fruit set. This increase was correlated with a doubling in the level of free putrescine but unaltered spermidine and spermine levels. Ornithine decarboxylase activity was found to peak at a level 140-fold that of arginine decarboxylase, and 90% of the total

content for all three polyamines was found in the bound form, probably as caffeoyl derivatives. Later, these same workers demonstrated the interference of tobacco ovary development by DFMO and DFMA (Slocum and Galston, 1985b). The effect of DFMA was believed to be the result of arginase mediated conversion of DFMA to DFMO and the subsequent inhibition of ornithine decarboxylase. The results of both studies suggested that further work is required to elucidate the interplay between the free and bound forms of the polyamines.

1.6.2 Polyamines in Fruit Development

There is much information to implicate a role for polyamines in fruit development. Costa and Bagni (1983) showed that application of mM concentrations of polyamines to flowers nine days after full bloom increased both fruit set and yield in apples. This effect was apparently the result of increasing fruit growth rate during the stage of rapid cell division. Flower bud formation was also increased. However, Volz and Knight (1986) applied exogenous putrescine to apples and recorded increased fruit set with one cultivar on one rootstock, but no effect on another rootstock or with two other cultivars. Rugini and Mencuccini (1985) reported an increase in fruit set in olives following application of high concentrations of putrescine during flowering. Cohen *et al.* (1982) showed that ornithine decarboxylase activity increased during cell proliferation in tomato fruits. They found that putrescine was necessary for fruit development. Later Teitel *et al.* (1985) correlated enzyme activity with cell division and the polyamine titre with DNA content, cell size and fresh weight in tomato fruits. Biasi *et al.* (1991) examined polyamine metabolism in fruit set and growth on 7-year old apple trees (*Malus domestica*). Their results indicated that there is a correlation between polyamine content, biosynthetic enzyme activities and fruit growth during the

initial stages of fruit development. Putrescine positively affected fruit set and size and increased polyamine content. More recently, Egea-Cortines and Mizrahi (1993) examined the effect of difluoromethylornithine on polyamine levels in pollinated and napthalenacetic acid (NAA)-induced young tomato fruits. They found that DFMO inhibited growth and cell division in tomato fruits after pollination. DFMO also increased free spermine, soluble conjugated and insoluble bound polyamines. It had no effect on the cell number of NAA-induced fruits five days after fruit set. The authors concluded that different morphogenic events are linked to the metabolism of different polyamine fractions in fruit. They suggested that soluble conjugated polyamines may be linked to cell division in the pericarp whereas the insoluble bound polyamine metabolism might be linked to the embryogenic process of seed formation. Law *et al.* (1991) demonstrated that putrescine, spermidine, spermine, diaminopropane, γ-aminobutyric acid and methionine increased the storage life of tomato fruits. This effect was not entirely due to an interference with ethylene synthesis and a direct action on the cell wall was proposed. This effect was also seen in 'Golden Delicious' and 'McIntosh' apples, where polyamine applications inhibited softening (Kramer *et al*, 1991). Again it was suggested that the polyamines were acting by rigidification of cell walls rather than through interactions with ethylene biosynthesis.

1.7 FUNGAL RESEARCH

Fungal research is another expanding field of polyamine experimentation. Hart *et al.* (1978) carried out an early investigation into polyamine distribution and AdoMetDC activity in a range of filamentous fungi. They found spermidine was the predominant polyamine, although putrescine and spermine were detectable in all the fungi examined. In addition, all the fungi contained a putrescine activated S-adenosylmethionine decarboxylase. Polyamine

metabolism has been extensively examined in the simple filamentous fungus, *Neurospora crassa,* which resembles mammalian cells in terms of polyamine metabolism and regulation. It takes up polyamines in a non-saturable way, which can be inhibited by the polyamines themselves and by MGBG, but is only weakly inhibited by ornithine and arginine (Davis and Ristow, 1988). Putrescine, in excess, is toxic to cells. In *N. crassa* the vacuole was shown to play a protective role by accumulating large excesses of the polyamine (Davis and Ristow, 1991). This fungus was also shown to excrete excess polyamines as a method of regulating intracellular levels. Davis (1990) concluded that the various elaborate regulation pathways utilised by *N. crassa* may be the reason that polyamines have not proved to be effective feed back inhibitors in this fungus.

Pfaller *et al.* (1987) showed that DFMO gave substantial growth-inhibition of *Candida albicans, C. tropicalis* and *C. parapsilosis.* Differences in susceptibility were noted between the three pathogenic yeasts. These same workers later showed that cyclohexylamine could diminish polyamine pools and inhibit growth of *C. albicans* and *C. tropicalis* (Pfaller *et al.*, 1988). They also discovered that using DFMO to deplete polyamine levels in the fungus improved the inhibitory activity of CHA. *Cryptococcus neoformans* is emerging as a major pathogen complicating the AIDS syndrome in patients. It has been shown to lack detectable spermine and to require high concentrations of spermidine, but not putrescine for growth. DFMO and cyclohexylamine depleted cellular polyamine levels and inhibited growth (Pfaller *et al.*, 1990).

Singhania *et al.* (1991) examined polyamine distribution in fifteen thermophilic moulds representing Zygomycetes, Ascomycetes and Deuteromycetes. They found that the three major polyamines were widely distributed but that putrescine was only sporadically distributed in the members of the

Zygomycetes. DFMO strongly inhibited growth of *Humicola lanuginosa, Talaromyces emersonii* and *Mucor pusillus*, with each fungus showing a different response pattern. Interestingly, the polyamine oxidase-polyamine system has been shown to kill *C. neoformans* and inhibit germ tube formation of *C. albicans* blastoconidia and hyphal growth of *Aspergillus fumigatus* conidia (Levitz *et al.*, 1990). In this system, polyamine breakdown by polyamine oxidase generates toxic acrolein, which kills the fungal cells.

Most fungal research however, has centred on the use of polyamine biosynthesis inhibitors against plant pathogens. This is based on the knowledge that most important crop pathogens studied contain only one pathway of polyamine biosynthesis, *via* ornithine decarboxylase, whereas plants can synthesises polyamines using both ornithine or arginine decarboxylase. In theory then, it should be possible to control fungal pathogens on plants without affecting growth of the plant itself. This hypothesis has been examined against a range of host-pathogen interactions. Walters (1986) showed that application of DFMO to broad bean (*Vicia faba* L.) controlled infection by the rust fungus, *Uromyces viciae-fabae* (Pers.) Schroet. DFMO did not affect plant growth or endogenous polyamine levels. Indeed, Rajam *et al.* (1991) found that spraying bean leaves (*Phaseolus vulgaris*) with DFMO and DFMA resulted in an increase in endogenous putrescine and spermidine. These workers suggested that this could be the result of the paradoxical stimulation of ODC by DFMA and ADC by DFMO. Activation of one pathway for putrescine synthesis may result form inhibition of the other.

Experiments carried out with polyamine biosynthesis inhibitors on plant pathogens can be roughly divided into those which were performed *in vivo* and those carried out *in vitro*. Much of the early *in vivo* work was carried out using DFMO, which protected *Phaseolus vulgaris* from the bean rust fungus

Uromyces phaseoli. Rajam *et al.* (1985) found that DFMO at 0.5 mM or above gave complete protection of the plant. At lower concentrations, post inoculation treatments were more effective and the protective effect appeared to be translocated within the plant, implying that DFMO may possess some systemic activity. The same workers carried out more detailed timing experiments with DFMO on the same host-pathogen interaction and found that application of DFMO 3 days after inoculation was the optimum time for fungal inhibition. They also found that spores failed to germinate on leaves treated 1 day previously with DFMO and that this inhibition could be partially reversed by the addition of spermidine to the leaves (Rajam *et al.*, 1986). Finally, these workers studied uredospore germination and germ tube growth *in vitro* and on the leaf surface. Both DFMO and DFMA inhibited uredospore germination *in vitro*, as well as delaying spore germination time by 15-30 minutes and restricting germ tube elongation. Putrescine and spermidine stimulated growth *in vitro* and partially reversed the inhibitor effects. On leaves, DFMA conferred no protection even at 5.0 mM (Rajam *et al.*, 1989).

A wide range of other pathogens have been examined *in vivo.* Thus, Galston and Weinstein (1988) showed that DFMO controlled rust infections very effectively and that, in general, the mildew infections examined were less sensitive to DFMO. They also showed that DFMA gave better control of southern corn late blight (*Helminthiosporium maydis*) than DFMO and that DFMO could control verticillium wilt (*Verticillium dahliae*) of tomato. Mussell *et al.* (1987) examined this soil borne pathogen, in more detail. *In vivo*, DFMO delayed disease symptom appearance and increasing concentrations of DFMO decreased disease symptoms.

Weinstein *et al.* (1987) examined the effect of DFMO and DFMA on *Puccinia recondita* (leaf rust), *P. graminis* f.sp. *tritici* (stem rust) and *Erysiphe graminis*

(powdery mildew) on wheat (*Trticum aestivum* L.). DFMO gave best control when applied after inoculation. Leaf and stem rusts were controlled at lower concentrations of DFMO than the powdery mildew fungus. DFMA gave some control, possibly due to conversion to DFMO. Polyamine levels were also examined in stem rust infected wheat leaves, using susceptible and resistant wheat varieties. On susceptible plants, DFMO concentrations as low as 0.0625 mM reduced uredospore production. DFMO had no effect on uredospore germination on healthy wheat plants (Machatschke *et al.*, 1990). More recently, Foster and Walters (1992) found that DFMO and MGBG could control *P. graminis tritici* infections of wheat. They found that best control was achieved using a combination of the two applied as a pre inoculation spray.

DFMO has been shown to control *Botrytis cinerea* (grey mould) on tomato, pepper, eggplant, bean and *Senecio* sp. leaves. Interestingly, spermidine itself also gave control of *B. cinerea*. In addition, 0.2 mM DFMO and 1.0 mM spermidine controlled *B. cinerea* of *Senecio* sp. and tomato additively better than either treatment alone, although this effect was not observed in leaves of lettuce and pepper. DFMO and spermidine also controlled white mold (*Sclerotinia sclerotiorum*) (Elad, 1991).

In a series of detailed experiments, West and Walters (1988a) examined the effect of a range of polyamine biosynthesis inhibitors on infection of *Hordeum vulgare* L. by *Erysiphe graminis* (powdery mildew). DFMO was shown to reduce mildew infection as effectively as the more recent ornithine analogues Δ-MFMO, Δ-MFMO.CH_3 and MAP. In addition DFMA, CHA and MGBG were tested and shown to control mildew. In general, post-inoculation applications proved more effective than pre inoculation ones. Greatest control was achieved using a combination of DFMO and MGBG, indicating a possible synergistic effect between these two inhibitors. Addition of polyamines with DFMO

produced a partial reversion of inhibition. Interestingly, DFMO in combination with film forming polymers has been shown to control powdery mildew infection more efficiently than either treatment alone (Walters, 1992). It was suggested that the synergism between DFMO and the film forming polymers could be related to enhanced uptake of DFMO by the germling.

In addition to the *in vivo* work described above a wide range of fungi have been examined *in vitro*. Thus, Rajam and Galston (1985) found that DFMO and DFMA affected growth and mycelial morphology of four phytopathogenic fungi: *Botrytis* sp., *B. cinerea, Rhizoctonia solani* and *Monilinia fructicola.* When polyamines were added to medium with the inhibitors, morphology was normal but cell length and diameters increased considerably. Birecka *et al.* (1986) showed that DFMO inhibited mycelial growth and sporulation of *Helminthosporium maydis* at 0.5 mM to 2.0 mM. Putrescine reversed this inhibition, and DFMA was found to have a very weak inhibitory effect on this fungus. Minocha and Khan (1989b) studied the effect of DFMO and DFMA on four phytopathogenic fungi, *H. maydis, H. carbonum, Fusarium oxysporum* and *Ceratocystis ulmi.* They found that all four fungi possessed ADC activity. Mycelial growth of all the fungi was inhibited by 1 to 5.0 mM DFMA or DFMO, except for *H. maydis,* which remained unaffected even by 5.0 mM DFMA. Inhibition was more pronounced with DFMO, but all inhibition was completely reversible by putrescine. Both inhibitors reduced putrescine and spermidine levels and increased spermine.

Trione *et al.* (1988) examined the wheat bunt fungi (*Tilletia* sp.) and found that of the inhibitors tested, only DFMO inhibited monokaryotic hyphal growth, but spermidine, alone or in combination with DFMO and DFMA, also strongly inhibited hyphal growth. Both DFMO and DFMA strongly inhibited growth of dikaryotic hyphae. However, they had only a moderate effect on spore

formation and little or no effect on spore germination. Singh *et al.* (1989) found that another bunt of wheat, *Neovossia indica*, could be inhibited *in vitro* by DFMO. DFMA had no effect on mycelial growth and sporidial formation.

West and Walters (1989) examined the effects of a range of polyamine biosynthesis inhibitors on the growth of four necrotrophic fungi: *Pyrenophora teres, Gaeumannomyces graminis, Fusarium culmorum* and *Septoria nodorum.* Species-dependent responses were observed, although DFMO was still proved to be the weakest inhibitor. Indeed, some of the inhibitors produced an increase in fungal growth. The authors suggested that this could possibly be the result of secondary enzyme production or overproduction of the target enzyme. MGBG was found to be the most powerful inhibitor examined and mixtures of DFMO and MGBG proved to be no more effective than MGBG alone. In contrast to these results, Foster and Walters (1990) showed that DFMO, MGBG and EMGBG inhibited mycelial growth of *Pyrenophora avenae.* DFMO proved less effective than MGBG and EMGBG and whilst it's inhibition could be almost completely reversed by putrescine and spermidine, that of MGBG and EMGBG could not. EMGBG reduced activity of AdoMetDC and spermidine levels. DFMO, alone and in combination with MGBG, reduced spermidine and spermine levels.

Smith *et al.* (1990) examined the growth inhibition of *B. cinerea* by compounds which interfered with polyamine metabolism. Growth was inhibited by DFMO, Δ-FMO, DFMA and Δ-FMA. A range of compounds were found to reverse this inhibition. They included cadaverine, diaminoheptane, putrescine, spermidine, butenediamine and spermine. Other compounds which had little inhibitory activity themselves, increased the growth inhibition produced by DFMO. These included MAP, aminooxyaminopropane, 2,2 difluoroputrescine and MGBG. These authors suggested that this synergistic effect could be the result of the

broad specificity of the membrane -based diamine uptake system, which becomes activated when endogenous putrescine is depleted. Such an approach, they suggested, could possibly provide new fungicides based on the inhibition of ODC. The same workers examined the effect of enzyme-activated inhibitors on ornithine decarboxylase from *B. cinerea* (Smith *et al.,* 1990). Interestingly two inhibitors which were growth inhibitors , DFMA and Δ-FMA, had no significant effect on ODC, while MAP and Δ-FMO , which were poor inhibitors of growth, were found to be effective inhibitors of ODC. More recently, Smith *et al.* (1992) examined two fungi, *Septoria tritici* and *Ustilagio maydis,* which were relatively insensitive to the inhibitors of ornithine decarboxylase, DFMO, Δ-FMO and MAP. Although growth was not inhibited, ODC activity was inhibited by all three. A 1.0 mM DFMO concentration in the medium reduced spermidine content in both fungi to between 30-40% of the control values. It would appear that spermidine levels need to be more substantially depleted if fungal growth is to be reduced

Biondi *et al.* (1992) found that DFMA was the most effective inhibitor of the fungus responsible for Dutch elm disease, *Ophiostoma ulmi* (Buism.) Nannf. The authors suggested that this was due to the presence of a predominant arginine decarboxylase pathway in this fungus.

Much of the *in vivo* and *in vitro* work reported has concentrated on the use of enzyme activated irreversible inhibitors e.g. DFMO. However, in recent work, Foster and Walters (1993) examined the fungicidal activity of two putrescine analogues, keto-putrescine and N-acetylputrescine. These two compounds were tested against a range of plant pathogens, both *in vivo* and *in vitro*. Keto-putrescine was shown to be effective *in vivo* against *E. graminis, U. viciae-fabae, B. fabae, P. hordei, P. infestans and Podosphaera leucotricha.* Although N-acetylputrescine had no effect on *in vitro* growth of *Pyrenophora avenae,*

Pyricularia oryzae and *Phytophthorainfestans,* Keto-putrescine did inhibit the growth of these three pathogens. In addition, keto-putrescine had only a small effect on ODC activity, but significantly reduced AdoMetDC activity in *P. avenae*. Putrescine levels were unaffected, but the spermidine pool was reduced by 43% and spermine levels were increased. Based on these results, the authors suggested that polyamine analogues might prove to be valuable new antifungal compounds.

The research described above has revealed that there is a great variation between species, in terms of polyamine biosynthesis and susceptibility to polyamine biosynthesis inhibition. In general, it appears that biotrophic fungal pathogens like rust and powdery mildew are very sensitive to inhibition of polyamine biosynthesis, while necrotrophic fungal pathogens are less sensitive. The reason for this apparent difference in fungal response is not known.

1.8 PATHOGENS

A brief description of the pathogens used in this project is given below.

1.8.1 *Eryisphe graminis* DC. ex Merat

Disease:

This pathogen causes powdery mildew of cereals and grasses. Symptoms are seen on aerial parts of leaves and consist of small, white fluffy pustules on the leaves and sheaths. These pustules darken as they age, acquiring first a pinkish tinge and finally becoming brown and matted. In the late growing season, cleistocarps may be found in the matted growth. These contain the ascospores which will be involved in producing new fungal strains in the growing season. Early sown winter crops may also be infected by ascospores in the autumn, but

more usually they are infected by conidia from volunteer plants. Resistant cereal varieties produce necrotic spotting when challenged by powdery mildew.

Economic Importance:

Yield losses as a result of powdery mildew are most severe in crops of barley, with a reduction of 27.4% of grain yield having been reported (CMI No. 153, 1967). The pathogen occurs worldwide and in the United Kingdom is the most widespread of cereal diseases.

Control:

Control measures fall into three categories. The first involves cultural practices. Such measures include avoiding early applications of excess nitrogen, as the fungus spreads more effectively in thick crops. Eradication of volunteer plants on which the fungus can overwinter is another cultural control measure, as is the avoidance of sowing spring crops close to diseased autumn sown crops. The second category is the use of resistant varieties. As this resistance may be controlled by only a few genes in the plant it is useful to grow different varieties in close proximity to one another. This will decrease the likelihood of the pathogen overcoming one gene and spreading across a large area of the growing crop. The third category is chemical control. A systemic seed treatment can protect the plant from an early attack of powdery mildew. Further applications to the crop will depend on disease levels present and regular inspection of growing crops is recommended. Examples of commonly used chemicals include : fenpropimorph, prochloraz, propiconazole and ethirimol. Recently, resistance has been reported to the triazole group of fungicides. As a result they are often used in combination with other active ingredients and the number of triazole applications in a growing season should be regulated and spaced out to avoid the build up of resistant strains of the fungus (Parry, 1990; ADAS, No. 579, 1982).

1.8.2 *Uromyces viciae-fabae* (Pers) Schroet.

Disease:

Uromyces viciae-fabae is the pathogen which causes rust on leaves and stems of broad beans. The disease symptoms often appear late in the growing season. Small red-brown rust pustules, often surrounded by chlorotic haloes, are randomly scattered over the leaf surfaces. The fungus can overwinter as uredospores or resting mycelium on living plant material, provided by self sown beans, old crop debris and autumn sown crops.

Economic Importance:

The pathogen is widespread both worldwide and within the United Kingdom. More serious damage has been reported in Germany, Egypt and the former Yugoslavia, where high atmospheric relative humidity and late applications of irrigation favour heavy infection. In the UK., damage is slight, although a yield loss of almost 30% has been recorded in an artificially infected trial.

Control:

Cultural control measures consist of eliminating volunteer plants and contaminated crop debris. Agrosan and thiram have been successfully used as seed treatments. Chemical control is used to stop disease build up. The most commonly applied chemical to the foliage is fenpropimorph. It has been shown recently that chlorothalonil-containing mixtures can give as good control of rust as fenpropimorph, at moderate to low infections. However under high disease pressure fenpropimorph was superior in terms of rust control and yield (Dobson and Giltrap, 1991; CMI No. 60, 1965; Parry, 1990; ADAS No. 2450, 1984).

1.8.3 *Botrytis fabae* Sardina

Disease:

This pathogen is responsible for chocolate spot of field (broad) beans. Two phases of this disease have been identified. The first phase is referred to as the non-aggressive phase. Symptoms appear in winter and early spring and consist of discrete reddish or chocolate brown spots. The second, aggressive phase appears in late spring-early summer when conditions are wet and humid. Symptoms include a coalescing of lesions and the death of large areas of tissue. Air borne conidia are still produced on dead bean foliage during wet weather and the fungus can overwinter on bean debris and as sclerotia in the soil. In addition, this pathogen can also be seed borne.

Economic Importance:

This disease is found in many parts of the world, including Europe and the United Kingdom. The timing and severity of attack influences economic significance. The non-aggressive phase does not usually cause significant yield loss, although an early attack of the aggressive phase can reduce yield by up to 50%.

Control:

Cultural control measures are aimed at reducing the chance of the aggressive phase developing. These measures include destroying all previous crop debris, sowing winter crops after mid October to avoid early infections, reducing crop humidity by using optimum seed rates and avoidance of oversheltered areas. Seed dressing with thiram, thiabendazole, captan and bromophos has been practised. Foliar applied fungicides are used at regular intervals depending on

disease levels and weather conditions. A number of active ingredients are recommended. These include benomyl, chlorothalonil, iprodione and carbendazim. Recent trials have shown two sprays at early and mid flower to be the most consistently profitable in terms of yield response. However during a three year trial period, prophylactic fungicide application to spring beans for chocolate spot control was not consistently profitable. There have been reports of resistance to the MBC fungicides and dicarboximides in *B. fabae* (Parry, 1990; Dobson and Giltrap, 1991; CMI No. 432, 1974).

1.8.4 *Podosphaera leucotricha* (Ell&Ev.) Salm

Disease:

Podosphaera leucotricha is the pathogen which causes powdery mildew of apple. Symptoms are long white conidial chains which develop on the underside of the leaf and then spread to cover both surfaces. The fungus also infects young shoots, fruits and blossom tissues. In some varieties the fruit can be attacked shortly after blossoming, but infections seldom persist after skin hardening in mid-summer. The disease is spread by wind borne conidia. The sole means of overwintering is by mycelium present within diseased fruit and vegetative buds.

Economic Importance:

The fungus has been reported in Africa, Asia, Australia and New Zealand, North and South America and Europe. In some countries it is of minor importance, but in the United Kingdom it is very serious. It has been shown to significantly reduce crop yield from severely infected trees, as well as causing a russeting on the fruit which reduces the market value.

Control:

Breeding of resistant varieties appears to be the most promising control measure. However, effective fungicides include benomyl, buprimate, captan and penconazole and carbendazim (Burchill, 1978; CMI No. 158, 1967; Ivens, 1993).

1.8.5 *Phytophthora infestans* (Montague) de Bary

Disease:

This fungus causes late blight on potato and tomato. Symptoms on potato haulm are the development of circular and irregular water soaked patches, usually at the edges or tips of lower leaves. This is followed by the development of large brown dead areas on the leaf and the appearance of white downy growth at the edge of the lesion on the underside of the leaf. Suitable conditions for disease development can result in the death of much of the potato haulm. Harvested tubers may become infected by zoospores from the infected haulm. Symptoms on the tuber surface are dark, slightly sunken irregular areas. Internally a reddish brown rot develops under these sunken surfaces. Blighted tubers are frequently colonised by secondary bacterial pathogens and can quickly be reduced to a semi-liquid state. The disease is spread by sporangia *via* wind and rain splash and overwinters in infected seed tubers, infected tubers from clamps and stores of the previous year and on volunteer plants.

Economic Importance:

The disease is widespread throughout potato growing areas in the world. Although the exact relationship between yield potential of the crop, damage and crop loss has not been established (Rotem *et al.*, 1983), it has been demonstrated that early attacks of blight can dramatically affect yield. Work done by Large in the 1950's (cited by Parry, 1990) showed that if 75% of the foliage was affected by blight by the end of July then further tuber development

would stop prematurely and there would be an approximately 50% yield loss. Growing seasons with high potato blight levels produce higher infections in glasshouse and field tomato crops.

Control:

Cultural control methods include siting potato clamps away from the site of future potato crops, destroying volunteer plants and checking seed tubers for infection prior to planting. In addition, the crop should be well ridged up to try and reduce the potential for yield reduction. Disease resistance has been bred into potatoes, but there are many races of *P. infestans* and this resistance is insufficient to protect the crop. Disease resistance is used with fungicide applications in an integrated programme. Fungicides may be protectants, e.g. dithiocarbamates, or systemic, e.g. phenylamides. Risk of infection is determined by environmental conditions and this in turn determines the number of fungicide applications. It is common for systemics to be used in the first part of the season and protectants towards the end (Parry, 1990; ADAS No. 271, 1985).

1.8.6 *Pyrenophora avenae* (Ito and Kuribayashi apud Ito)

Disease:

Pyrenophora avenae Ito and Kurabayashi apud Ito (conidial state *Drechslera avenae* [Eidam] Sharif) is the pathogen which causes leaf stripe, blotch or spot and seedling blight of oats. Symptoms produced vary in seedlings from pre emergence death, to streaking of coleoptiles. Infection of emerging leaves by mycelium causes distortion and spotting. Conidia produced in primary leaves causes secondary spread to upper leaves, producing light reddish-brown irregular streaks or blotches.

Economic Importance:

The fungus is found in most of the oat growing areas of the world. In the United Kingdom it poses a threat in Scotland, Wales, Northern England and Northern Ireland, where cooler, wetter conditions dominate during the early seedling stage. Severe outbreaks can cause spikelet drop and stem-break in older plants.

Control:

The early stage of this disease can be controlled by seed dressing. Thiram and dithiocarbamate type seed dressings have been shown to give control. Disease symptoms on leaves can be controlled by systemic fungcides , such as triadimefon and propiconazole (Smedegaard-Peterson, 1988; CMI No. 389, 1973).

1.8.7 *Pyricularia oryzae* Cav.

Disease:

The fungus causes blast disease of rice, on leaves, leaf sheaths, rachis, pedicel of spike, glumes and joints of culms. Symptoms are most conspicuous on leaf blades, where minute brown spots appear which enlarge to become spindle shaped, pointed at both ends. Several spots on a leaf may coalesce and the leaf then withers. The most serious damage occurs when the neck region of the flowering stem is attacked. Black necrotic regions form near the uppermost node, the stem rots and the panicle falls over giving the typical broken neck phase of the disease. The disease is spread by simple conidiophores, which are readily detached and disseminated by air movement.

Economic Importance:

Blast occurs in all rice growing areas, but is a major problem in Japan, Taiwan, U.S.A and many other countries. Losses of up to 95% have been recorded. Blast is becoming an increasing problem as rice production expands and intensifies.

Control:

Cultural control measures include reducing nitrogen input, since this makes plants more susceptible to blast. Planting nurseries in small, narrow beds rather than large blocks reduced infection, as no areas of high humidity occurred in such narrow beds. Resistance genes have been identified in rice plants, but unfortunately different pathogenic races exist in different localities and varieties have to be picked on the basis of their growing location. Some resistant varieties are still susceptible to neck rot and it appears that different pathogen races attack the plant at different stages. Chemical control has been provided mainly by organomercury compounds, although these compounds have the disadvantage of being toxic to indica varieties of rice. In Japan, antibiotic sprays of Blasticidin-S have been used. These compounds have a therapeutic effect and can act against mycelium of *P. oryzae* within the leaf. Intensive rice production uses a combination of control methods and includes 5 separate sprays to control blast (Pans Manual No. 3, 1970; CMI No. 169, 1968).

1.8.8 *Botrytis cinerea* Pers. ex Pers

Disease:

Botrytis cinerea is the conidial state of the fungus *Scelrotinia fuckeliana* (de Bary) Fuckel. This fungus causes 'grey mould', a blight or rot of immature, fleshy or senescent tissues, on a wide range of host plants. Symptoms of the disease include the development of tan or brown water soaked lesions, which

become greyish on drying out. The disease is spread by airborne conidia and on the surface of rain splash droplets. Sporulation on diseased plants is prolific in wet weather resulting in disease epidemics. The fungus can overwinter as sclerotia or mycelium in old plant debris and can be carried on certain seeds, e.g. flax, as spores and mycelium.

Economic Importance:

The fungus is found worldwide, but is prevalent as a disease in humid or sub-tropical areas. In the United Kingdom the disease is common but severe epidemics are rare.

Control:

Chemical control measures depend on the crop attacked. Many broad spectrum fungicides applied to crops also control *B. cinerea* e.g. carbendazim and chlorothalonil will control the disease on oilseed rape. Resistance to some chemicals has been observed on some crops e.g. dichlofluanid and benomyl on strawberries and tomatoes (Parry, 1990; CMI No. 431, 1974).

1.9 AIMS OF THE PROJECT

Based on the earlier results of Foster and Walters (1993), which showed that Keto-putrescine exerted powerful fungicidal activity, a project was established in conjunction with Professor D.J. Robins of the Chemistry Department at the University of Glasgow, to synthesise novel putrescine analogues. These analogues were then sent down to the Plant Science Department at SAC

Auchincruive for biological evaluation. In specific terms the aims of the project were :

i) To assess the fungicidal activity of these novel putrescine analogues against a range of fungal pathogens both *in vitro* and *in vivo*.

ii) To examine the effects of the novel putrescine analogues on polyamine levels and biosynthetic enzymes in the oat infecting fungus *Pyrenophora avenae.*

iii) To determine the potential of these novel putrescine analogues as possible new fungicides against *Erysiphe graminis* in field conditions.

The initial year of this project was undertaken by Dr S.A. Foster as part of a post doctoral position within this department, funded by the British Technology Group Ltd. Her initial results for the screening of E-BED and Z-BED against a range of pathogens, and the effect of E-BED and Z-BED on polyamine levels and ODC and AdoMetDC activity in *P. avenae,* are included in this thesis in order to give a complete overview of the fungicidal activity of these compounds.

SECTION TWO

MATERIALS AND METHODS

2.1 EFFECT OF NOVEL COMPOUNDS ON INFECTION OF BARLEY BY *ERYSIPHE GRAMINIS* F.SP. *HORDEI*

2.1.1 Glasshouse Experiments

Most of the experiments carried out on *Erysiphe graminis* infection of barley were carried out in the glasshouse.

2.1.1.1 Maintenance of the Pathogen

E. graminis f.sp. *hordei* Marchal was maintained on barley plants (*Hordeum vulgare L.*, cv. Golden Promise) which were kept in a separate glasshouse compartment under natural daylight supplemented for 16h daily by 400W mercury vapour lamps. The maximum temperature was 24°C during the day and fell to a minimum of 9°C at night. Barley seeds were sown and plants were sequentially inoculated on a fortnightly basis at about the third leaf stage.

2.1.1.2 Plant Material

Healthy plant material was obtained by sowing seeds of barley (cv. Golden Promise) in Fison's Levington compost in 36 cm seed trays. The plants were grown in a separate glasshouse compartment under the conditions described above (2.1.1.1).

Plants at growth stage 13 (third leaf unfolded, Zadok's scale) were used for experiments. Plants were inoculated simply by dusting them with mildew conidia.

2.1.1.3 Treatment with Novel Compounds

Solutions of the novel compounds were applied to plants as aqueous solutions containing 0.01% (v/v) Tween 20 (BDH Ltd, Poole, Dorset). In glasshouse comparisons with active ingredients, 0.01% (v/v) Agral 90 (ICI) was used instead of Tween 20. Solutions were adjusted to pH 7.0 using sodium hydroxide. Solutions were applied to plants until run off using a Shandon spray unit. The only exception to this was in experiments where solutions were applied to the lower leaves of the plant. This was achieved by painting the solutions onto the lower leaves with a camel hair brush. Solutions used in root drench experiments did not contain Tween 20. In these experiments, a volume of 187.5 ml of solution was added to each tray. This volume was calculated as 1/8th of the tray volume and was based on the method of Ouimette and Coffey (1989).

Full details of the compounds used are given in section 2.6. Treatments and concentrations used against powdery mildew were as follows :

1. E-BED dihydrochloride; 1.0 mM; pre- and post-inoculation treatments.
2. Z-BED dihydrochloride; 1.0 mM; pre- and post-inoculation treatments.
3. E-BED dibromide; 1.0 mM; post-inoculation treatment.
4. E-BED dihydrobenzoate; 1.0 mM; post-inoculation treatment.
5. E-BED phosphate; 1.0 mM; post-inoculation treatment.
6. E-BED fumarate; 1.0 mM; post-inoculation treatment.
7. E-BED dihydropropionate; 1.0 mM; post-inoculation treatment.
8. Dipip-E-BED; 1.0 mM; post-inoculation treatment.
9. Dichex-E-BED; 1.0 mM; post-inoculation treatment.
10. E-DED; 1.0 mM; post-inoculation treatment.
11. E-DPD; 1.0 mM; post-inoculation treatment.
12. E-DBD; 1.0 mM; post-inoculation treatment.
13. E-UDED; 1.0 mM; post-inoculation treatment.

14. 0.1% (v/v) Tridemorph; 0.1% (v/v) Propiconazole; 0.1% (v/v) Flutriafol; 0.1% (v/v) Fenpropidin; All post-inoculation treatments.
15. TED-1 dihydrobromide; 1.0 mM; pre- and post-inoculation treatments.
16. E-TED benzoate; 1.0 mM; post-inoculation treatment.
17. E-TED phosphate; 1.0 mM; post-inoculation treatment.
18. E-TED fumarate; 1.0 mM; post-inoculation treatment.
19. E-TED propionate; 1.0 mM; post-inoculation treatment.
20. DMD dihydrobromide; 1.0 mM; pre- and post-inoculation treatments.
21. cis TED-1 dihydrobromide; 1.0 mM; pre- and post-inoculation treatments.
22. BAD dihydrochloride; 1.0 and 5.0 mM; pre- and post-inoculation treatments.
23. trans-BAD dihydrochloride; 1.0 mM; post-inoculation treatment.
24. cis-BAD dihydrochloride; 1.0 mM; post-inoculation treatment.
25. BAC; 1.0mM; post-inoculation treatment.
26. DPH; 1.0 mM; pre- and post-inoculation treatment.
27. TCHBM dihydrochloride; 1.0 mM; post-inoculation treatment.
28. TCCBM dihydrochloride; 1.0 mM; post-inoculation treatment.
29. BACP dihydrochloride; 1.0 mM; post-inoculation treatment.
30. BDDMC; 1.0 mM; post-inoculation treatment.
31. E-BED + BAD; 1.0 mM; post-inoculation treatment.
32. E-BED + trans-BAD; 1.0 mM; post-inoculation treatment.
33. E-BED + DMD; 1.0mM; post-inoculation treatment.
34. E-BED + TED-1; 1.0 mM; post-inoculation treatment.

2.1.1.4 Timing of Compound Applications

Barley seedlings were sprayed with compounds 3 h before inoculation for pre-inoculation treatments and 3 d after inoculation for post-inoculation treatments. In root drench and systemic experiments, solutions were applied 5 d, 2 d and 1 d

pre-inoculation or 1 d, 2 d or 5 d post-inoculation. Visual assessments of infection were carried out 6, 8 and 10 days after inoculation using a standard area diagram, (MAFF, 1988).

2.1.2 Field Experiments

In order to examine the effects of these novel compounds on *Erysiphe graminis* under field conditions, a small scale field trial was carried out in 1991.

2.1.2.1 Growth of Plants

Spring barley (cv. Golden Promise) was sown on 17 April 1991 at 12 cm row spacing with a seed rate of 190 kg ha^{-1}. Seed was sown in plots (4 x 2 m) in a randomised block design with four replicates (see Figure 4). Plots were fertilised at the rate of 190 kg ha^{-1} with 20: 10: 10 (N: P: K) fertiliser. Plots were sprayed with the herbicide 'Advance' (ICI) (bromoxynil, fluoroxypyr and ioxynil). This herbicide controls cleavers, chickweed, hemp-nettle, speedwells and annual dicotyledons.

2.1.2.2 Treatment with Novel Compounds

Plots were sprayed with the novel compound E-1,4-diaminobut-2-ene (E-BED) (1.0 and 5.0 mM), the ODC inhibitor, α-difluouromethylornithine (DFMO) (1.0 and 5.0 mM) and the commercial fungicide 'Early Impact' (ICI) (flutriafol and carbendazim; 120 and 192g / litre SC). E-BED and DFMO were made up in water with Agral 90 (ICI) (0.5 litres ha^{-1}). Treatments were applied using an AZO compressed air sprayer with a delivery rate of 1 litre in 20 seconds. All treatments were applied at the rate of 200 litres ha^{-1} using a sprayer boom width of 1.8 m.

FIGURE 4. Randomized block design of field trial carried out in 1991.

1	2	9	5	10	6	11	7	3	4	8
4	7	10	2	8	3	1	9	11	5	6
7	10	5	6	11	8	1	2	9	4	3
9	4	7	5	6	10	11	3	1	8	2

Teatments were as follows;

1. 1.0 mM E-BED, One Spray.
2. 5.0 mM E-BED, One Spray.
3. 1.0 mM DFMO, One Spray.
4. 5.0 mM DFMO, One Spray.
5. Early Impact, One Spray.
6. 1.0 mM E-BED, Two Sprays.
7. 5.0 mM E-BED, Two Sprays.
8. 1.0 mM DFMO, Two Sprays.
9. 5.0 mM DFMO, Two Sprays.
10. Early Impact, Two Sprays
11. Untreated.

2.1.2.3 Timing of Compound Applications

Some plots were sprayed with fungicide once, at the first sign of mildew (Growth Stage 25; 1 June), while other plots were sprayed twice (Growth Stage 25 and 59; 1 June and 1 July). Powdery mildew infection was assessed on the third leaf on 7 and 14 June (Growth Stage 28 and 30) and on the flag leaf on 9 and 16 July (Growth Stage 60 and 61). At the end of the field trial, 10 plants were harvested from each plot and measurements made of plant height, plant dry weight and grain weight.

2.2 EFFECT OF NOVEL COMPOUNDS ON INFECTION OF BROAD BEAN BY *UROMYCES VICIAE-FABAE* AND *BOTRYTIS FABAE*

2.2.1 Maintenance of the Pathogens

Uromyces viciae-fabae (Pers) Schroet was maintained on broad beans (*Vicia faba* cv. Bunyards Exhibition) which were kept in a glasshouse under the conditions described previously (2.1.1.1). Plants were sown on a monthly basis and sequentially inoculated when plants were at the third leaf stage.

Botrytis fabae Sardina was maintained on agar in 90 mm single vent, plastic Petri dishes. The medium used was as described by Last and Hamley (1956) and comprised the following : 1 litre of water supplemented with 10% sucrose w/v: 1.52 g KH_2PO_4, 0.52 g $MgSO_4.7H_2O$. 6.0 g $NaNO_3$, 10 g dextrose, 2.0 g peptone, 3.0 g casein hydrolysate, 0.5 g yeast nucleic acids and 20 g agar. After incubation for 12 days on this medium at 22 ^oC, spores were ready for use.

FIGURE 5. Picture showing the field trial set up to evaluate the effects of E-BED and DFMO against powdery mildew. Spring barley (c.v. Golden Promise) was sown in 20 metre strips with a drill width of 2 metres. The strips were then subdivided into 4 x 2 metre plots and marked out using 1.5 metre glass fibre rods.

FIGURE 6. The field trial was subdivided into 4 blocks and the treatments randomised within each block (see Figure 4). Each block contained all the treatments and an untreated control plot. A 1 metre discard was left between the plots to ensure no overspray occurred.

2.2.2 Plant Material

Seeds of broad bean (cv. Bunyards Exhibition) were sown in Fisons' Levington compost in 15 cm plastic pots. Plants were grown in a ventilated glasshouse as described in Section 2.1.1.1 and were used in experiments at the 3-4 leaf stage.

Plants were inoculated with *Uromyces viciae-fabae* by painting a spore suspension (25mg/100 ml distilled water) onto the leaves with a camel hair brush. Plants were then covered with clear plastic bags for 48 h, in order to maintain the high relative humidity necessary for spore germination.

Plants were inoculated with *Botrytis fabae* by painting leaves with a spore suspension (approximately 4 x 10^5 conidia per ml of distilled water) using a camel hair brush. Plants were then covered with clear plastic bags for 48 h in order to provide the high relative humidity required for spore germination.

2.2.3 Treatment with Novel Compounds

Solutions of the novel compounds were made up in 0.01% (v/v) Tween 20 and adjusted to pH 7.0 using sodium hydroxide. Sprays were applied until run-off using a Shandon spray unit.

The following treatments were used against *Uromyces viciae-fabae*:

1. E-BED dihydrochloride; 1.0 mM; pre- and post inoculation treatments.
2. Z-BED dihydrochloride; 1.0 mM; pre- and post-inoculation treatments.
3. TED-1 dihydrobromide; 1.0 mM; post-inoculation treatment.

4. cis TED-1 dihydrobromide; 1.0 mM; post-inoculation treatment.
5. DMD dihydrobromide; 1.0 mM; post-inoculation treatment.
6. BAD dihydrochloride; 1.0 and 5.0 mM; pre- and post-inoculation treatments.
7. trans BAD dihydrochloride; 1.0 mM; post-inoculation treatment.
8. DPH; 1.0 mM; post-inoculation treatment.

The following treatments were used against *Botrytis fabae*:

1. E-BED dihydrochloride; 1.0 mM; pre- and post-inoculation treatments.
2. Z-BED dihydrochloride; 1.0 mM; pre- and post-inoculation treatments.
3. TED-1 dihydrobromide; 1.0 mM; post-inoculation treatment.
4. DMD dihydrobromide; 1.0 mM; post-inoculation treatment.
5. BAD dihydrochloride; 1.0 mM; post-inoculation treatment.

2.2.4 Timing of Novel Compound Applications

Pre- and post-inoculation treatments were the same for both fungi. In pre-inoculation treatments the plants were sprayed 3 hours before inoculation and in post-inoculation treatments the plants were sprayed 3 days after inoculation.

Infection intensity of *Uromyces viciae-fabae* was assessed 17 d after inoculation by estimating the percentage leaf area infected with rust pustules using a standard area diagram. Values are the means of six replicates. Infection intensity of *Botrytis fabae* was assessed 7 d after inoculation by estimating the percentage leaf area infected using a standard area diagram. Values are the means of six replicates.

2.3 EFFECT OF NOVEL COMPOUNDS ON INFECTION OF APPLE SEEDLINGS BY *PODOSPHAERA LEUCOTRICHA*

2.3.1 Maintenance of the Pathogen

P. leucotricha (Ell & Ev.) Salm was maintained on apple seedlings (*Malus pumila* cv. Malus bitenfelder) which were kept in a glasshouse under the conditions described previously (Section 2.1.1.1). Plants were sown and inoculated on a monthly basis to ensure a continual supply of inoculum.

2.3.2 Plant Material

Seeds of apple (cv. Malus bitenfelder) were stratified by placing them in cold storage (-10 ^{o}C) for 14 weeks in 36 cm trays of Fisons' Levington compost and horticultural sand (3:1 mixture). After this time the seeds were removed from cold storage and placed in a heated, ventilated glasshouse under the conditions described previously (Section 2.1.1.1). After a further 22 d, the seedlings were inoculated by gently brushing mildew conidia onto the leaves using a camel hair brush.

2.3.3 Treatment with Novel Compounds

Solutions of the novel compounds were made up in 0.01% (v/v) Tween 20 and adjusted to pH 7.0 using sodium hydroxide. The treatments used were as follows:

1. E-BED dihydrochloride; 1.0 mM; post-inoculation treatment.

2. Z-BED dihydrochloride; 1.0 mM; post-inoculation treatment.
3. TED-1 dihydrobromide; 1.0 mM; post-inoculation treatment.
4. cis TED-1 dihydrobromide; 1.0 mM; post-inoculation treatment.
5. DMD dihydrobromide; 1.0 mM; post-inoculation treatment.
6. BAD dihydrochloride; 1.0 and 5.0 mM; post-inoculation treatment.
7. DPH; 1.0 mM; post-inoculation treatment.

2.3.4 Timing of Novel Compound Applications

Seedlings were sprayed until run-off 3 d after inoculation using a Shandon spray unit. Infection intensity was assessed 17 d after inoculation using an infection key. In a later experiment with DPH and cis TED-1, a visual estimate of the percentage leaf area infected was made.

2.4 EFFECT OF NOVEL COMPOUNDS ON INFECTION OF POTATO LEAF DISCS BY *PHYTOPHTHORA INFESTANS*

2.4.1 Maintenance of the Pathogen

Stock cultures of *P. infestans* were grown on Potato Dextrose Agar (PDA) (Oxoid). Media was autoclaved at 120 ^{o}C for 15 minutes and the cultures were kept in a darkened incubator at a temperature of 22 ^{o}C. Fresh cultures were initiated on a 21 day rota.

2.4.2 Plant Material

Healthy plant material was obtained by sowing seed tubers (*Solanum tuberosum* cv. King Edward) in Fisons' Levington compost in 8 cm pots. The pots were

placed in a glasshouse under the conditions described previously (Section 2.1.1.1). After 3 weeks, 15 mm diameter discs were cut from plant leaves and floated, upside down, in solutions of the novel compounds in 45 mm single vent plastic petri dishes. The leaf discs were inoculated with *P. infestans* by pipetting 0.1 ml of a sporangial suspension onto the surface of each leaf disc.

2.4.3 Treatment with Novel Compounds

The treatments used were as follows:

1. E-BED dihydrochloride; 1.0 mM.
2. Z-BED dihydrochloride; 1.0 mM.
3. TED-1 dihydrobromide; 1.0 mM.
4. DMD dihydrochloride; 1.0 mM.
5. trans-BAD dihydrochloride; 1.0 and 5.0 mM.

Infection intensity was assessed 6 days later using an infection key.

2.5 EFFECT OF NOVEL COMPOUNDS ON GROWTH OF FUNGI *IN VITRO*

2.5.1 Maintenance of the Pathogens

Stock cultures of *P. avenae* (IMI Culture number 296817) and *P. oryzae* (obtained from Shell Research Ltd, Sittingbourne) were grown on Potato Dextrose Agar (PDA) (Oxoid). *B. cinerea* was grown on Czapek Dox Agar (Oxoid). All media were autoclaved at 120 ^{o}C for 15 minutes. The cultures were kept in a darkened incubator at a temperature of 24 ^{o}C. Fresh culture plates were initiated on a 7-21 day rota depending on the growth of each fungus.

2.5.2 Treatment with the Novel Compounds

The following treatments were used in *in vitro* experiments:

1. E-BED dihydrochloride; 0.1, 0.5 and 1.0 mM.
2. Z-BED dihydrochloride; 0.5 and 1.0 mM.
3. TED-1 dihydrobromide; 1.0, 0.5, 0.1 and 0.01 mM.
4. TED-1 dihydrobromide + Ascorbic Acid; 0.5 mM.
5. TED-1 dihydrobromide + Calcium chloride; 0.5 mM.
6. BAD dihydrochloride; 1.0 mM.

2.5.2.1 Growth on Solid Media

Filter sterilised aqueous solutions (10 ml) containing the novel compounds were added to 140 ml of sterile Potato Dextrose Agar (PDA) (Oxoid) at 45-47 ^{o}C to obtain the final concentrations required. Filter sterilisation was carried out using Sartorius Minisart filters (pore size 0.2 μm). Control plates contained culture medium only.

Sterile medium (20 ml) containing the novel compounds was added aseptically to each 90 mm single-vent plastic Petri dish. To obtain inoculum, a sterile 10 mm diameter cork borer was used to remove plugs of mycelium from the edges of stock cultures. The mycelial plugs were inverted and one placed in the centre of each Petri dish. Inoculated plates were incubated in the dark at 24 ^{o}C. Colony diameters were measured in millimetres, excluding the 10 mm plug of inoculum, 3, 6 and 8 days after inoculation. Three measurements were made from each Petri dish and all results are the means of six replicates.

2.5.2.2 Growth in Liquid Media

Filter sterilised solutions (10 ml) containing novel compounds were added to 140 ml of sterile liquid PDA, in 250 ml flasks, to obtain the desired concentrations. Liquid PDA consisted of 200 grams of potato, scrubbed and cut into cubes, then boiled in 1 litre of water until soft. The mashed potato pulp was squeezed through a fine mesh sieve. To this, 20 grams of dextrose was added and the final volume adjusted to 1 litre (Commonwealth Mycological Institute, 1983).

The flasks were then inoculated with a 10 mm disc of mycelium of the particular fungus under investigation. Flasks were placed in a Gallenkamp orbital shaker at 140 rpm, at 24 ^{o}C, with a mean irradiance of 150 μmol m^{-2} s^{-1}. After 4 days the fungus was removed from the flasks and washed with distilled water on a 250 μ m mesh sieve (Henry Simon) and centrifuged at 16,000 g for 10 minutes. The pellet obtained was used for enzyme and polyamine analysis.

2.5.2.3 Enzyme Assays

For ODC and AdoMetDC assays, crude enzyme extracts were prepared by grinding 500 mg fungus with 1 ml buffer using a pre-chilled pestle and mortar. This buffer contained 10 mM potassium phosphate, pH 7.6, 2 mM 1,4-dithiothreitol (DTT), 1 mM magnesium chloride, 0.1 mM ethylenediaminetetra-acetic acid (EDTA) and 0.1 mM pyridoxal phosphate (PLP) (Stevens *et al.*, 1976). The suspensions were sonicated using a Soniprep 150 for 10 cycles of 10 seconds on / 20 seconds off. Test tubes were kept on ice during sonication. Each sample was centrifuged at 24,000 g for 15 minutes at 0 ^{o}C.

For ODC assays the supernatant (cytosolic fraction) was dialysed against 30 volumes of buffer for 24 hours in the dark at 4 ^{o}C, using dialysis tubing with a molecular cut off of 12,000.

For AdoMetDC assays, 430 mg of ammonium sulphate was added per millilitre of supernatant (cytosolic fraction). The suspension was then centrifuged at 24,000 g for 20 minutes at 0 ^{o}C. The pellet obtained was redissolved in the original volume of buffer and dialysed as described for ODC assays above.

ODC and AdoMetDC activities were assayed by measuring the $^{14}CO_2$ released after incubation with [1-^{14}C] ornithine and S-adenosyl-[1-^{14}C] methionine respectively. Radioisotopes were obtained from Amersham International plc. Reaction mixtures used were as described by Stevens *et al.* (1976). For ODC assays the reaction mixture consisted of 50 mM Tris/HCl, pH 8.0, 0.05 mM L-ornithine monohydrochloride, 0.031 mM pyridoxal-l-phosphate, 0.125 μCi of DL-[1-^{14}C] ornithine hydrochloride (58 mCi/mol) and 0.1 ml of enzyme extract in a total volume of 0.4 ml. For AdoMetDC assays the reaction mixture contained 0.1M sodium phosphate, pH 7.4, 0.2 mM S-adenosyl-L-methionine, 1.0 mM putrescine, 0.025 μCi of S-adenosyl-L-[1-^{14}C-] methionine (45 mCi/mmol) and 0.1 ml of enzyme extract in a total volume of 0.4 ml.

To study the effects of the novel inhibitors on *in vitro* enzyme activity, the enzyme extract was mixed in a 1:1 ratio with an aqueous solution of the novel compounds. This was then added to the ODC or AdoMetDC rection mixtures described above. The following treatments were used with enzyme extract from *P. avenae* for *in vitro* assays:

1. ODC; Z-BED dihydrochloride; 0.5 and 1.0 mM.
2. AdoMetDC; Z-BED dihydrochloride; 0.5 and 1.0 mM.
3. ODC; E-BED dihydrochloride; 0.5, 1.0, 5.0, 10.0 and 20.0 mM.
4. AdoMetDC; E-BED dihydrochloride; 0.5 and 1.0 mM.

Assays were carried out in 100 mm glass test-tubes fitted with silicone rubber stoppers (Vacutainer, U.K.) and 35 mm long, 22 gauge needles. A 10 mm filter paper square was impregnated with 10 µl of 2 M KOH and 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% (v/v) perchloric acid was added to each tube and the tubes were incubated for a further 30 minutes. The filter paper was then removed and placed in a scintillation vial containing 12 ml Packard Emulsifier-safe scintillant. Samples were counted for radioactivity using a Packard 1900 TR liquid scintillation counter. Values obtained as disintegrations per minute were converted and expressed as ρmol $^{14}CO_2$ (mg protein) $^{-1}$ hour^{-1}. Protein assays were carried out using the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) as a standard (see Appendix I). All results are the mean of four replicates.

For diamine oxidase assays a modification of the method of Okayama and Kobayashi (1961) was used. The fungus was macerated in a prechilled mortar and pestle. 300 mg of fungus was ground up with 2 mls of buffer. The buffer contained 100 mM potassium phosphate, pH 8.0, and 2.0 mM dithiothreitol. Samples were then centrifuged at 20,000 g for 20 minutes at 4 °C. 0.5 mls of the supernatant obtained was then added to 0.5 mls of reaction medium in 100 mm glass test tubes. This medium contained 100 mM potassium phosphate, pH 8.0, 1.0 mM putrescine and 30 µg catalase. 0.15 µCi of ^{14}C-putrescine (108 mCi/mmol; Amersham) was then added to each reaction vessel and the test tubes were placed in a water bath at 37 °C for 30 minutes. The reaction was stopped by adding 1 ml of 4 M KOH and shaking. The enzyme products were then extracted by addition of 2 ml toluene and vortexing for 10 seconds. A 2 ml aliquot of the toluene phase was then added to a scintillation vial containing 10 ml Emulsifier Safe scintillation fluid (Packard) and counted on a Packard 1900 TR liquid scintillation counter. Protein was assayed by the Lowry method.

Counts obtained as disintegrations per minute (dpm) were converted and expressed as ηmol product (mg protein)$^{-1}$ hour^{-1}. All results are the mean of four replicates.

2.5.2.4 Polyamine Analysis

This analysis determined the concentration of free polyamines in the fungal tissue.

600 mg of tissue was macerated with 1 ml of 4 % percloric acid in a chilled pestle and mortar. Suspensions were then sonicated for 10 cycles of 10 seconds on / 20 seconds off using a Soniprep 150. The samples were then centrifuged at 12,000 g for 25 minutes at 0 ^{o}C. To 0.1 ml of the supernatant, the following were added; 0.2 ml of saturated sodium carbonate and 0.4 ml of dansyl chloride (30 mg per ml in acetone). The samples were vortexed gently and incubated in darkness at 60 ^{o}C for 20 minutes. Excess dansyl chloride was then converted to dansyl proline by incubating the samples with 0.1 ml of L-proline (100 mg per ml).

The dansylated polyamines were then extracted in 0.5 ml toluene. After vortexing, 25 µl aliquots of the toluene extract were spotted on to activated (1 hour at 110 ^{o}C) Whatman LK6D silica-gel thin layer chromatography (TLC) plates. The plates were left to develop in a TLC tank containing chloroform: triethylamine (5:1 (v/v)) until the solvent front reached the top of the plate. The spots were traced using a Gallenkamp ultra violet lamp and identified against standards. Spots were scraped off the plate into 20 ml glass scintillation vials and the dansylated derivatives extracted in 5 ml ethyl acetate. Standards between 0.2 and 10.0 µg were measured and standard curves produced (see Appendix I). Fluorescence was measured in a Perkin-Elmer LS5 luminescence spectrometer at excitation 365 ηm, emission 506 ηm. Values obtained as units

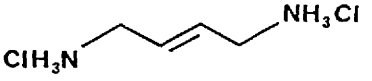
of fluorescence were converted and expressed as μmol g^{-1} fresh weight. All results are the means of four replicates.

2.5.2.5 Uptake of ^{14}C-putrescine by *Pyrenophora avenae* in the presence of TED-1

In an attempt to determine wether TED-1 replaces endogenous putrescine from binding sites, the following experiment was performed. The idea was that the fungus is grown in the presence of TED-1, which binds to endogenous putrescine binding sites. The fungus is then allowed to take up labelled putrescine. However, this labelled putrescine, unable to bind to intracellular binding sites, diffuses out of the cells, into the external medium. This method was based on an experiment described by Davis and Ristow (1988).

P. avenae was grown in liquid culture as described previously. Two flasks contained liquid Potato Dextrose Broth (PDB) only, while two others contained 0.5 mM TED-1 in liquid PDB. After 4 days the fungus was removed from the flasks and washed with distilled water on a 250 μm mesh sieve (Henry Simon) and centrifuged at 16,000 g for 10 minutes. 500 mg of fungal tissue was then placed into a 100 ml beaker containing 50 ml of fresh liquid PDB. 0.6 μCi of ^{14}C-putrescine (108 mCi/mol; Amersham) was added to each flask and the flasks placed on a Denly orbital shaker for 2 hours. The fungus was then removed and washed with distilled water containing 0.25 M NaCl (to remove externally bound putrescine) through a 250 μm mesh sieve (Henry Simon). The fungus was then placed in flasks containing fresh liquid medium only and replaced on the shaker for a further 2 hours. After this period 2 ml samples were taken from the medium and added to 10 mls Emulsifier Safe scintillation fluid (Packard) and counted on a Packard 1900 TR liquid scintillation counter. Figures are the mean of five replicates.

2.6 DETAILS OF COMPOUNDS USED

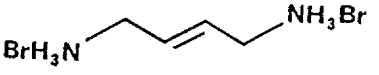


E-BED

E-1,4-diaminobut-2-ene dihydrochloride

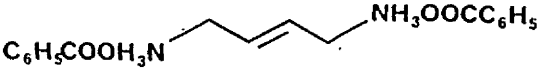
Z-BED

Z-1,4-diaminobut-2-ene dihydrochloride



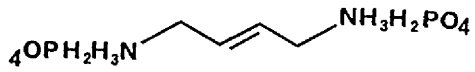
E-BED dibromide

E-1,4-diaminobut-2-ene dibromide



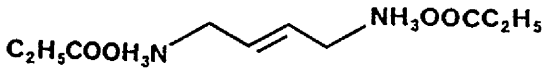
E-BED benzoate

E-1,4-diaminobut-2-ene benzoate



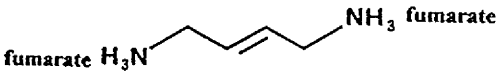
E-BED phosphate

E-1,4-diaminobut-2-ene phosphate



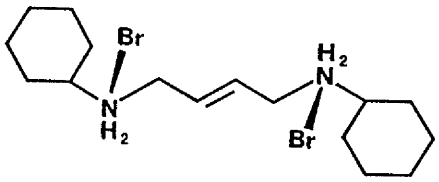
E-BED propionate

E-1,4-diaminobut-2-ene propionate



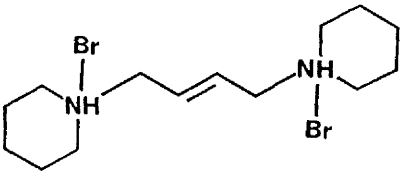
E-BED fumarate

E-1,4-diaminobut-2-ene fumarate



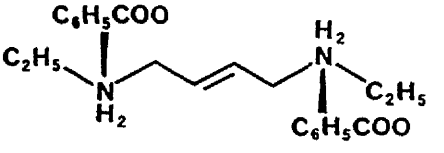
Dichex-E-BED

1,4-bis-N-cyclohexylamino-E-but-2-ene dihydrobromide



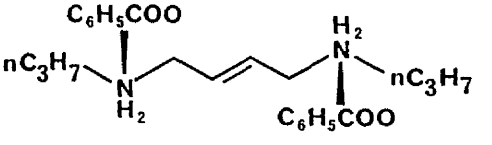
Dipip-E-BED

1,4-bis-N-pipridyl-E-but-2-ene dihydrobromide



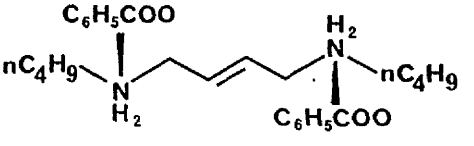
E-DED

E-(N,N-diethyl 1,4-diaminobut-2-ene) benzoate



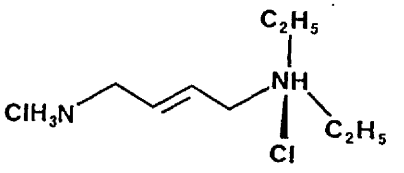
E-DPD

E-(N,N-dipropyl 1,4-diaminobut-2-ene) benzoate



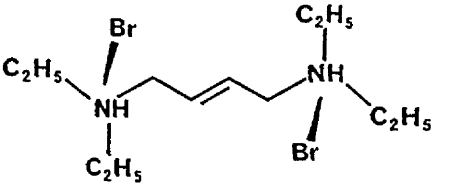
E-DBD

E-(N,N-dibutyl 1,4-diaminobut-2-ene) benzoate



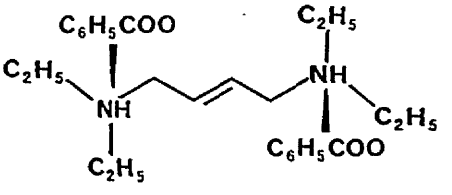
E-UDED

(E)-N,N-diethyl-1,4-diaminobut-2-ene dihydrochloride



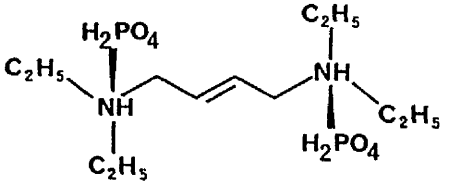
TED-1

E-(N,N,N',N'-tetraethyl)-1,4-diaminobut-2-ene dihydrobromide



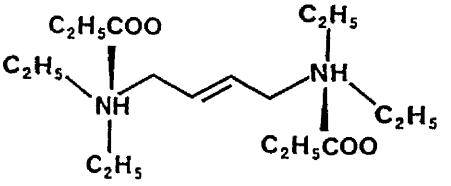
E-TED benzoate

E-(N,N,N',N'-tetraethyl)-1,4-diaminobut-2-ene benzoate



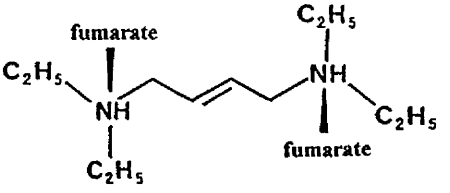
E-TED phosphate

E-(N,N,N',N'-tetraethyl)-1,4-diaminobut-2-ene phosphate



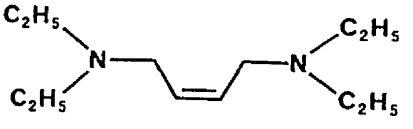
E-TED propionate

E-(N,N,N',N'-tetraethyl)-1,4-diaminobut-2-ene propionate



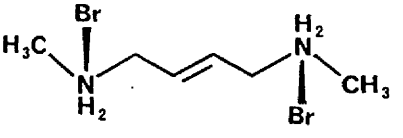
E-TED fumarate

E-(N,N,N',N'-tetraethyl)-1,4-diaminobut-2-ene fumarate



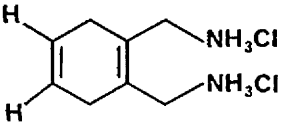
<u>cis</u> TED-1

Z-(N,N,N',N'-tetraethyl)-1,4-diaminobut-2-ene dihydrobromide



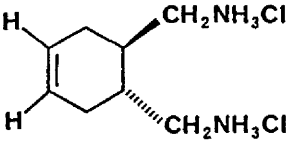
DMD

E-(N,N'-dimethyl)-1,4-diaminobut-2-ene dihydrobromide



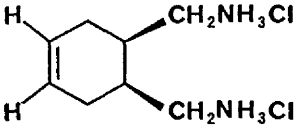
BAD

1,2-bis(aminomethyl)-4,5 dimethylcyclohexa-1,4-diene dihydrochloride



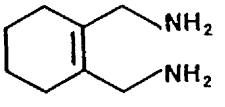
trans-BAD

trans-4,5-bis(aminomethyl)-1,2 dimethylcyclohex-1-ene dihydrochloride



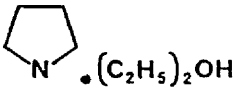
cis-BAD

cis-4,5-bis(aminomethyl)-1,2 dimethylcyclohex-1-ene dihydrochloride



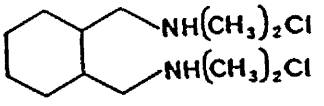
BAC

1,2-bis(aminoethyl)-4,5-dimethylcyclohexa-1,4-diene



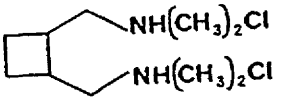
DPH

N,N-diethylpyrrolidinium hydroxide



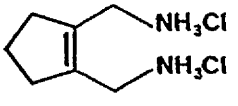
TCHBM

trans-N,N,N',N'-tetramethyl-1,2-cyclohexane bis(methaneamine) dihydrochloride



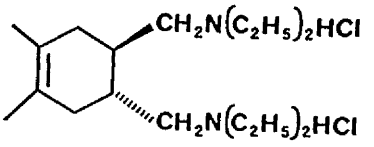
TCCBM

trans-N,N,N',N'-tetramethyl-1,2-cyclobutane bis(methaneamine) dihydrochloride



BACP

1,2 bis(aminomethyl) cyclopentene dihydrochloride



BDDMC

trans-4,5-bis(diethylaminomethyl) 1,2-dimethyl cyclohexene

SECTION THREE

RESULTS

3.1 EFFECT OF E-BED AND IT'S DERIVATIVES ON FUNGAL PATHOGENS

3.1.1 Statistical Analysis

A one way analysis of variance was carried out on all the results, using Minitab and Genstat statistical programs. Residual mean squares were calculated using the formula taken from Parker (1983).

$$\text{Residual Mean Square} = \frac{(\Sigma x^2 - C) - (\Sigma T^2/n - C)}{k(n-1)}$$

Σx^2 = Sum of squares of observations

C = Correction term [$(GT)^2/kn$]

k = Number of treatments

n = Number of replicates

GT = Grand Total of treatments

ΣT^2 = Sum of the totals squared

The residual mean square was then used to calculate the standard error of differences using the formula:

$$\text{s.e.d.} = \sqrt{[R.m.s._1/n_1 + R.m.s._2/n_2]}$$

$R.m.s._1$ = Mean square of errors from first group of observations

$R.m.s._2$ = Mean square of errors from second group of observations

n_1 = Number of observations in first group

n_2 = Number of observations in second group

Least significant differences were then calculated using the formula:

l.s.d. = t (5%, for k(n-1) degrees of freedom) x s.e.d.

Least significant differences were determined between treatments and the control.

3.1.2 Effect of E-BED and it's Salts and Derivatives on Infection of Barley by *E. graminis*

E-BED and it's cis-isomer Z-BED, applied as their dihydrochloride salts both gave substantial control of *E. graminis* when used as pre- and post-inoculation sprays (Table 2). E-BED gave the most effective control when applied 1 or 2 days post-inoculation (Figure 7). Experiments to study the systemic activity of E-BED showed that mildew infection was significantly reduced if E-BED was applied as a root drench 1 or 2 days post-inoculation, but not if applied pre-inoculation (Figures 9 and 10). Application of E-BED to lower leaves gave greatest control of mildew on upper leaves, when carried out 1 day pre-inoculation (Figure 8).

Apart from the dihydrochloride salt, other salts of E-BED were examined for their effectiveness against *E. graminis* (Figures 11 and 12). Best control was achieved using the fumarate salt, which reduced mildew infection by 62% ten days post-inoculation. Of the derivatives tested for activity against *E. graminis*, Dichex-E-BED was found to possess the most fungicidal activity (Figures 13 and 14). In glasshouse comparisons with active ingredients, 1.0 mM E-BED performed favourably and gave better control than a 0.1% solution of flutriafol (Figure 15).

In the field trial experiment, E-BED gave early season control of mildew comparable to that achieved using the commercial fungicide 'Early Impact'. However, later in the season, although E-BED gave significant control of *E. graminis* compared to the untreated plots, it was outperformed by the commercial standard (Table 3). E-BED was also shown to be more effective against *E. graminis* in this field scale experiment than the irreversible ODC inhibitor, DFMO. Percentage green leaf area was higher in the E-BED treated plots than the untreated plots (Table 4). Plots sprayed twice with E-BED and 'Early Impact' produced significantly taller plants, which had increased dry weights and grain weights with respect to the untreated plots (Table 5).

3.1.3 Effect of E-BED and it's Derivatives on Fungal Pathogens *In Vivo*

E-BED and it's cis-isomer Z-BED were shown to give significant control of a range of fungal pathogens *in vivo*. In general post-inoculation treatments gave better control than pre-inoculation ones and E-BED was also more effective than Z-BED (Table 2). Both compounds gave significant control of *Uromyces viciae-fabae, Botrytis fabae, Phytophthora infestans* and *Podosphaera leucotricha.* Greatest disease control was achieved by a post-inoculation spray of E-BED against *Botrytis fabae* (91%; Table 2).

TABLE 2. Fungicidal Activity of E-BED and Z-BED. E-BED and Z-BED were applied at 1.0 mM. Late Blight on potato leaf discs was assessed using the following key; 1 = A few isolated sporophores, 2 = <50% infection, 3 = >50% infection. Powdery Mildew on apples was assessed using the following key; 0 = No infection, 1 = A few isolated spores, 2 = <50% infection, 3 = >50% infection. Treatments to *P. infestans* and *P. leucotricha* were applied post-inoculation. All treatments differed significantly from the control at P = 0.001.

A

Plant pathogen interaction	**% leaf area infected**			**% disease control**	
	Control	**E-BED**	**Z-BED**	**E-BED**	**Z-BED**
Erysiphe graminis/barley					
pre-inoculation	18.9 ± 2.75	5.8 ± 0.63	8.9 ± 0.99	70	53
post-inoculation	18.9 ± 2.75	3.8 ± 0.41	11.1 ± 1.12	80	42
Uromyces viciae-fabae/broad bean					
pre-inoculation	22.5 ± 2.60	10.1 ± 2.00	12.9 ± 3.10	56	43
post-inoculation	22.5 ± 2.60	6.3 ± 2.60	10.0 ± 1.11	72	56
Botrytis fabae/broad bean					
pre-inoculation	3.2 ± 0.68	0.5 ± 0.14	0.6 ± 0.11	85	82
post-inoculation	3.2 ± 0.68	0.3 ± 0.12	0.7 ± 0.39	91	79

B

Plant pathogen interaction	**Mean disease score**		
	Control	**E-BED**	**Z-BED**
Phytophthora infestans/potato	3	1	1
Podosphaera leucotricha/apples	3	1	1

TABLE 3. Effect of E-BED, DFMO and 'Early Impact' on powdery mildew infection of barley in a Field Trial. Least significant differences from the control at P = 0.05 are shown in the table. Figures are the means of 40 replicates.

Treatment	**% Leaf area infected with mildew**			
	1st Assessment 7 June	**2nd Assesment 14 June**	**3rd Assessment 9 July**	**4th Assessment 16 July**
Untreated	9.48	20.22	6.62	19.43
One Spray				
1 mM E-BED	4.18	11.95	11.45	15.28
5 mM E-BED	3.54	10.73	8.95	17.15
1 mM DFMO	5.57	20.44	8.48	19.37
5 mM DFMO	3.05	12.60	9.98	25.20
Early Impact	4.21	11.30	8.55	17.95
Two Sprays				
1 mM E-BED			7.08	13.55
5 mM E-BED			3.50	10.50
1 mM DFMO			8.82	15.20
5 mM DFMO			7.05	15.25
Early Impact			2.68	4.83
L.S.D (P=0.05)	2.48	3.69	4.79	5.98

TABLE 4. Effect of E-BED, DFMO and 'Early Impact' on green leaf area of spring barley in a Field Trial. Least significant differences from the control at P = 0.05 are shown in the table. Figures are the means of 40 replicates.

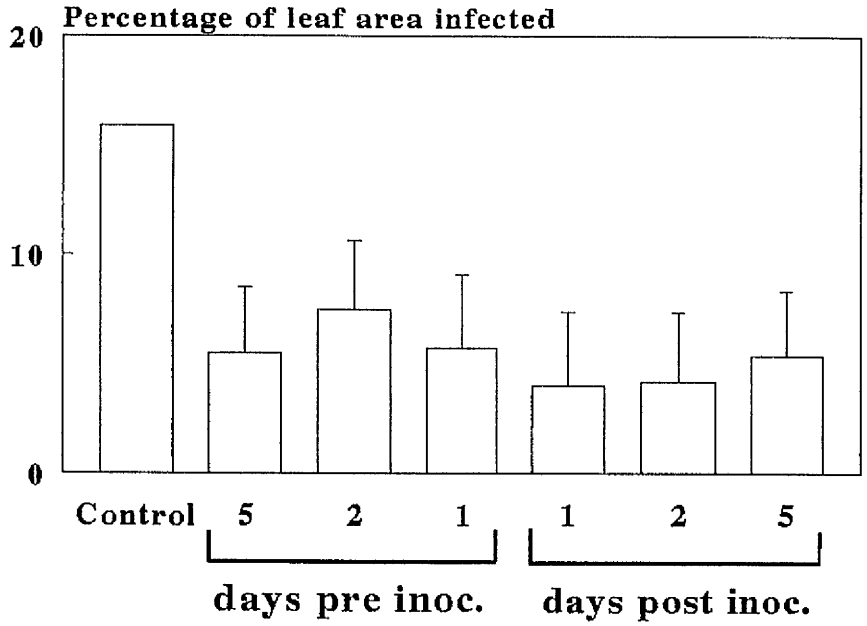
Treatment	% Green leaf area			
	1st Assessment 7 June	2nd Assessment 14 June	3rd Assessment 9 July	4th Assessment 16 July
Untreated	88.85	67.20	88.58	76.56
One Spray				
1 mM E-BED	93.89	82.47	86.95	80.50
5 mM E-BED	95.78	81.30	89.37	79.72
1 mM DFMO	93.60	70.56	88.52	75.50
5 mM DFMO	95.80	81.28	89.15	70.70
Early Impact	95.59	84.56	89.05	79.35
Two Sprays				
1 mM E-BED			91.15	83.07
5 mM E-BED			94.0	86.47
1 mM DFMO			90.31	80.46
5 mM DFMO			91.07	80.78
Early Impact			94.72	91.22
L.S.D (P=0.05)	3.74	7.90	4.95	6.08

TABLE 5. Effect of E-BED, DFMO and 'Early Impact' on Plant Height, Plant Dry Weight and Grain Weight in a Field Trial. Least significant differences from the control at P = 0.05 are shown in the table. Figures are the means of 40 replicates.

Treatment	Plant Height (cm)	Plant Dry Weight (g)	Grain Weight (g)
Untreated	75.68	7.60	6.84
One Spray			
1.0mM E-BED	75.41	7.85	6.35
5.0mM E-BED	78.19	10.50	7.60
1.0mM DFMO	73.81	9.24	6.81
5.0mM DFMO	78.05	9.01	7.16
Early Impact	78.87	9.69	7.12
Two Sprays			
1.0mM E-BED	75.76	8.50	7.14
5.0mM E-BED	81.05	11.79	7.94
1.0mM DFMO	74.08	8.24	7.06
5.0mM DFMO	77.82	10.69	7.58
Early Impact	80.87	9.78	7.93
L.S.D (P=0.05)	4.98	2.54	1.07

FIGURE 7. Effect of application of 1.0 mM E-BED to barley seedlings at various times, both pre- and post-inoculation, on powdery mildew infection. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of ten replicates.

FIGURE 8. Effect of application of 1.0 mM E-BED to lower barley leaves on infection by powdery mildew on upper leaves. E-BED was applied at various times pre- and post-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of ten replicates.


Percentage of leaf area infected
20
10
0
Control
5
2
1
1
2
5
days pre inoc.
days post inoc.

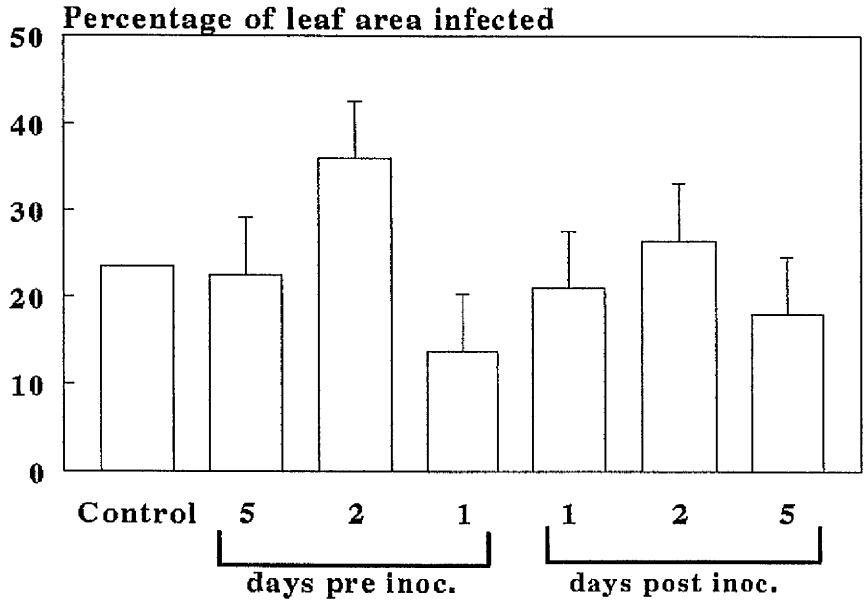
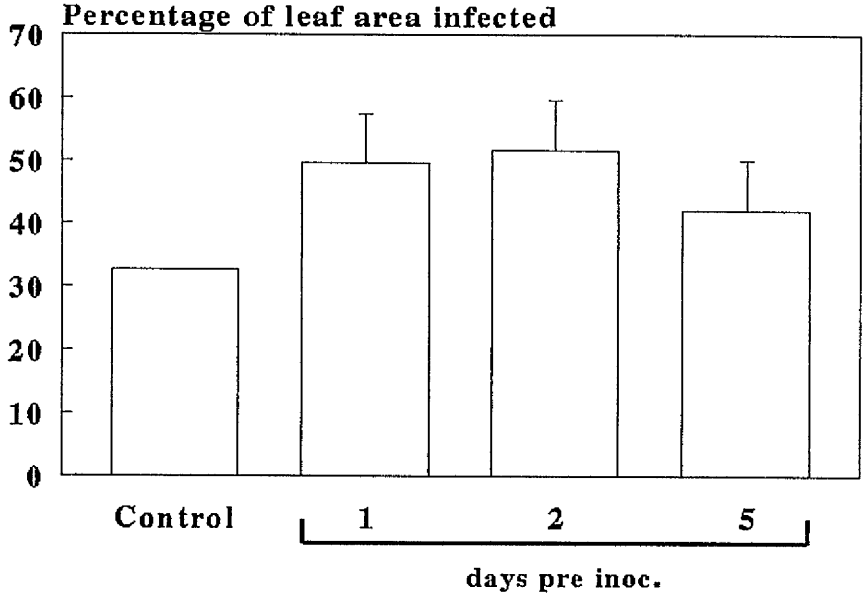

Percentage of leaf area infected
50
40
30
20
10
0
Control
5
2
1
1
2
5
days pre inoc.
days post inoc.

FIGURE 9. Effect of application of 1.0 mM E-BED as a root drench to barley seedlings on infection of leaves by powdery mildew. E-BED was applied at various times pre-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of ten replicates.

FIGURE 10. Effect of application of 1.0 mM E-BED as a root drench to barley seedlings on infection of leaves by powdery mildew. E-BED was applied at various times post-inoculation. Results shown are for the final assessment of leaves, 10 days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of ten replicates.


Percentage of leaf area infected
70
60
50
40
30
20
10
0
Control
1
2
5
days pre inoc.

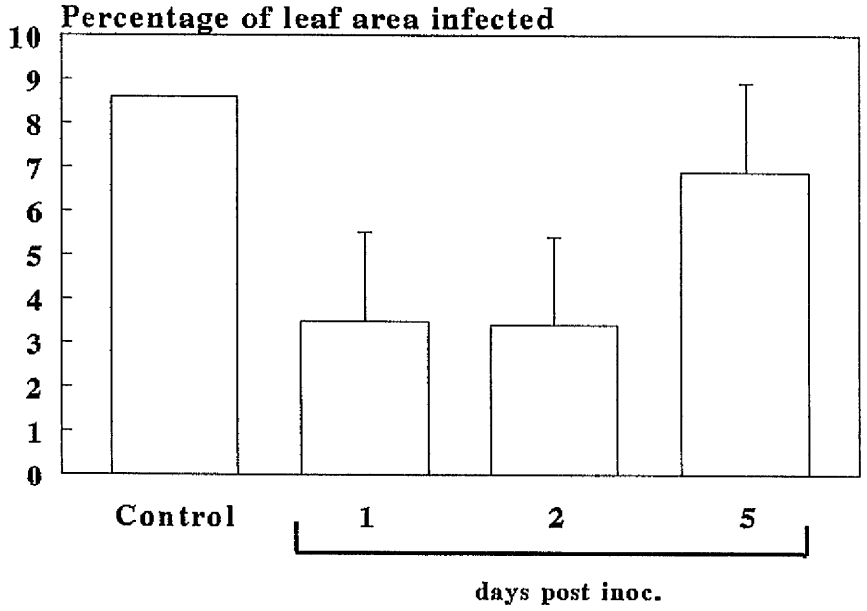
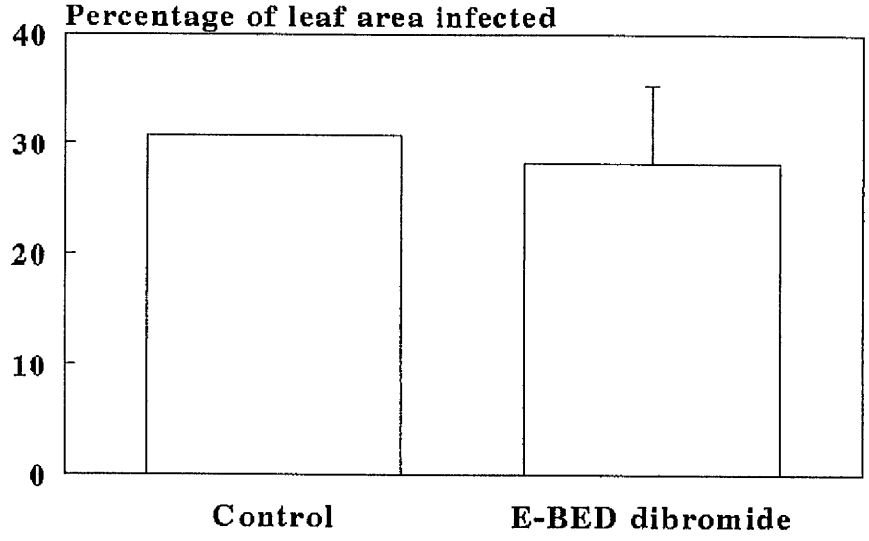

Percentage of leaf area infected
10
9
8
7
6
5
4
3
2
1
0
Control
1
2
5
days post inoc.

FIGURE 11. Effect of the dibromide salt of E-BED on powdery mildew infection of barley, when applied three days post-inoculation at 1.0 mM concentration. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bar represents the least significant difference from the control at $\underline{P} = 0.05$. Figures are the mean of twenty replicates.

FIGURE 12. Effect of four salts of E-BED on powdery mildew infection of barley, when applied three days post-inoculation at 1.0 mM concentrations. Salts used were E-BED benzoate, E-BED phosphate, E-BED fumarate and E-BED propionate. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of twenty replicates.


Percentage of leaf area infected
40
30
20
10
0
Control
E-BED dibromide

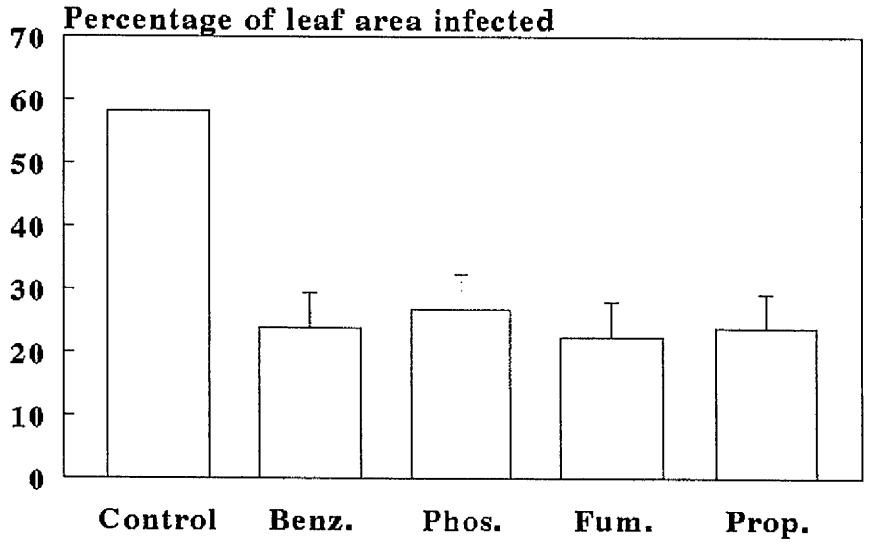
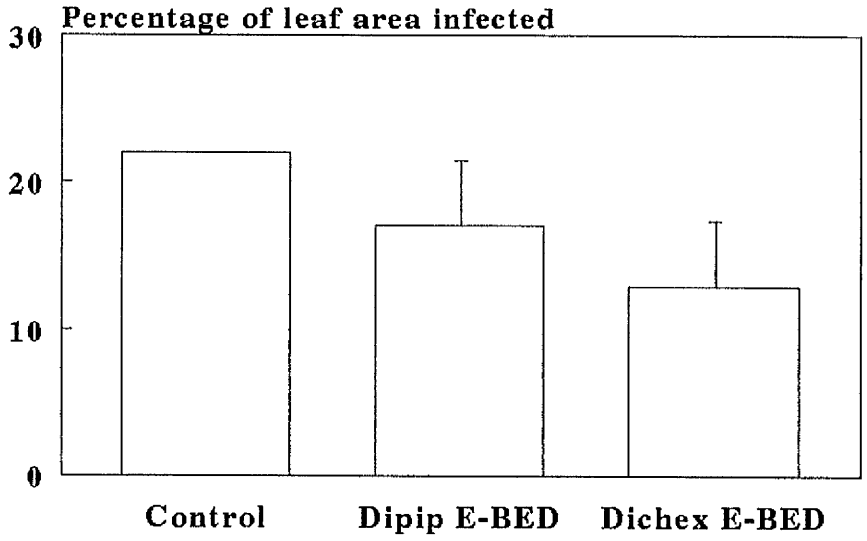

Percentage of leaf area infected
70
60
50
40
30
20
10
0
Control
Benz.
Phos.
Fum.
Prop.

FIGURE 13. Effect of application of two E-BED derivatives on powdery mildew infection of barley, when applied three days post-inoculation at 1.0 mM concentrations. Compounds used were Dipip-E-BED and Dichex-E-BED. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of twenty replicates.

FIGURE 14. Effect of application of four E-BED derivatives on powdery mildew infection of barley, when applied three days post-inoculation at 1.0 mM concentrations.. Compounds used were E-UDED, E-DED, E-DPD and E-DBD. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of twenty replicates.


Percentage of leaf area infected
30
20
10
0
Control
Dipip E-BED
Dichex E-BED

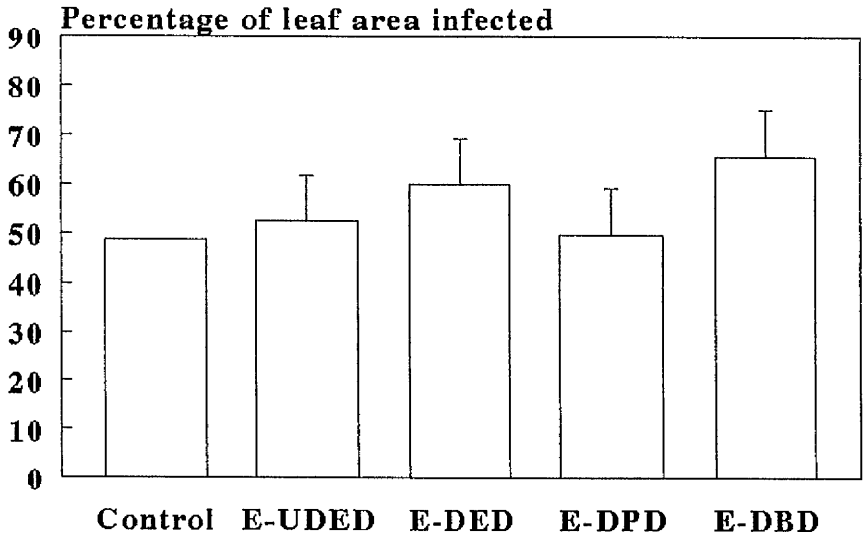
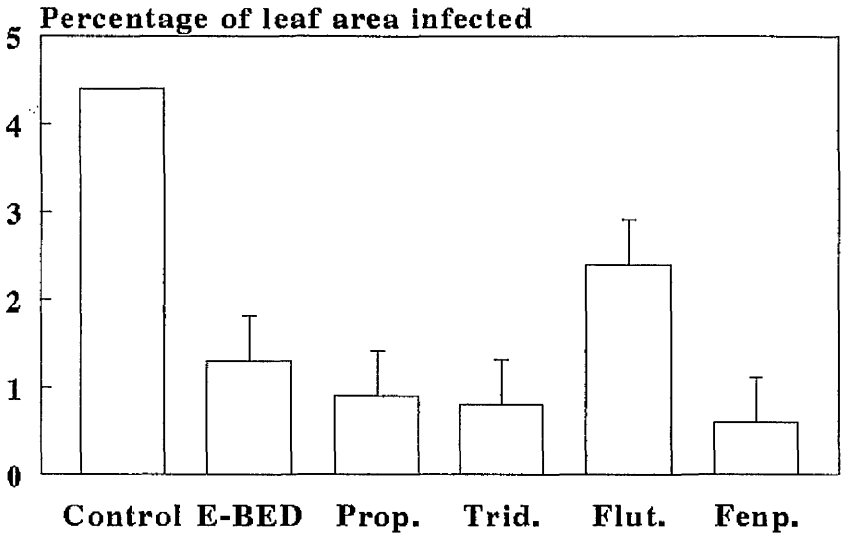

Percentage of leaf area infected
90
80
70
60
50
40
30
20
10
0
Control
E-UDED
E-DED
E-DPD
E-DBD

FIGURE 15. Effect of application of E-BED and four commercial active ingredients on powdery mildew infection of barley, when applied three days post-inoculation. E-BED was applied at 1.0 mM concentration. Active ingredients used were Propiconazole, Tridemorph, Flutriafol and Fenpropidin. All active ingredients were used as 0.1% solutions. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of twenty replicates.


Percentage of leaf area infected
5
4
3
2
1
0
Control
E-BED
Prop.
Trid.
Flut.
Fenp.

3.1.4 Effect of E-BED and it's Derivatives on Fungi grown *In Vitro*

E-BED and Z-BED were shown to significantly inhibit the growth of *Botrytis cinerea, Pyricularia oryzae* and *Pyrenophora avenae in vitro* (Table 6). A 1.0 mM concentration of E-BED reduced the growth of *B. cinerea* by 87%. E-BED gave greater growth inhibition of *B. cinerea* and *P. avenae*, whilst Z-BED gave greater inhibition of *P. oryzae* (Table 6).

3.1.5 Effect of E-BED and it's Derivatives on Enzyme Activities

Enzyme activities were examined in *P. avenae* grown in the presence of E-BED and Z-BED (Table 7). 0.5 mM E-BED and Z-BED reduced ornithine decarboxylase (ODC) activity by 87 and 31% respectively. However, although E-BED reduced S-adenosylmethionine decarboxylase (AdoMetDC) activity, Z-BED produced a substantial increase in enzyme activity. When the effect of Z-BED and E-BED on enzyme activities was examined *in vitro*, Z-BED at 1.0 mM produced a decrease in ODC and AdoMetDC activities (Figures 16 and 17). E-BED at 1.0 mM caused a decrease in ODC activity and a slight increase in AdoMetDC activity, although neither of these changes were significant (Figures 18 and 20). Higher concentrations of E-BED produced larger reductions in *in vitro* ODC activity (Figure 19). *P. avenae* grown in the presence of E-BED was found to have reduced diamine oxidase activity (Figure 21).

TABLE 6. Effect of E-BED and Z-BED on *in vitro* growth of *Botrytis cinerea, Pyricularia oryzae* and *Pyrenophora avenae*. E-BED and Z-BED were used at 1.0 mM against *B. cinerea* and *P.oryzae* and at 0.5 mM against *P. avenae*. Mycelial growth of *P. avenae* was harvested from growth in liquid culture. Significant differences from the control were shown at $\underline{P} = 0.001$ a.

Treatment	**Mycelial growth (mm)**		**Mycelial weight (g)**
	B. cinerea	***P. oryzae***	***P. avenae***
Control	17.0 ±1.2	27.9 ± 0.20	3.8 ± 0.22
E-BED	2.2 ± 0.3 [a]	25.6 ± 0.20 [a]	1.6 ± 0.03 [a]
Z-BED	7.4 ± 0.9 [a]	24.9 ± 0.30 [a]	3.4 ± 0.26 [a]

3.1.6 Effect of E-BED and it's Derivatives on Polyamine Levels

E-BED and it's cis-isomer, Z-BED, exhibited vastly different effects on polyamine levels in *P. avenae*. Z-BED significantly reduced spermidine levels but left putrescine and spermine levels relatively unaltered (Table 7). In contrast, E-BED produced a large increase in putrescine concentration, a significant decrease in spermidine and significant increase in spermine (Table 7).

TABLE 7. Effects of E-BED and Z-BED on ODC and AdoMetDC activity *in vivo* and on polyamine concentrations in *Pyrenophora avenae*. *P avenae* was grown in the presence of 0.5 mM E-BED and Z-BED. Significant differences from the control are shown at P = 0.001 a; P = 0.01 b.

A

Treatment	**Enzyme Activity (pmol CO_2 [mg protein]$^{-1}$ hour^{-1})**	
	ODC	**AdoMetDC**
Control	7.7 ± 0.62	7.4 ± 1.50
E-BED	1.0 ± 0.08 [a]	1.3 ± 0.66 [a]
Z-BED	5.3 ± 0.43 [b]	13.5 ± 2.09 [a]

B

Treatment	**Polyamine concentration (μmol g^{-1} fresh weight)**		
	Putrescine	**Spermidine**	**Spermine**
Control	62.1 ± 11.10	199.6 ± 11.50	47.8 ±3.40
E-BED	538.3 ± 25.20 [a]	135.5 ± 6.30 [a]	76.5 ± 6.91 [a]
Z-BED	62.1 ± 19.6	116.8 ± 4.91 [a]	57.4 ± 3.80

FIGURE 16. Effect of Z-BED on the *in vitro* activity of ornithine decarboxylase from the fungus *Pyrenophora avenae*. Z-BED was used at 1.0 and 0.5 mM concentrations. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of four replicates.

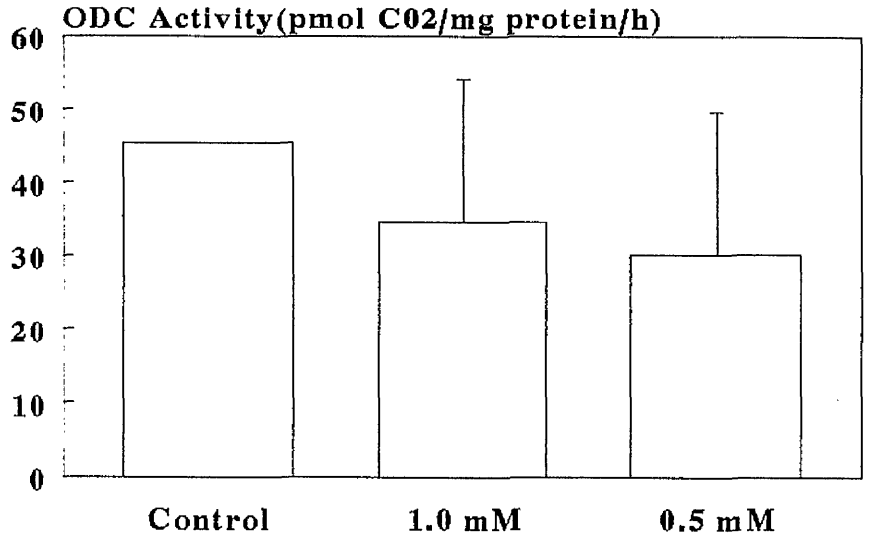
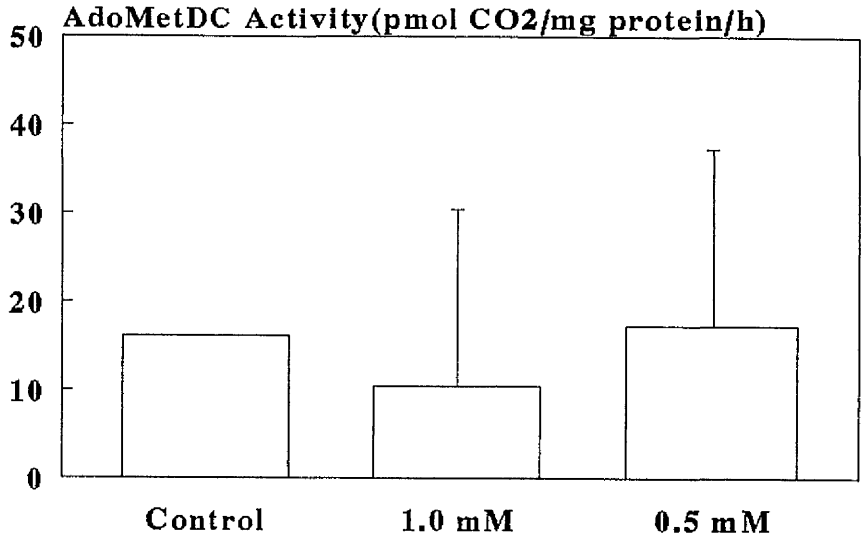

ODC Activity(pmol C02/mg protein/h)
60
50
40
30
20
10
0
Control
1.0 mM
0.5 mM

FIGURE 17. Effect of Z-BED on the *in vitro* activity of S-adenosylmethionine decarboxylase activity from the fungus *Pyrenophora avenae*. Z-BED was used at 1.0 and 0.5 mM concentrations. Vertical bars represent the least significant difference form the control at $\underline{P} = 0.05$. Figures are the means of four replicates.

FIGURE 18. Effect of E-BED on the *in vitro* activity of ornithine decarboxylase from the fungus *Pyrenophora avenae*. E-BED was used at 1.0 and 0.5 mM concentrations. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of four replicates.


AdoMetDC Activity(pmol CO2/mg protein/h)
50
40
30
20
10
0
Control
1.0 mM
0.5 mM

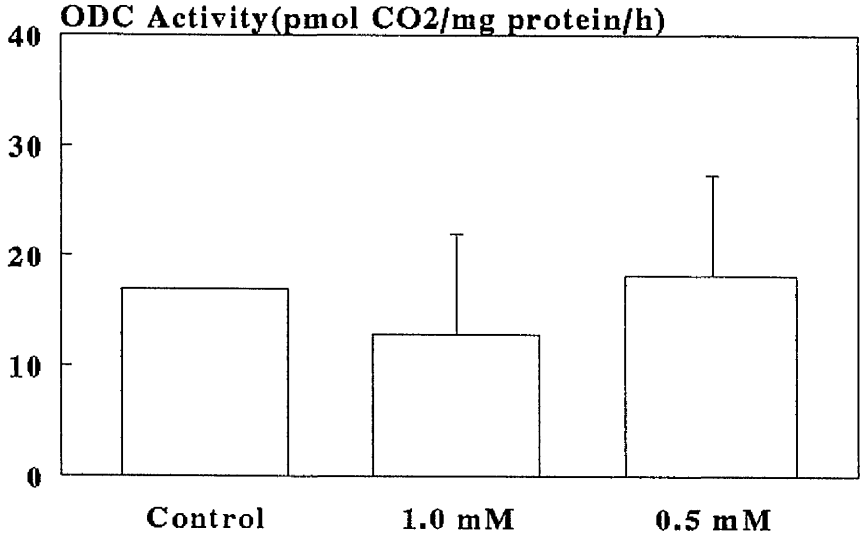
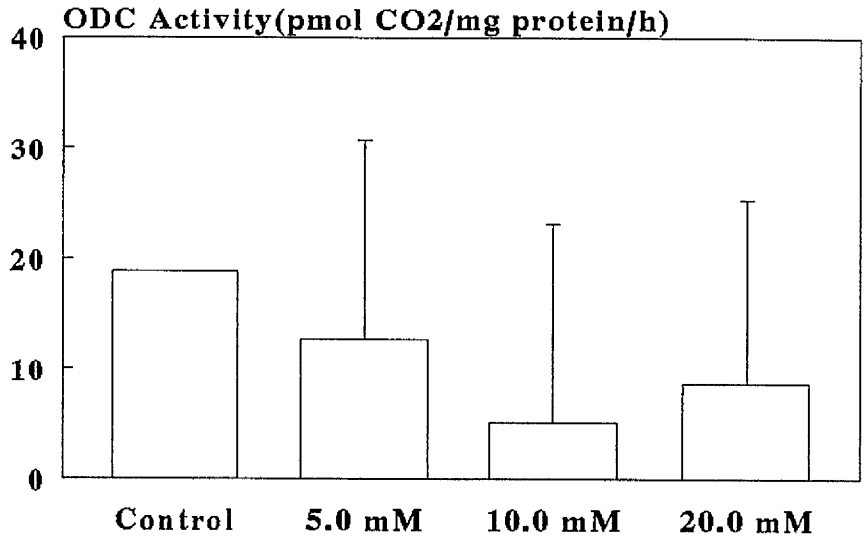

ODC Activity(pmol CO2/mg protein/h)
40
30
20
10
0
Control
1.0 mM
0.5 mM

FIGURE 19. Effect of higher concentrations of E-BED on the *in vitro* activity of ornithine decarboxylase from the fungus *Pyrenophora avenae.* E-BED was used at 5.0, 10.0 and 20.0 mM concentrations. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of four replicates.

FIGURE 20. Effect of E-BED on the *in vitro* activity of S-adenosylmethionine decarboxylase activity from the fungus *Pyrenophora avenae*. E-BED was used at 1.0 and 0.5 mM concentrations. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of four replicates.


ODC Activity(pmol CO2/mg protein/h)
40
30
20
10
0
Control
5.0 mM
10.0 mM
20.0 mM

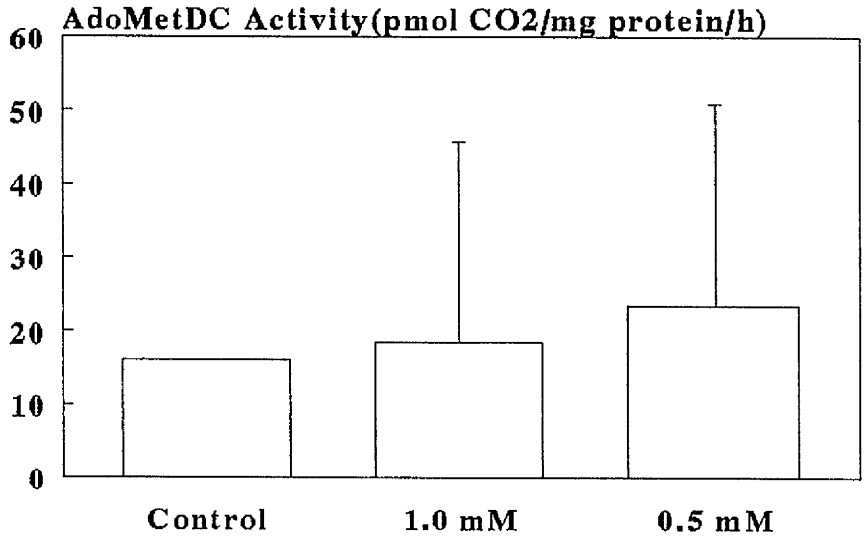
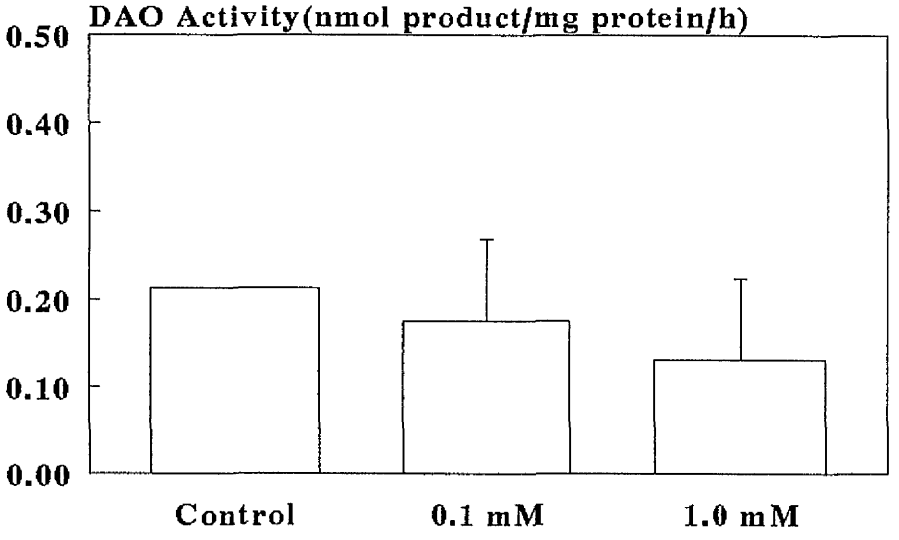

AdoMetDC Activity(pmol CO2/mg protein/h)
60
50
40
30
20
10
0
Control
1.0 mM
0.5 mM

FIGURE 21. Effect of E-BED on the *in vivo* activity of diamine oxidase from the fungus *Pyrenophora avenae*. *P. avenae* was grown in the presence of 0.1 and 1.0 mM E-BED. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of four replicates.


DAO Activity(nmol product/mg protein/h)
0.50
0.40
0.30
0.20
0.10
0.00
Control
0.1 mM
1.0 mM

3.2 EFFECT OF TED-1 AND IT'S DERIVATIVES ON FUNGAL PATHOGENS

3.2.1 Statistical Analysis

The results were analysed as described previously in Section 3.1.1. Least significant differences from the control were calculated using the formulae given previously.

3.2.2 Effect of TED-1 and it's Salts and Derivatives on Infection of barley by *E. graminis*

A post-inoculation spray of 1.0 mM TED-1 was shown to give 80% control of the pathogen when assessed ten days after inoculation (Table 8). Greatest control of infection was achieved when whole plants were sprayed two days post-inoculation (Figure 22 and 23). TED-1 applied as a root drench produced only small, insignificant reductions in infection of barley leaves (Figure 24). When applied to lower leaves, greatest control on upper leaves was achieved when TED-1 was applied two days pre-inoculation (Figure 25). In a glasshouse comparison, 5.0 mM TED-1 compared favourably to a 1% propiconazole solution (85 and 90% control respectively; Figure 26; Figure 29). Of the TED-1 salts examined TED-1 phosphate and TED-1 fumarate gave the best control of *E. graminis* infection (Figure 27).

A derivative of TED-1, DMD, produced a substantial reduction in mildew infection when applied as a post-inoculation treatment (Figure 31). DMD was most effective when applied one day post-inoculation (Figures 32 and 33). As a root drench, DMD proved most effective when applied five days post-inoculation (Figure 34). DMD applied five days pre-inoculation to lower leaves

gave a significant reduction in mildew infection on upper leaves (Figure 35). The cis-isomer of TED-1 also possessed fungicidal activity. A post-inoculation treatment reduced mildew infection by 62% (Figure 37).

3.2.3 Effect of TED-1 and it's Derivatives on Fungal Pathogens *In Vivo*

TED-1 also provided substantial control of *U. viciae-fabae, B. fabae, P. infestans* and *P.leucotricha in vivo* (Table 8; Figure 42). Greatest control was achieved against infection of broad beans by the rust *U. viciae-fabae* (60%). DMD was also shown to inhibit infection by *U. viciae-fabae* (Figure 38), *B. fabae* (Figure 40), *P. infestans* and *P. leucotricha* (Table 9; Figure 43). The cis-isomer of TED-1, cis TED-1, reduced infection of broad bean seedlings by *U. viciae-fabae* (Figure 39) and infection of apple seedlings by *P. leucotricha* (Figure 41).

3.2.4 Effect of TED-1 on Fungi Grown *In Vitro*

TED-1 was found to have only a small, insignificant effect on growth of *P. avenae* and *P. oryzae* on plates (Figures 44 and 45). However, at the same concentration (1.0 mM), TED-1 completely inhibited the growth of *P. avenae* in liquid culture and growth was also significantly reduced at lower concentrations (Figure 46). TED-1 had no significant effect on the growth of *P. oryzae* in liquid culture (Figure 47).

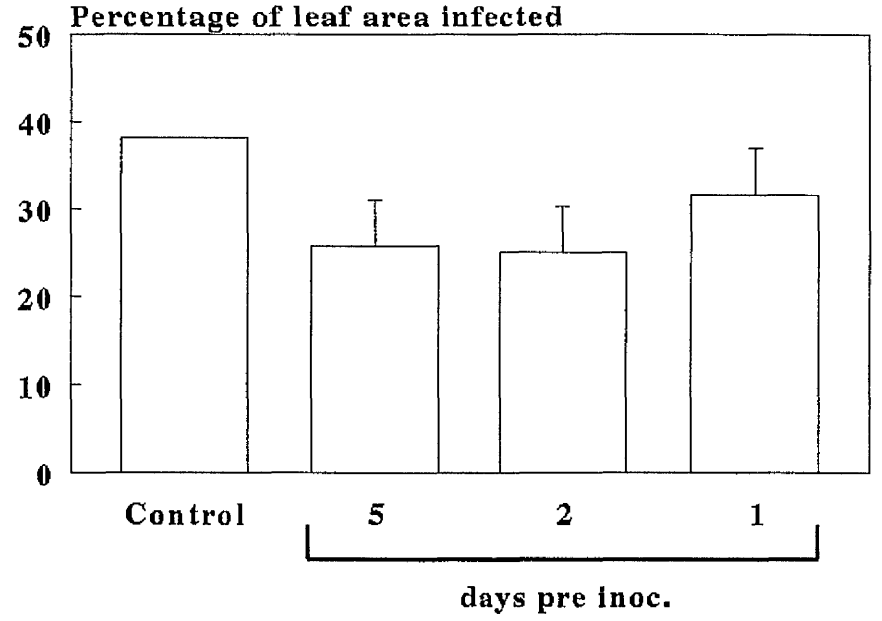
TABLE 8. Fungicidal activity of TED-1. TED-1 was used at 1 mM in all experiments. Potato Leaf Blight was assessed using the following key; 0 = No growth, up to 5 = Leaf disc entirely covered. Apple powdery mildew was assessed using the following key; 0 = No infection, 1 = A few isolated spores, 2 = <50% infection, 3 = >50% infection. Least significant differences from the control at P = 0.05 are shown in the table.

A				
Plant pathogen interaction	**% leaf area infected**		**% disease control**	**L.S.D (P = 0.05)**
	Control	**TED-1**		
Erysiphe graminis / barley post-inoculation	13.9	3.9	80	1.74
Uromyces viciae-fabae / broad bean post-inoculation	9.6	3.8	60	3.81
Botrytis fabae / broad bean post-inoculation	26.9	15.0	45	11.10

B		
Plant pathogen interaction	**Mean disease score**	
	Control	**TED-1**
Phytophthora infestans / potato	5.0	2.5
Podosphaera leucotricha / apples	2.0	1.0

FIGURE 22. Effect of application of 1.0 mM TED-1 to barley seedlings at various times pre-inoculation, on powdery mildew infection. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of ten replicates.

FIGURE 23. Effect of application of 1.0 mM TED-1 to barley seedlings at various times post-inoculation on powdery mildew infection. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of ten replicates.


Percentage of leaf area infected
50
40
30
20
10
0
Control
5
2
1
days pre inoc.

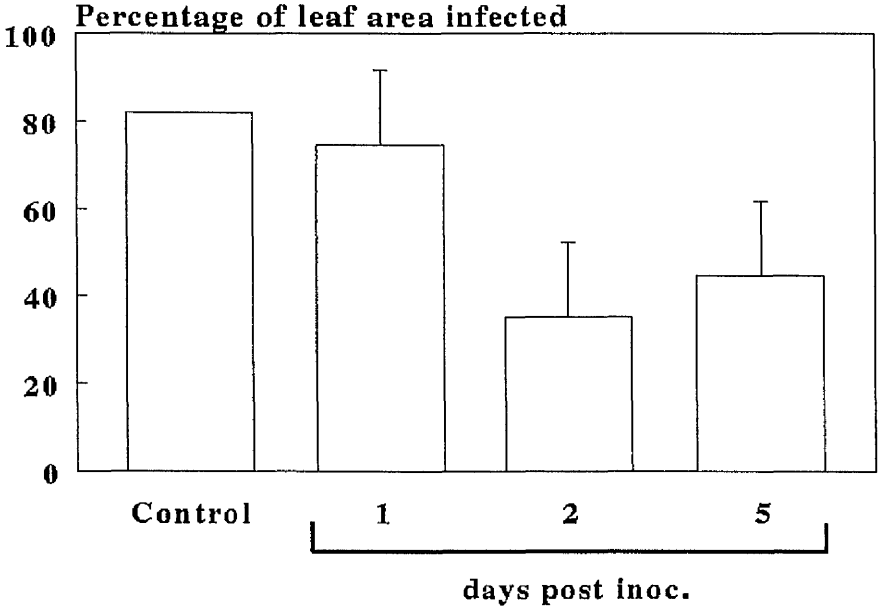
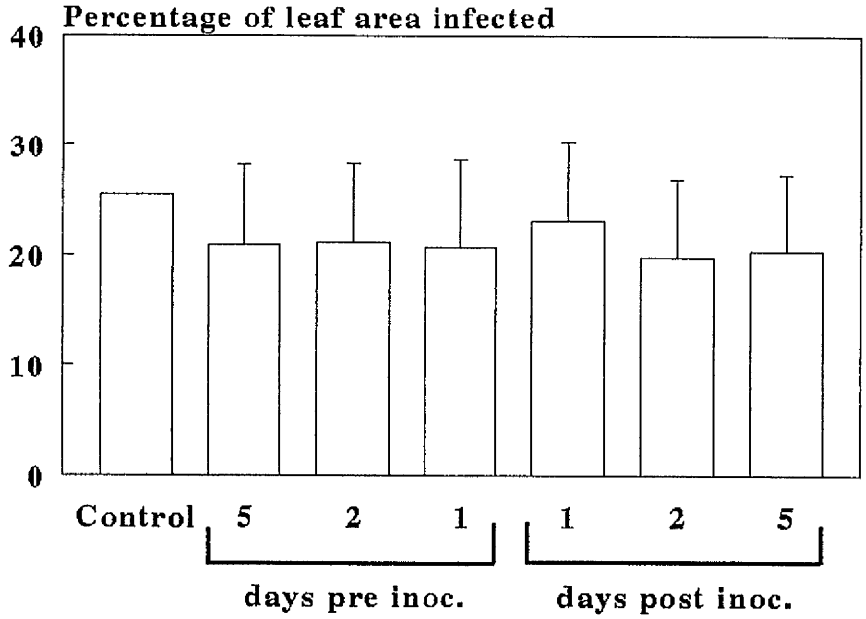

Percentage of leaf area infected
100
80
60
40
20
0
Control
1
2
5
days post inoc.

FIGURE 24. Effect of application of 1.0 mM TED-1 as a root drench to barley seedlings on infection of leaves by powdery mildew. TED-1 was applied at various times both pre- and post-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of ten replicates.

FIGURE 25. Effect of application of 1.0 mM TED-1 to lower barley leaves on infection by powdery mildew on upper leaves. TED-1 was applied at various times both pre- and post-inoculation. Results shown are for the final assessment, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of ten replicates.


Percentage of leaf area infected
40
30
20
10
0
Control
5
2
1
1
2
5
days pre inoc.
days post inoc.

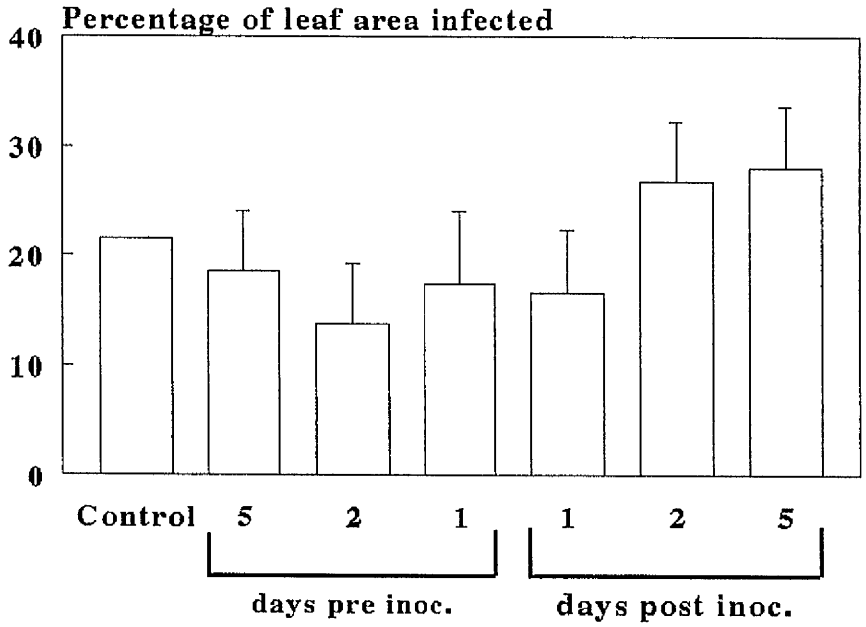
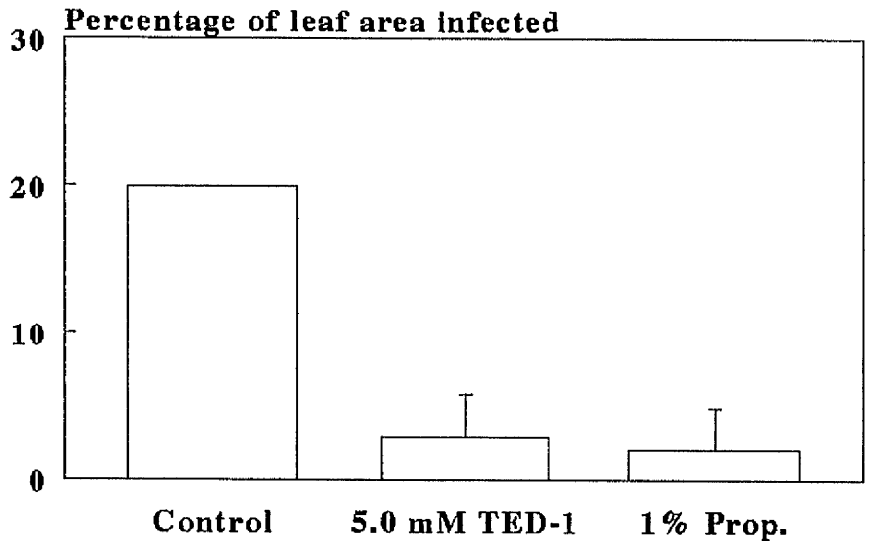

Percentage of leaf area infected
40
30
20
10
0
Control
5
2
1
1
2
5
days pre inoc.
days post inoc.

FIGURE 26. Effect of application of 5.0 mM TED-1 and a 1% solution of the commercial active ingredient, Propiconazole, on powdery infection of barley, when applied three days post-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of twenty replicates.

FIGURE 27. Effect of application of four salts of TED-1 on powdery mildew infection of barley, when applied three days post-inoculation at 1.0 mM concentrations. Salts used were E-TED benzoate, E-TED phosphate, E-TED fumarate and E-TED propionate. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of twenty replicates.


Percentage of leaf area infected
30
20
10
0
Control
5.0 mM TED-1
1% Prop.

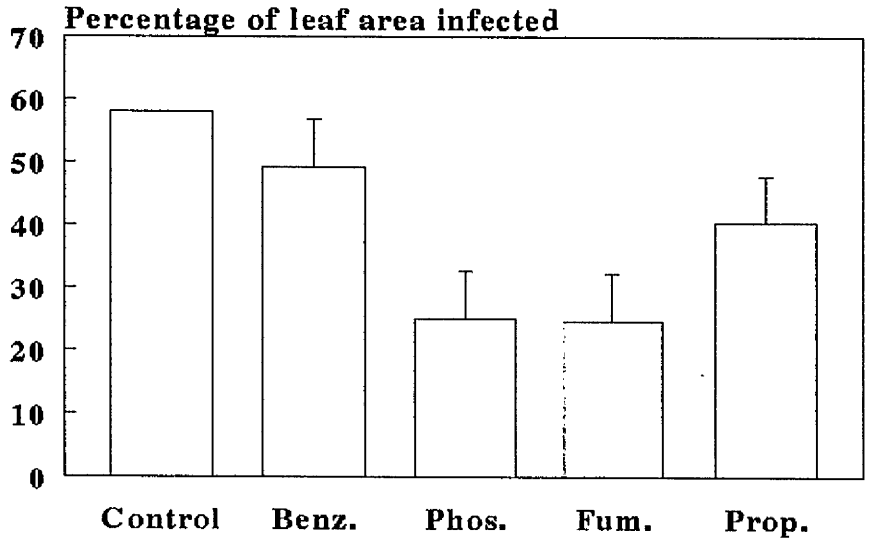
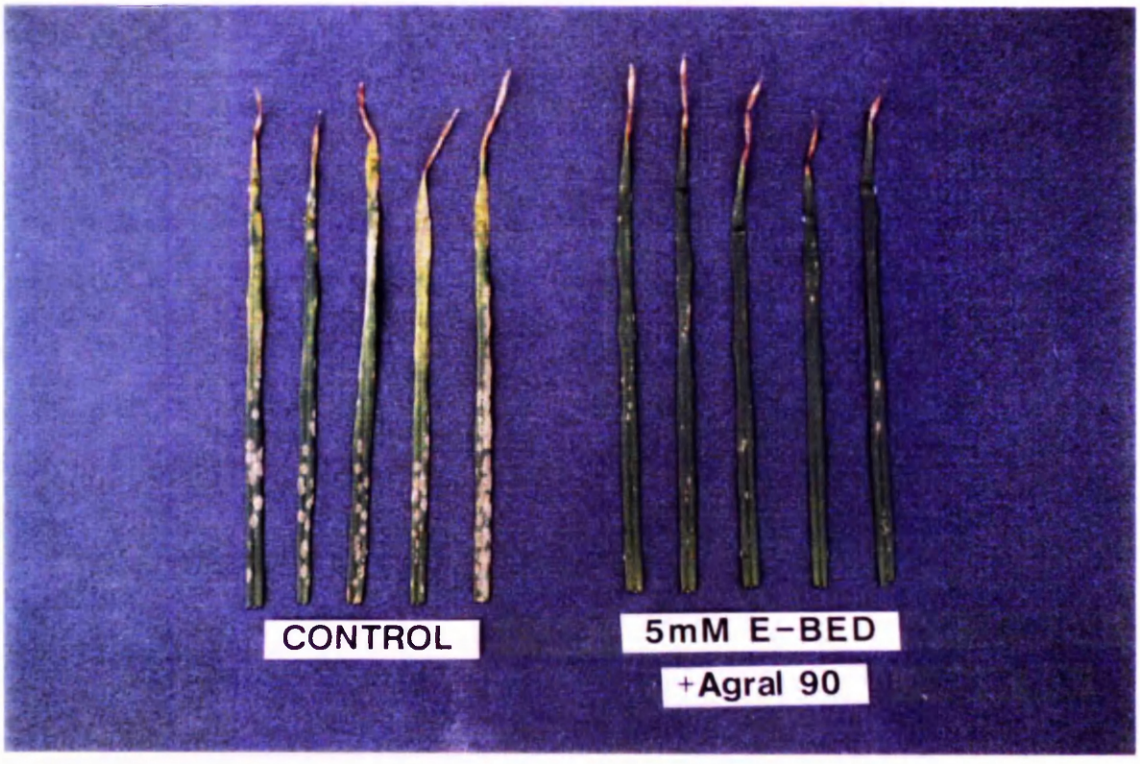

Percentage of leaf area infected
70
60
50
40
30
20
10
0
Control
Benz.
Phos.
Fum.
Prop.

FIGURE 28. Effect of 5.0 mM E-BED + 0.01% (v/v) Agral 90 on powdery mildew infection of barley seedlings, when applied three days post-inoculation. Leaves were detached and photographed ten days post-inoculation.

FIGURE 29. Effect of 5.0 mM TED-1 + 0.01% (v/v) Agral 90 on powdery mildew infection of barley seedlings, when applied three days post-inoculation. Leaves were detached and photographed ten days post-inoculation.


CONTROL
5mM E-BED
+Agral 90

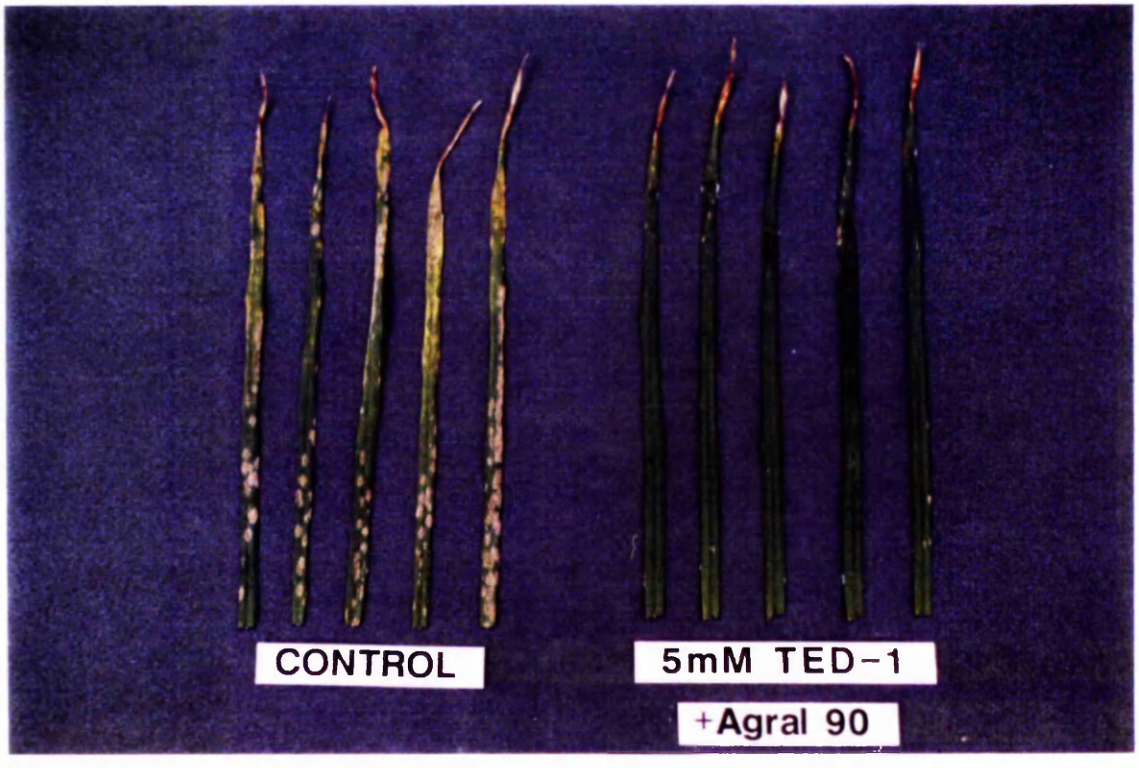
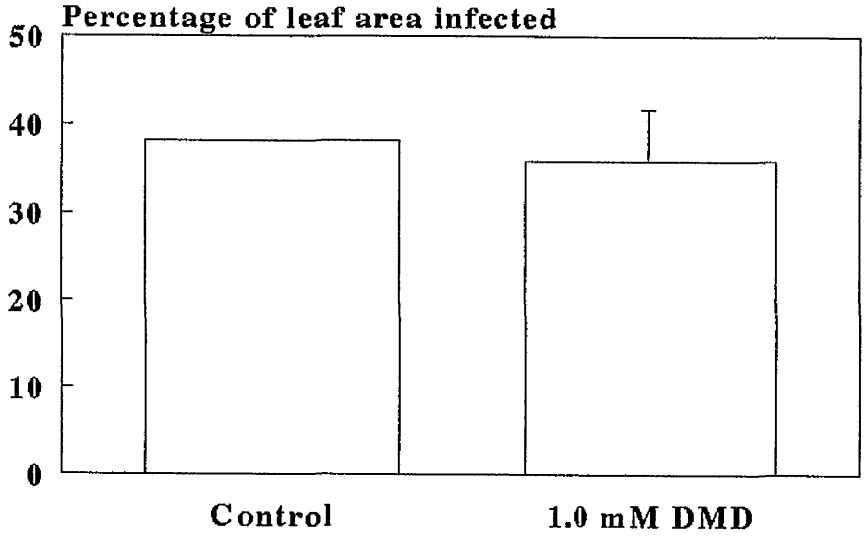

CONTROL
5mM TED-1
+Agral 90

FIGURE 30. Effect of application of 1.0 mM DMD on powdery mildew infection of barley, when applied three hours pre-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of twenty replicates.

FIGURE 31. Effect of application of 1.0 mM DMD on powdery mildew infection of barley, when applied three days post-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of ten replicates.


Percentage of leaf area infected
50
40
30
20
10
0
Control
1.0 mM DMD

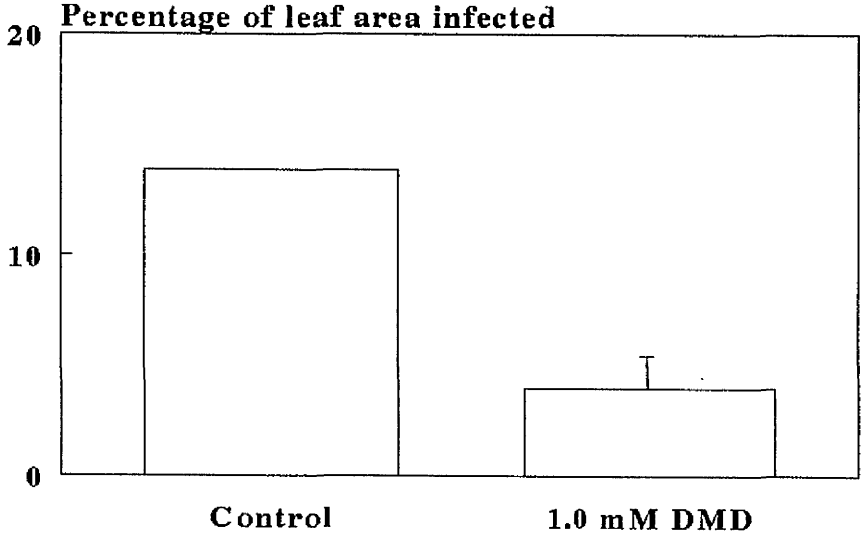
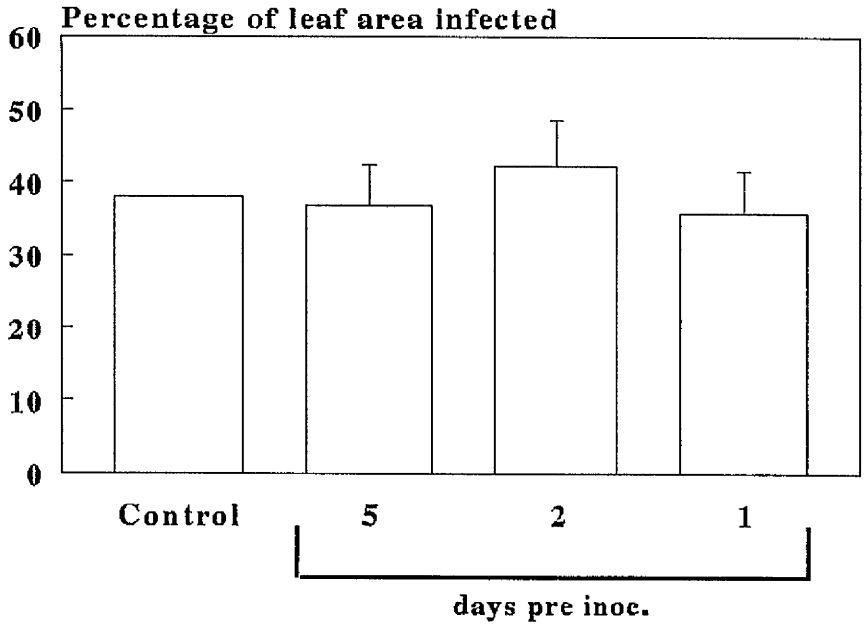

Percentage of leaf area infected
20
10
0
Control
1.0 mM DMD

FIGURE 32. Effect of application of 1.0 mM DMD to barley seedlings at various times pre-inoculation on powdery mildew infection. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the leaves at $\underline{P} = 0.05$. Figures are the means of ten replicates.

FIGURE 33. Effect of application of 1.0 mM DMD to barley seedlings at various times post-inoculation on powdery mildew infection. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference form the control at $\underline{P} = 0.05$. Figures are the means of ten replicates.


Percentage of leaf area infected
60
50
40
30
20
10
0
Control
5
2
1
days pre inoc.

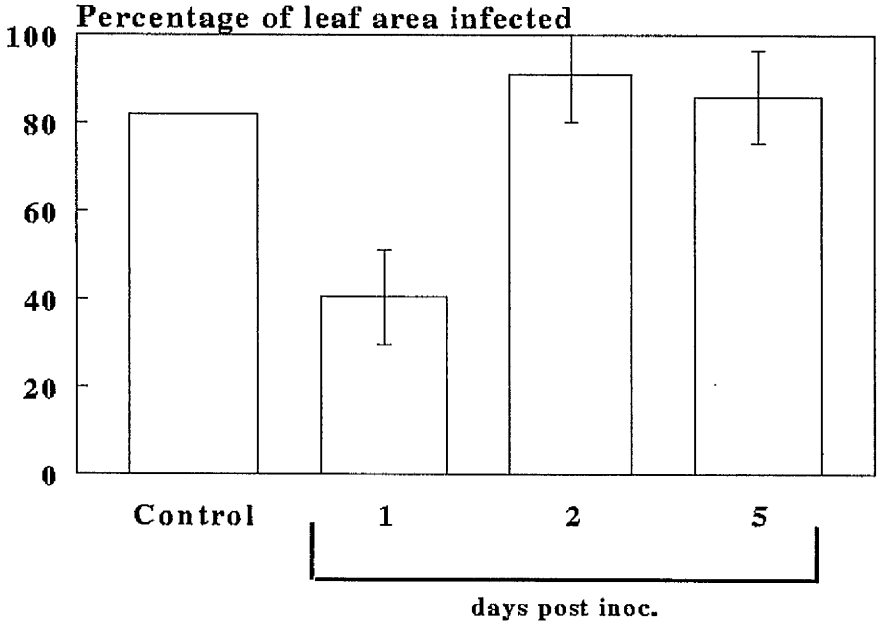
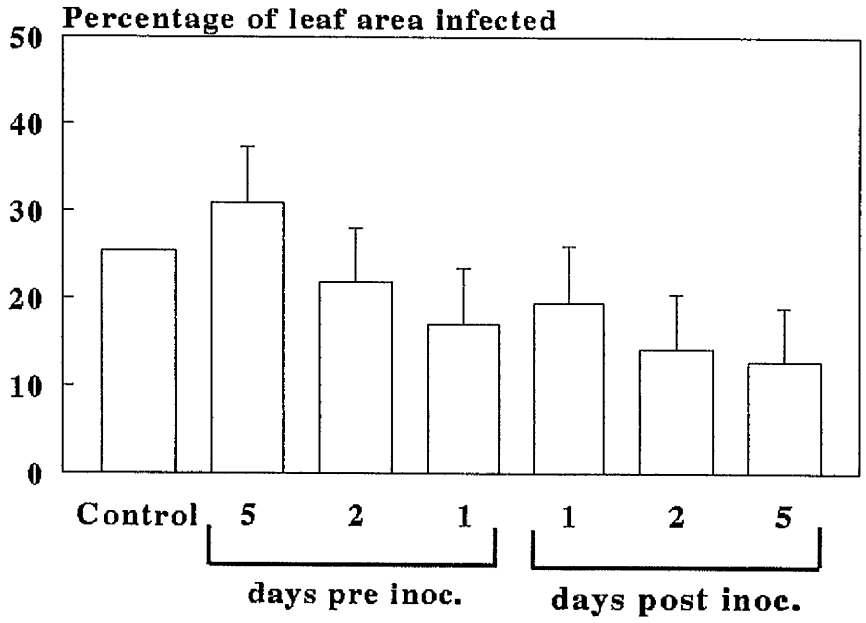

Percentage of leaf area infected
100
80
60
40
20
0
Control
1
2
5
days post inoc.

FIGURE 34. Effect of application of 1.0 mM DMD as a root drench to barley seedlings on infection of leaves by powdery mildew. DMD was applied at various times both pre- and post-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of ten replicates.

FIGURE 35. Effect of application of 1.0 mM DMD to lower barley leaves on infection by powdery mildew on upper leaves. DMD was applied at various times pre- and post-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of ten replicates.


Percentage of leaf area infected
50
40
30
20
10
0
Control
5
2
1
1
2
5
days pre inoc.
days post inoc.

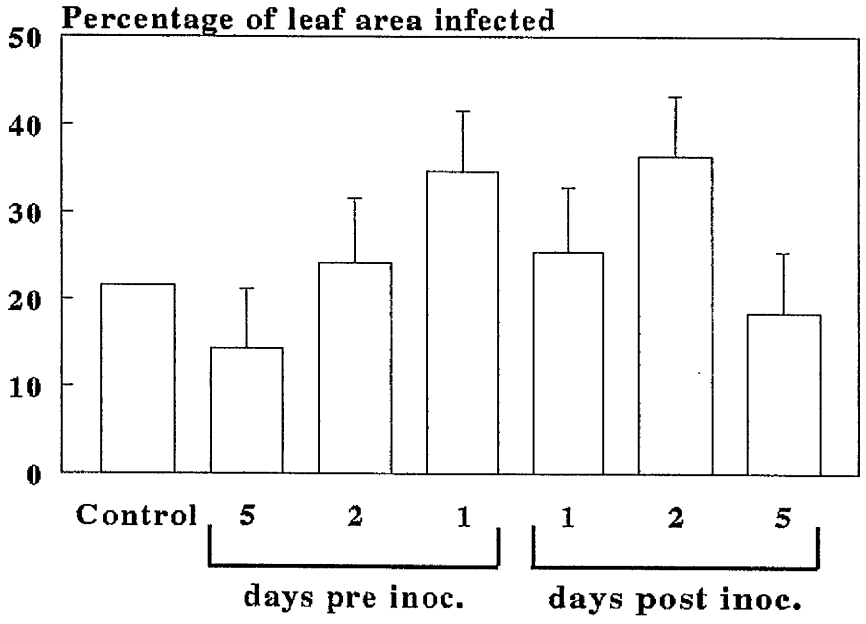
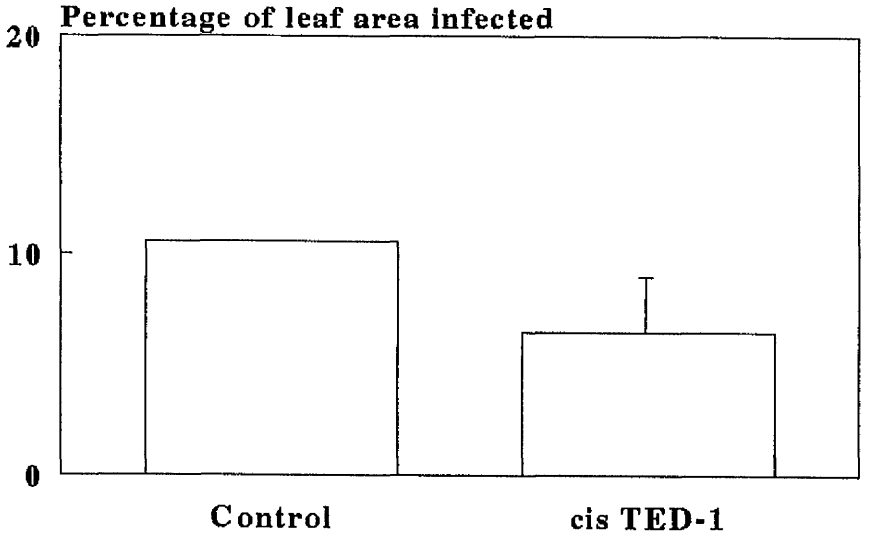

Percentage of leaf area infected
50
40
30
20
10
0
Control
5
2
1
1
2
5
days pre inoc.
days post inoc.

FIGURE 36. Effect of 1.0 mM cis TED-1 on powdery mildew infection of barley when applied three hours pre-inoculation. Results shown are for the final assessment of barley leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at P = 0.05. Figures are the means of ten replicates.

FIGURE 37. Effect of 1.0 mM cis TED-1 on powdery mildew infection of barley, when applied three days post-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at P = 0.05. Figures are the means of twenty replicates.


Percentage of leaf area infected
20
10
0
Control
cis TED-1

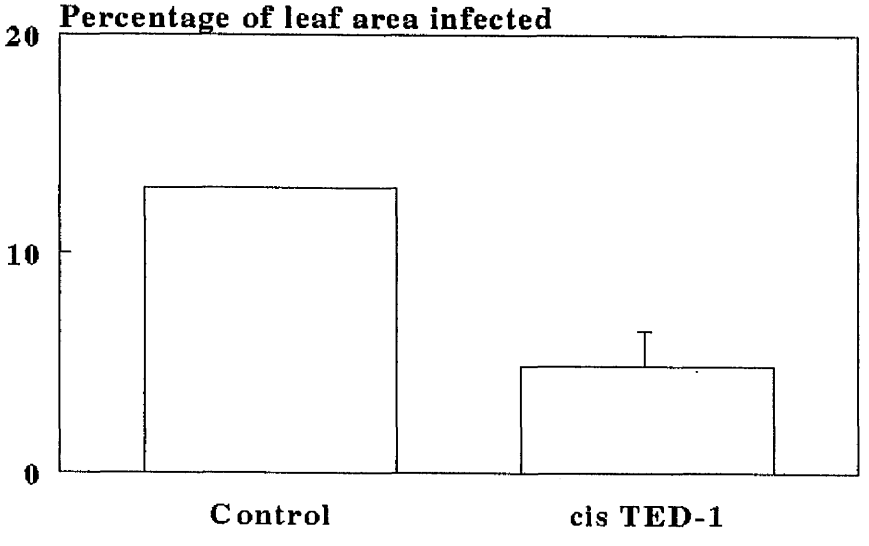
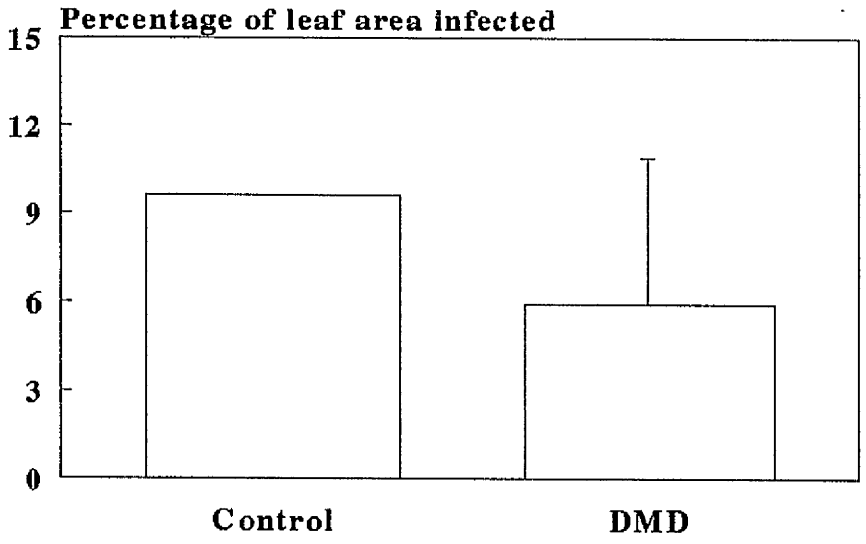

Percentage of leaf area infected
20
10
0
Control
cis TED-1

FIGURE 38. Effect of 1.0 mM DMD on rust infection of broad bean leaves when applied three days post-inoculation. Results shown are for the final assessment of leaves, seventeen days post-inoculation. Vertical bars represent the least significant difference form the control at $\underline{P} = 0.05$. Figures are the means of six replicates.

FIGURE 39. Effect of 1.0 mM cis TED-1 on rust infection of broad bean leaves , when applied three days post-inoculation. Results shown are for the final assessment of leaves, seventeen days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of six replicates.


Percentage of leaf area infected
15
12
9
6
3
0
Control
DMD

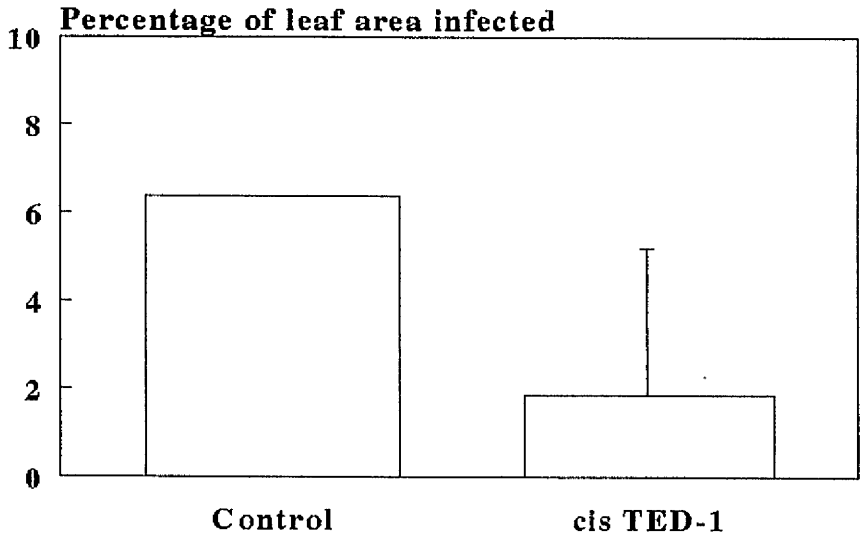
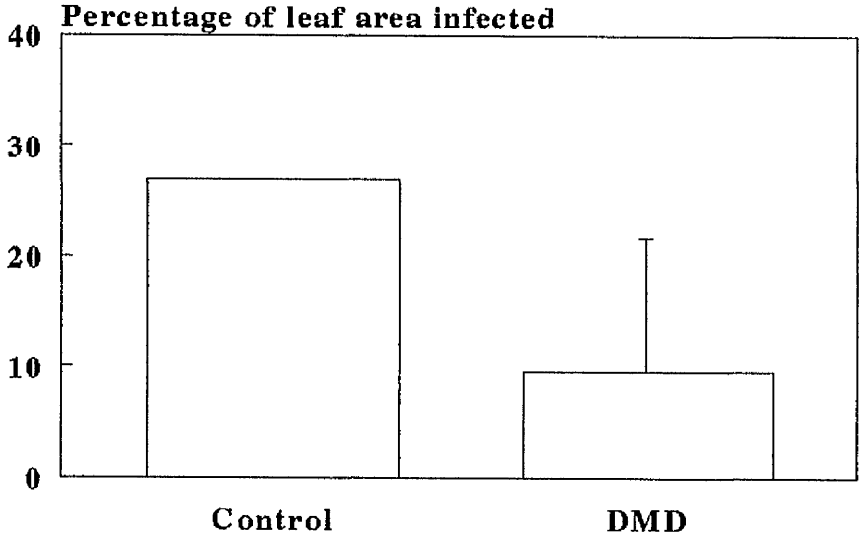

Percentage of leaf area infected
10
8
6
4
2
0
Control
cis TED-1

FIGURE 40. Effect of 1.0 mM DMD on chocolate spot infection of broad bean leaves, when applied three days post-inoculation. Results shown are for the final assessment of leaves, seven days post-inoculation. Vertical bars represent the least significant difference from the control at P = 0.05. Figures are the means of six replicates.

FIGURE 41. Effect of 1.0 mM cis TED-1 on powdery mildew infection of apple seedlings, when applied three days post-inoculation. Results shown are for the final assessment of leaves, seventeen days post-inoculation. Vertical bars represent the least significant difference from the control at P = 0.05 . Figures are the means of six replicates.


Percentage of leaf area infected
40
30
20
10
0
Control
DMD

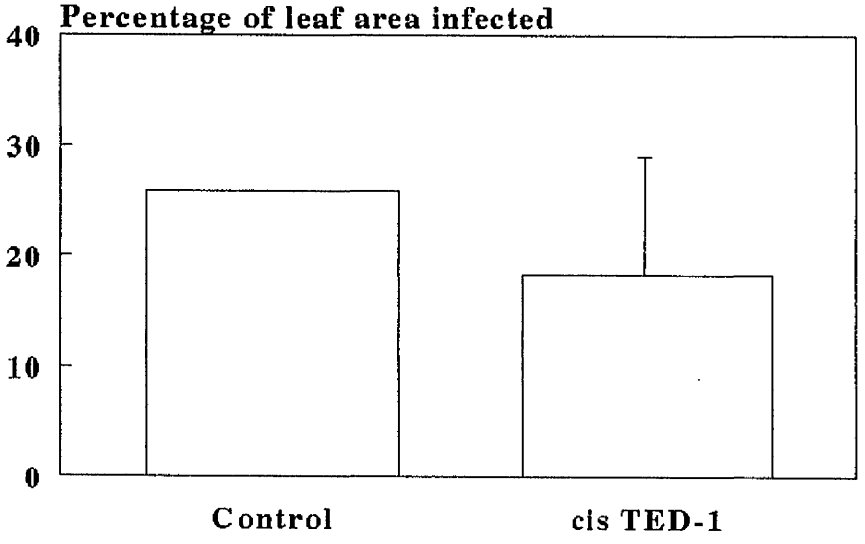

Percentage of leaf area infected
40
30
20
10
0
Control
cis TED-1

TABLE 9. Effect of DMD on *Phytophthorainfestans* and *Podosphaera leucotricha.* Potato leaf blight was assessed using the following key; 0 = No infection, up to 5 = Leaf disc entirely covered. Apple powdery mildew was assessed using the following key; 0 = No infection, 1 = A few isolated spores, 2 = <50% leaf area infected, 3 = >50% leaf area infected. All figures are the means of six replicates.

Plant-pathogen interaction	**Mean Disease Score**		
	Control	**1.0 mM DMD**	**5.0 mM DMD**
Phytophthora infestans/ potato	5.0	5.0	3.5
Podosphaera leucotricha/ apple	1.83	1.67	

FIGURE 42. Effect of 1.0 mM TED-1 + 0.01% (v/v) Tween 20, on mildew infection of apple seedlings, when applied three days post-inoculation. Photographs were taken seventeen days post-inoculation.

FIGURE 43. Effect of 1.0 mM DMD + 0.01% (v/v) Tween 20, on mildew infection of apple seedlings, when applied three days post-inoculation. Photographs were taken seventeen days post-inoculation.


1mM TED-1
CONTROL
+ Tween 20


1mM DMD
CONTROL
+ Tween 20

3.2.5 Effect of TED-1 on Polyamine Levels

TED-1 at two different concentrations affected polyamine concentrations in *P. avenae*. Both 0.5 mM and 0.1 mM concentrations significantly reduced putrescine and spermidine levels, with a greater effect produced by 0.5 mM TED-1. Spermine levels remained unaffected by exposure to TED-1 (Table 10).

3.2.6 Effect of TED-1 on Enzyme Activity

P. avenae grown in the presence of TED-1 at 0.1 mM concentration exhibited increased ODC activity and a reduced AdoMetDC activity, although neither of these changes were significant (Table 11). The fungus also exhibited a greatly reduced diamine oxidase activity (Figure 50).

3.2.7 Further Attempts to Elucidate Biochemical Mode of Action of TED-1

Since it appeared unlikely that perturbation of polyamine metabolism could account fully for the fungicidal activity of TED-1, further experiments were conducted to determine whether TED-1 might also possess other modes of action. As described in Section 2.5.2.5, an experiment was performed to determine whether TED-1 replaces putrescine from intracellular binding sites. In *P. avenae* grown in the presence of 0.5 mM TED-1 and allowed to take up ^{14}C-Putrescine, more label appeared in the external medium taken from *P. avenae* control (Figure 49).

Experiments were also performed in which 0.5 mM Ascorbic acid or 0.5 mM Calcium Chloride were added to the growth media, along with 0.5 mM TED-1. Ascorbic acid mops up free radicals and was used to test the hypothesis that fungal diamine oxidase might use TED-1 (a putrescine analogue) as a substrate,

generating hydrogen peroxide and free radicals. The latter would damage fungal membranes and reduce growth. Calcium chloride was used to test wether TED-1 displaces Ca^{2+} from endogenous binding sites. Addition of 0.5 mM Ascorbic acid to the medium further inhibits fungal growth (Figure 48), while 0.5 mM $CaCl_2$ produces a small increase in fungal growth, although mycelial yields were still significantly less than in control flasks (Figure 48).

TABLE 10. Effect of TED-1 on *in vivo* ODC and AdoMetDC activities in *Pyrenophora avenae.* Least significant differences from the control are shown at P = 0.05 in the table. Figures are the means of four replicates.

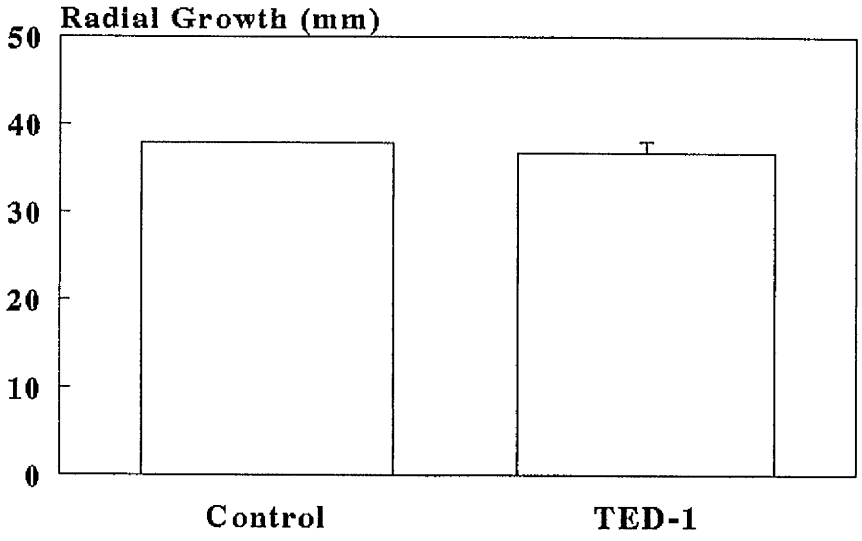
Treatment	Enzyme Activity (pmol CO_2 [mg protein]$^{-1}$ hour^{-1})	
	ODC	AdoMetDC
Control	8.90	31.80
0.1 mM TED-1	16.14	12.07
L.S.D at P = 0.05	9.63	41.49

TABLE 11. Effect of TED-1 on polyamine concentrations in *Pyrenophora avenae.* Least significant differences from the control are shown at P = 0.05. Figures are the means of four replicates.

Treatment	Polyamine concentration (µmol g^{-1} fresh weight)		
	Putrescine	Spermidine	Spermine
Control	8.38	16.55	41.93
0.5 mM TED-1	3.55	10.78	38.40
0.1 mM TED-1	4.22	13.45	40.07
L.S.D at P = 0.05	1.91	2.10	17.81

FIGURE 44. Effect of 1.0 mM TED-1 on the growth of the fungus, *Pyrenophora avenae* on plates. Results shown are for the final measurements of the plates, six days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of six replicates.

FIGURE 45. Effect of 1.0 mM TED-1 on the growth of the fungus, *Pyricularia oryzae* on plates. Results shown are for the final measurements of the plates, eight days post-inoculation. Vertical bars represent the least significant difference form the control at $\underline{P} = 0.05$. Figures are the means of six replicates.


Radial Growth (mm)
50
40
30
20
10
0
Control
TED-1

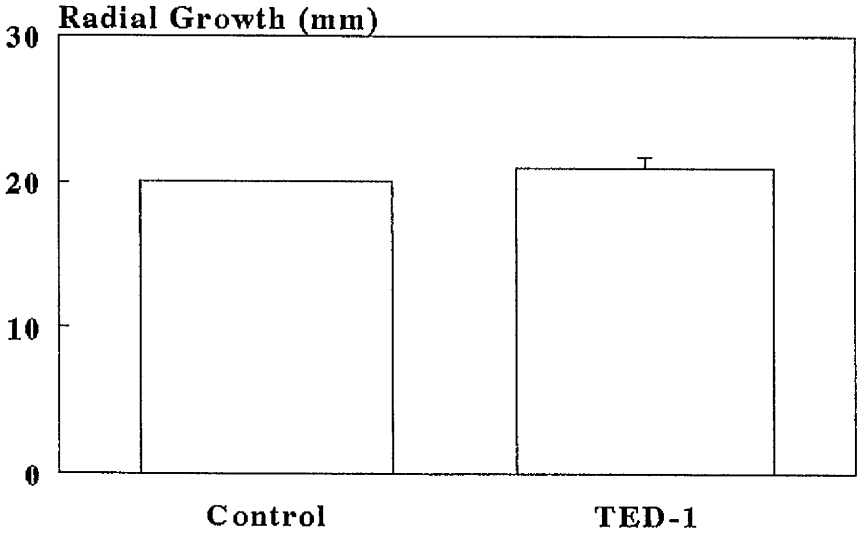
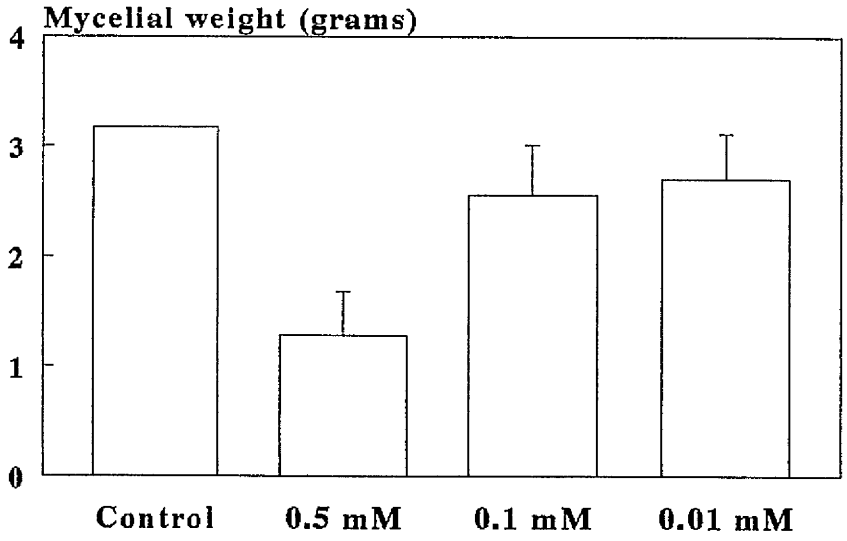

Radial Growth (mm)
30
20
10
0
Control
TED-1

FIGURE 46. Effect of TED-1 at various concentrations, on the growth of the fungus, *Pyrenophora avenae* in liquid culture. Results shown are for the fungus harvested after 4 days. TED-1 was used at 0.5, 0.1 and 0.01 mM concentrations. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of four replicates.

FIGURE 47. Effect of 1.0 mM TED-1 on the growth of the fungus, *Pyricularia oryzae* in liquid culture. Results shown are for the fungus harvested after four days. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of four replicates.


Mycelial weight (grams)
4
3
2
1
0
Control
0.5 mM
0.1 mM
0.01 mM

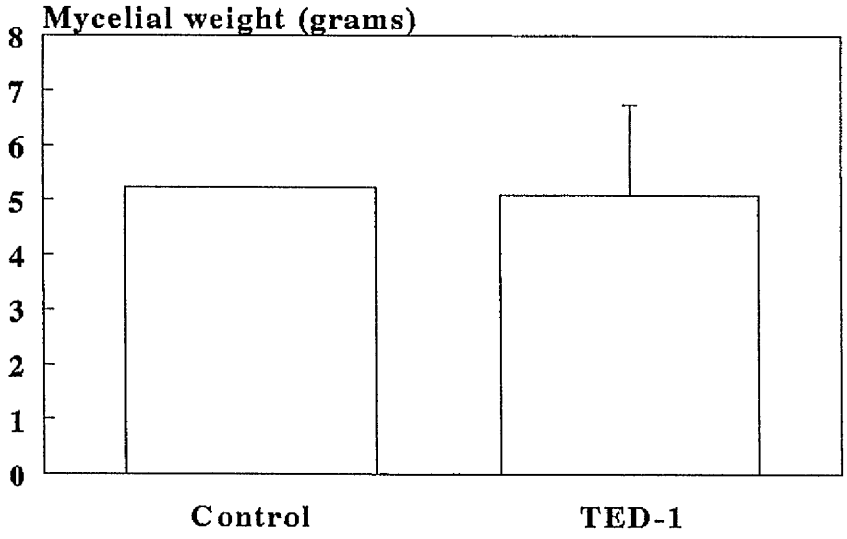
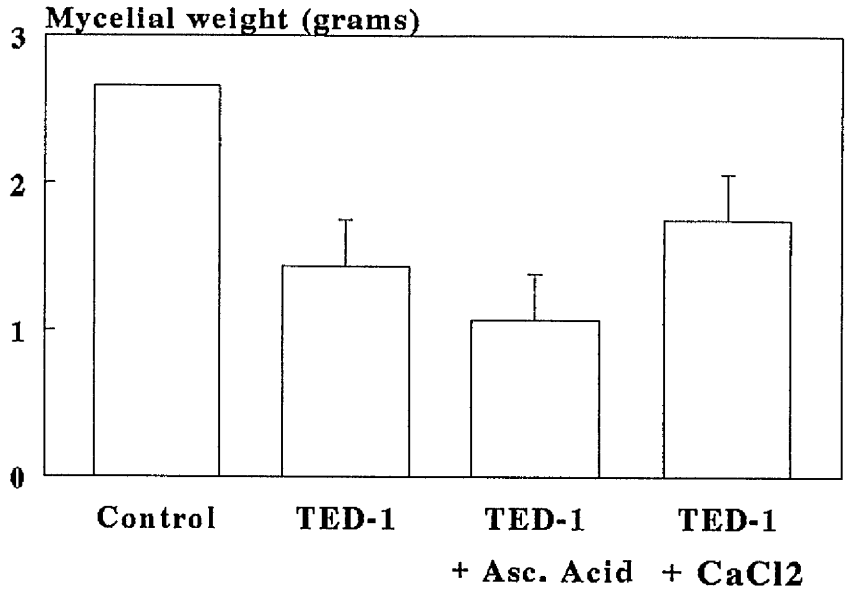

Mycelial weight (grams)
8
7
6
5
4
3
2
1
0
Control
TED-1

FIGURE 48. Effect of TED-1 alone and in combination with Ascorbic Acid or Calcium chloride, on the growth of the fungus, *Pyrenophora avenae* in liquid culture. Results shown are for the fungus harvested after four days. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of four replicates.

FIGURE 49. Efflux of ^{14}-C Putrescine into the external media from *P. avenae* grown previously in 0.5 mM TED-1 (see Section 3.2.7). Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of five replicates.


Mycelial weight (grams)
3
2
1
0
Control
TED-1
TED-1
+ Asc. Acid
TED-1
+ CaCl2

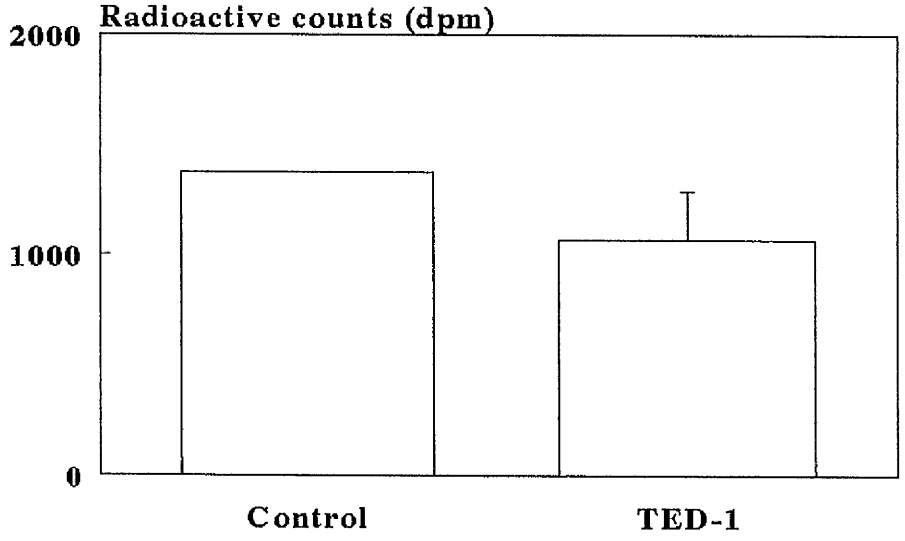
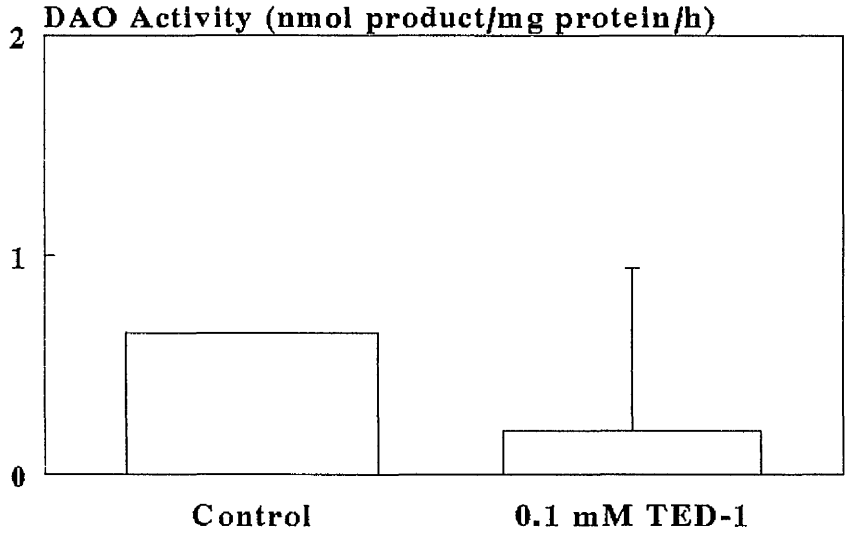

Radioactive counts (dpm)
2000
1000
0
Control
TED-1

FIGURE 50. Effect of 0.1 mM TED-1 on diamine oxidase activity in the fungus, *Pyrenophora avenae*. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of four replicates.


DAO Activity (nmol product/mg protein/h)
2
1
0
Control
0.1 mM TED-1

3.3 EFFECT OF BAD AND OTHER CYCLIC COMPOUNDS ON FUNGAL PATHOGENS

3.3.1 Statistical Analysis

Results were analysed as described in Section 3.1.1 i.e. least significant differences from the control were calculated using the formulae given previously.

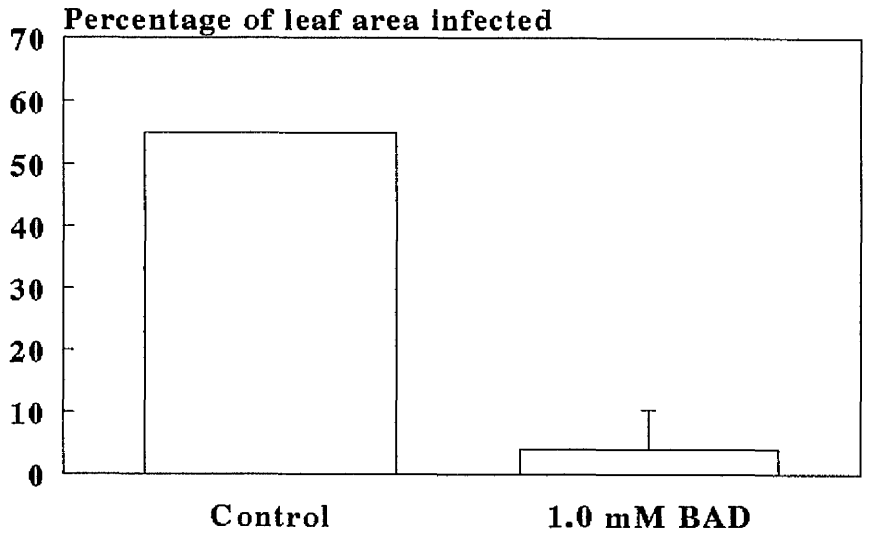
3.3.2 Effect of BAD and other Cyclic Compounds on Infection of Barley by *E. graminis*

Post-inoculation application of BAD gave substantial control of powdery mildew (Figures 51 and 52). Thus, a 1.0 mM post-inoculation treatment of BAD gave 93% control of *E. graminis* infection of barley seedlings (Figure 51). Pre-inoculation treatments also gave good control of *E. graminis* (Figure 53). However, BAD gave the most effective control of *E. graminis* when applied two days post-inoculation (Figure 54). When BAD was applied to barley seedlings as a root drench, only post-inoculation treatments gave significant control of *E. graminis* infection (Figure 56). Application of BAD to lower leaves gave no control of infection of upper leaves (Figure 57). . In a glasshouse comparison with active ingredients, 1.0 mM BAD achieved comparable control to 0.1% solutions of four commercial active ingredients and outperformed one, flutriafol (Figures 58-60).

Other cyclic compounds also controlled infection by *E. graminis*. The most effective control was given by post-inoculation treatments of trans-BAD (66%; Figure 61), DPH (78%; Figure 64) and TCCBM (87%; Figure 65).

FIGURE 51. Effect of 1.0 mM BAD on powdery mildew infection of barley seedlings, when applied three days post-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference form the control at $\underline{P} = 0.05$. Figures are the means of twenty replicates.

FIGURE 52. Effect of 5.0 mM BAD on powdery mildew infection of barley seedlings, when applied three days post-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of twenty replicates.


Percentage of leaf area infected
70
60
50
40
30
20
10
0
Control
1.0 mM BAD

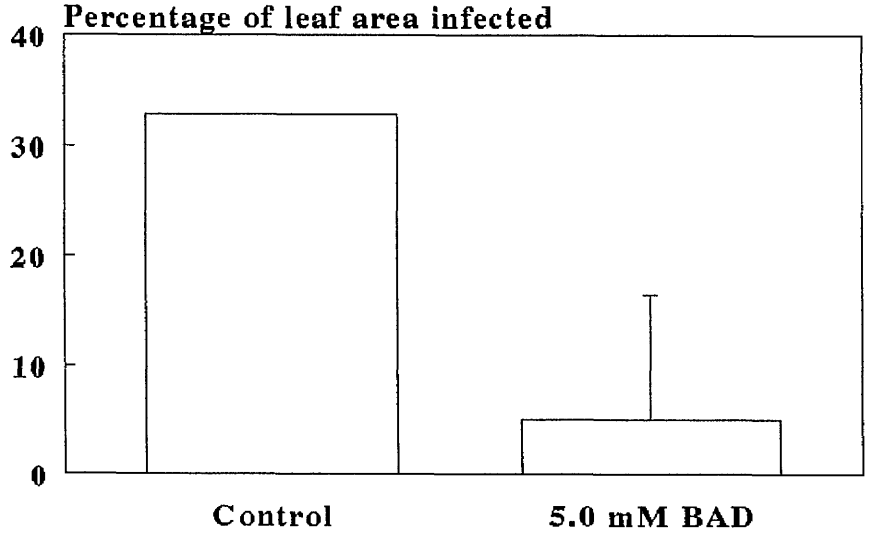
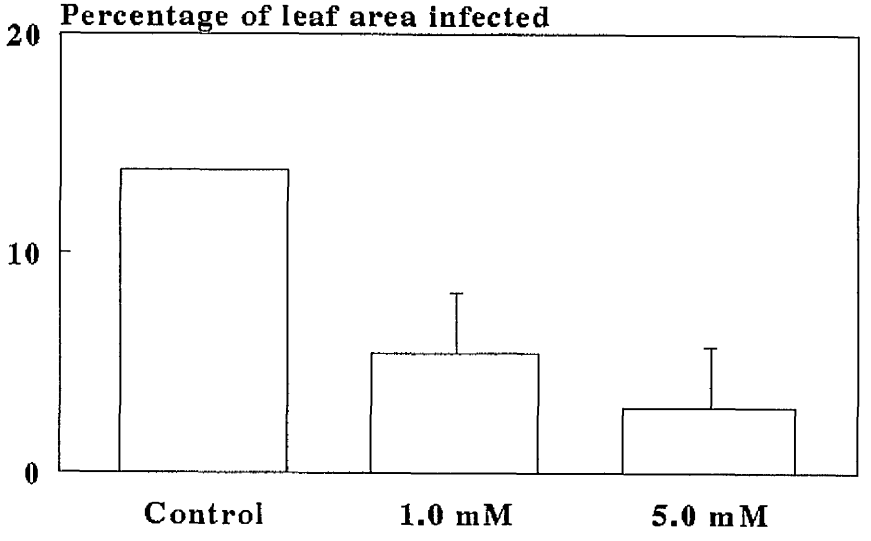

Percentage of leaf area infected
40
30
20
10
0
Control
5.0 mM BAD

FIGURE 53. Effect of 1.0 and 5.0 mM BAD on powdery mildew infection of barley seedlings, when applied three hours pre-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of twenty replicates.

FIGURE 54. Effect of application of 1.0 mM BAD to barley seedlings at various times, both pre- and post-inoculation, on powdery mildew infection of leaves. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of ten replicates.


Percentage of leaf area infected
20
10
0
Control
1.0 mM
5.0 mM

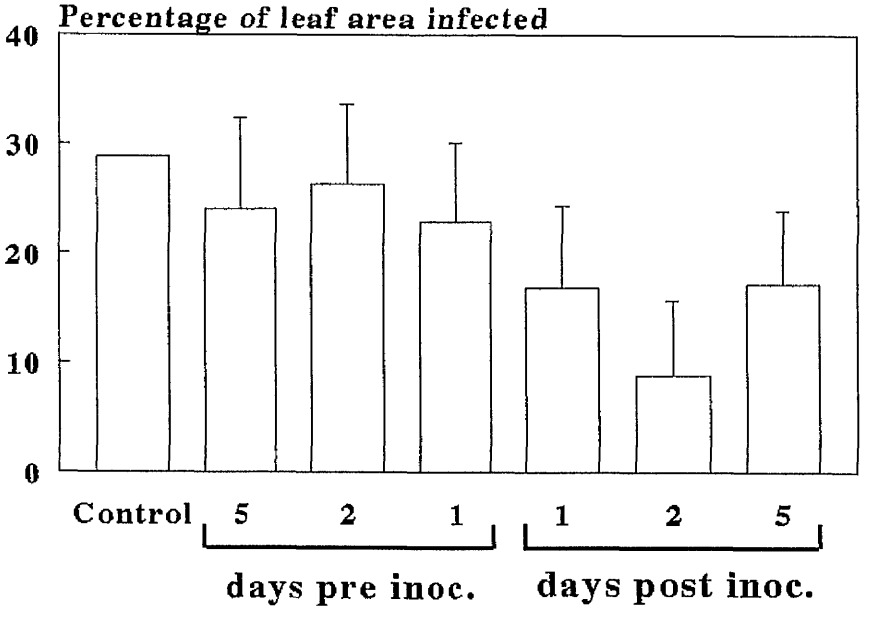
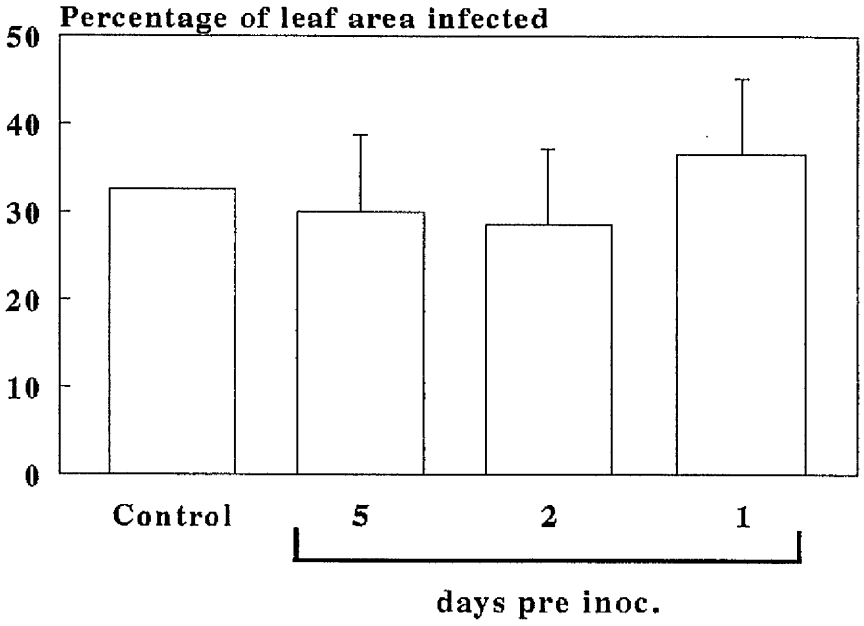

Percentage of leaf area infected
40
30
20
10
0
Control
5
2
1
1
2
5
days pre inoc.
days post inoc.

FIGURE 55. Effect of application of 1.0 mM BAD as a root drench to barley seedlings, on infection of leaves by powdery mildew. BAD was applied at various times pre-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at P = 0.05. Figures are the means of ten replicates.

FIGURE 56. Effect of application of 1.0 mM BAD as a root drench to barley seedlings, on infection of leaves by powdery mildew. BAD was applied at various times post-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at P = 0.05. Figures are the means of ten replicates.


Percentage of leaf area infected
50
40
30
20
10
0
Control
5
2
1
days pre inoc.

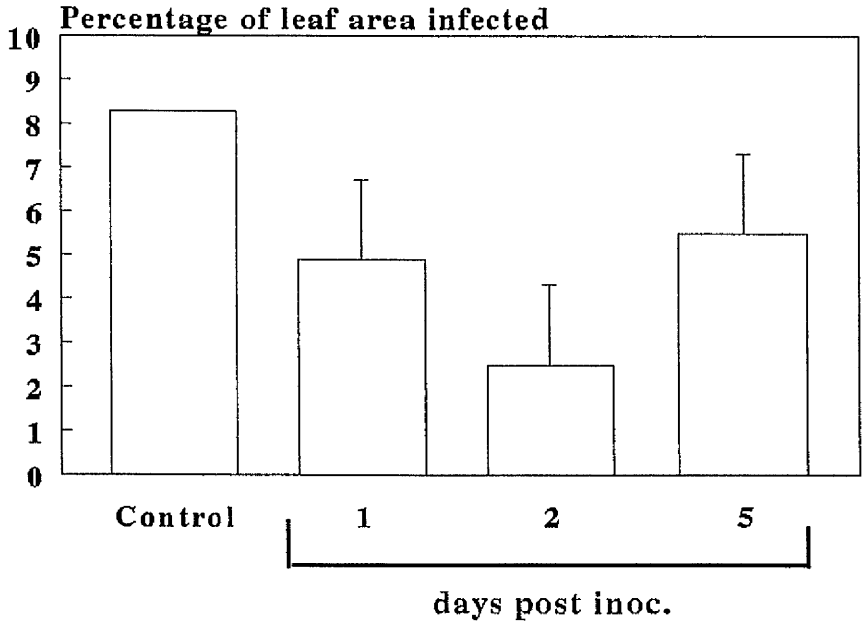
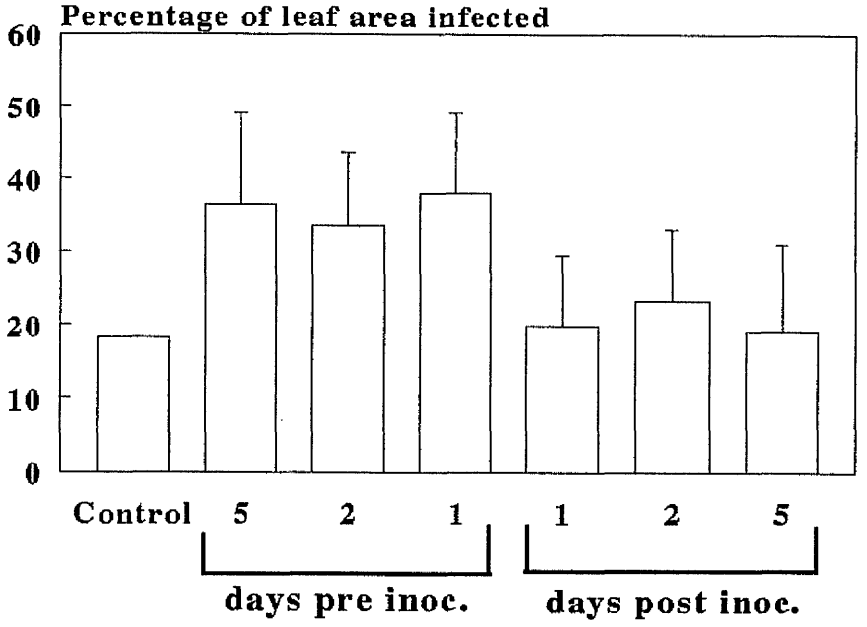

Percentage of leaf area infected
10
9
8
7
6
5
4
3
2
1
0
Control
1
2
5
days post inoc.

FIGURE 57. Effect of application of 1.0 mM BAD to lower barley leaves on infection of upper leaves by powdery mildew. BAD was applied at various times pre- and post-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at P = 0.05. Figures are the means of ten replicates.

FIGURE 58. Effect of application of BAD and 4 commercial active ingredients on powdery mildew infection of barley, when applied three days post-inoculation. BAD was applied at 1.0 mM concentration. Active ingredients used were Propiconazole, Tridemorph, Flutriafol and Fenpropidin. All active ingredients were used as 0.1% solutions. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at P = 0.05. Figures are the means of twenty replicates.


Percentage of leaf area infected
60
50
40
30
20
10
0
Control
5
2
1
1
2
5
days pre inoc.
days post inoc.

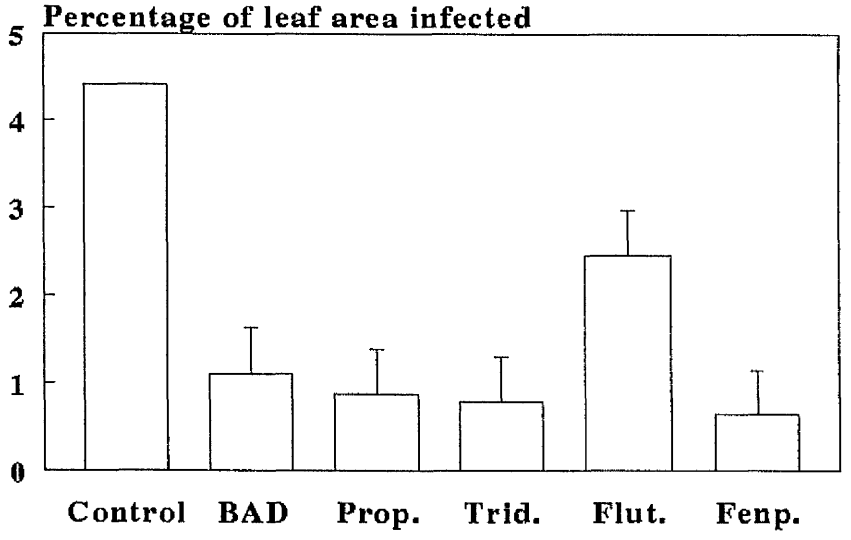
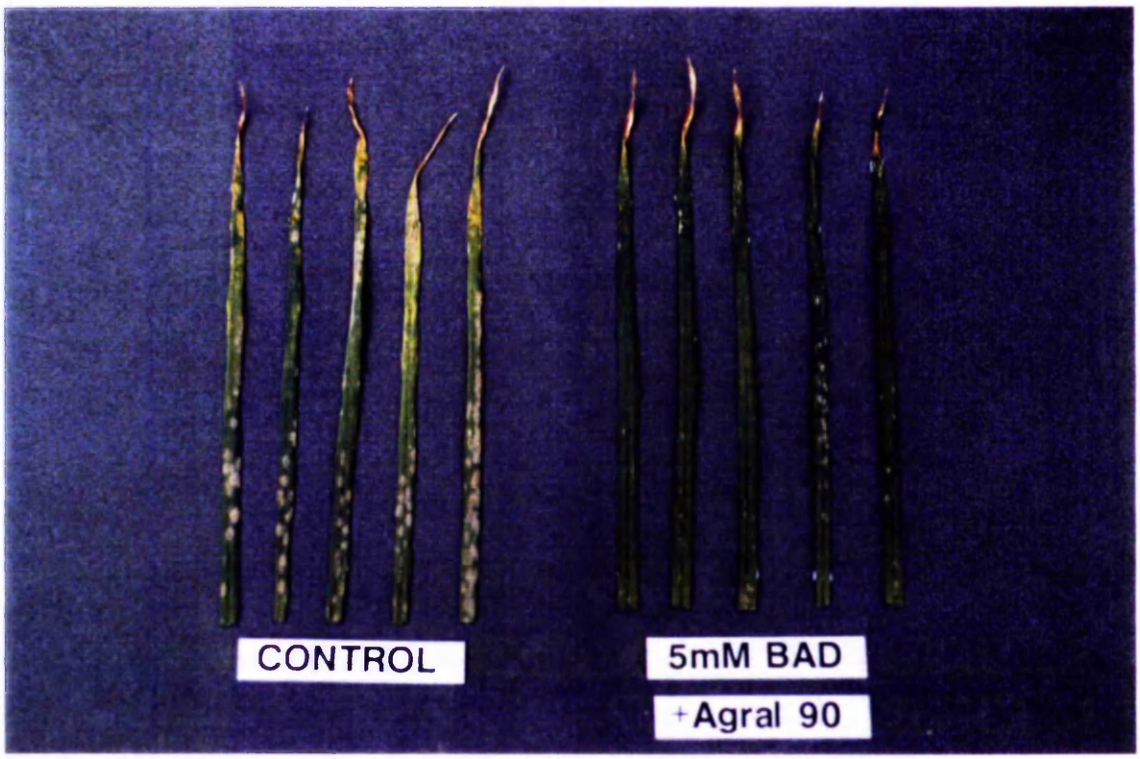

Percentage of leaf area infected
5
4
3
2
1
0
Control
BAD
Prop.
Trid.
Flut.
Fenp.

FIGURE 59. Effect of 5.0 mM BAD + 0.01% (v/v) Agral 90 on powdery mildew infection of barley seedlings, when applied three days post-inoculation. Leaves were detached and photographed ten days post-inoculation.

FIGURE 60. Effect of 1.0% Propiconazole on powdery mildew infection of barley seedlings, when applied three days post-inoculation. Leaves were detached and photographed ten days post-inoculation.


CONTROL
5mM BAD
+Agral 90

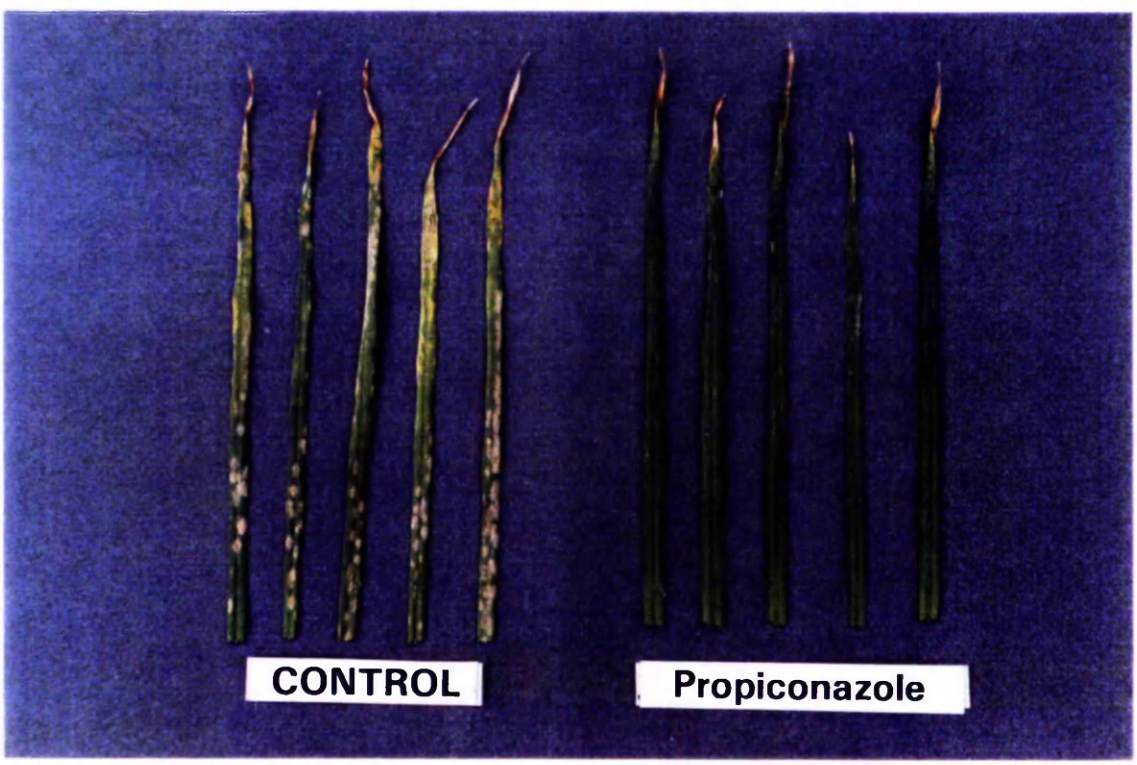
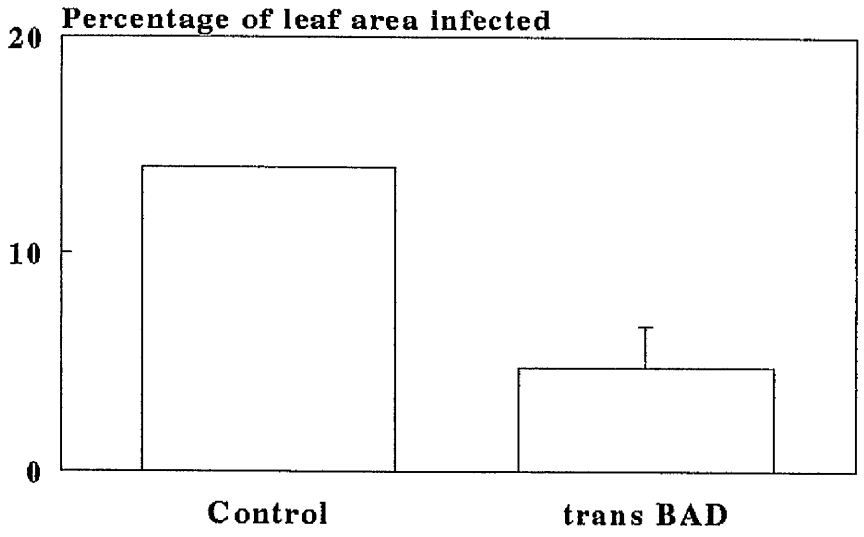

CONTROL
Propiconazole

FIGURE 61. Effect of 1.0 mM trans BAD on powdery mildew infection of barley seedlings, when applied three days post-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at P = 0.05. Figures are the means of twenty replicates.

FIGURE 62. Effect of 1.0 mM cis BAD and 1.0 mM BAC on powdery mildew infection of barley seedlings, when applied three days post-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at P = 0.05. Figures are the means of twenty replicates.


Percentage of leaf area infected
20
10
0
Control
trans BAD

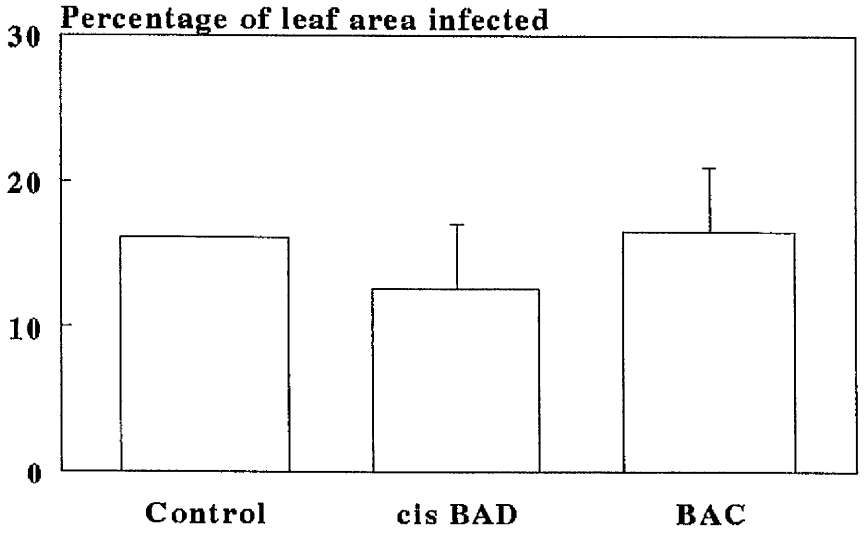
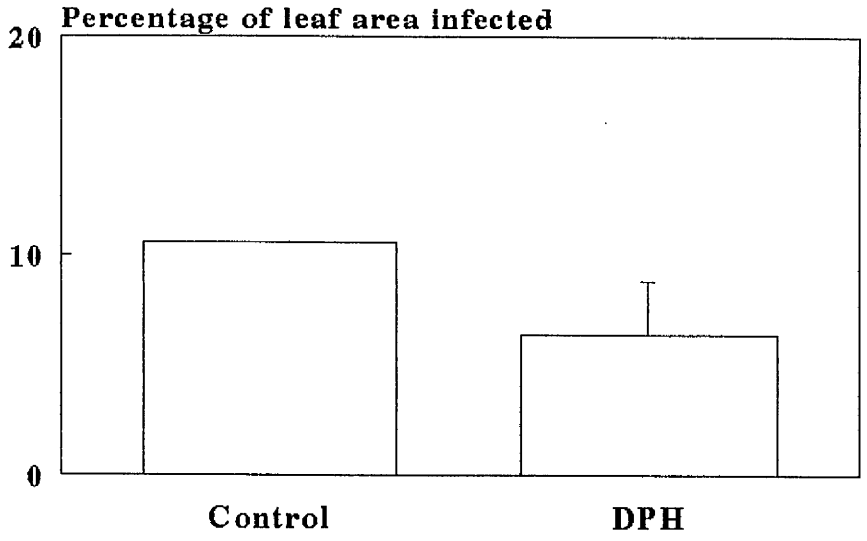

Percentage of leaf area infected
30
20
10
0
Control
cis BAD
BAC

FIGURE 63. Effect of 1.0 mM DPH on powdery mildew infection of barley seedlings, when applied three hours pre-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of twenty replicates.

FIGURE 64. Effect of 1.0 mM DPH on powdery mildew infection of barley seedlings, when applied three days post-inoculation. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of twenty replicates.


Percentage of leaf area infected
20
10
0
Control
DPH

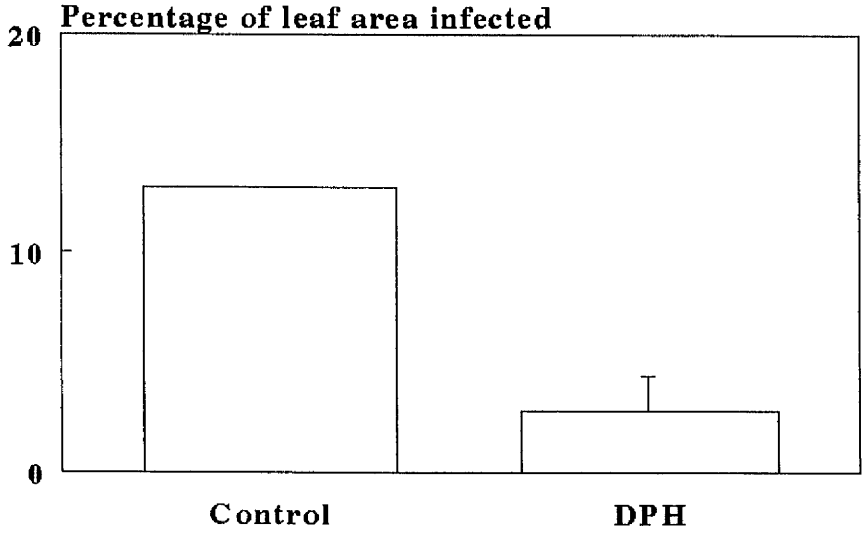
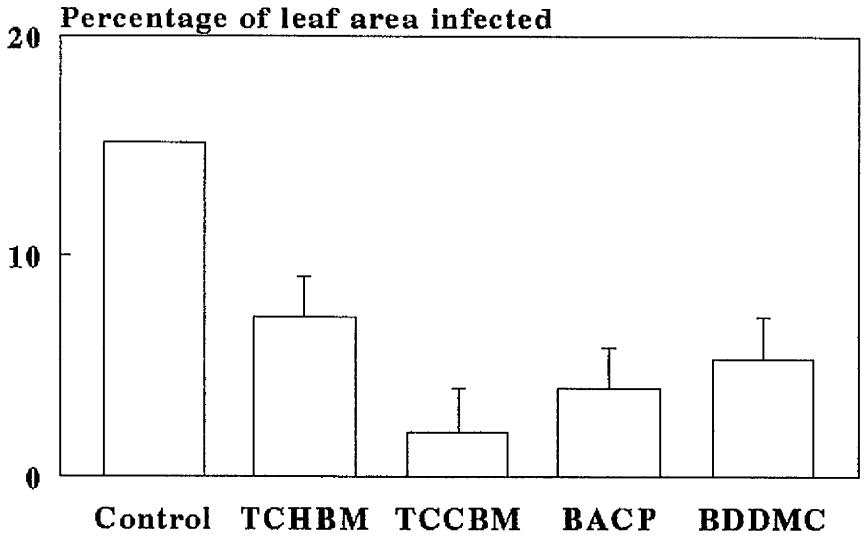
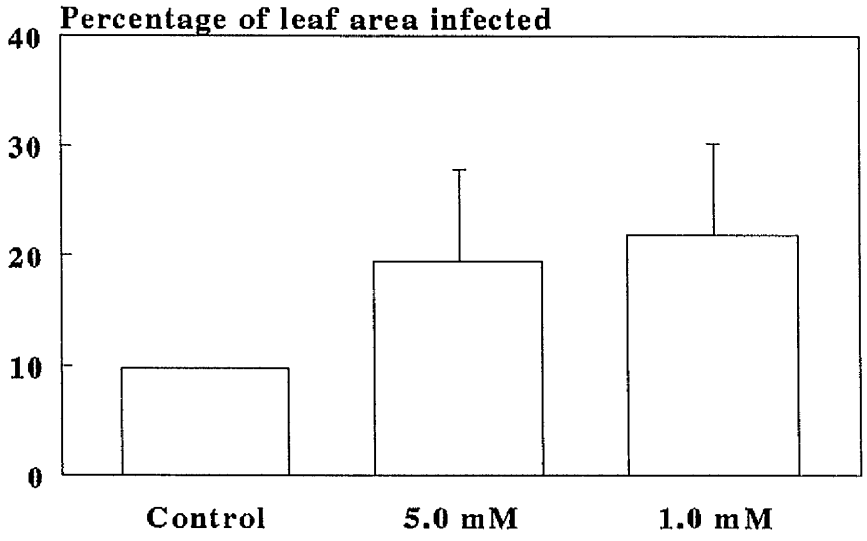

Percentage of leaf area infected
20
10
0
Control
DPH

FIGURE 65. Effect of four cyclic compounds on powdery mildew infection of barley seedlings, when applied three days post-inoculation. Compounds used were 1.23 mM TCHBM, 1.0 mM TCCBM, 1.0 mM BACP and 0.238 mM BDDMC. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of twenty replicates.

FIGURE 66. Effect of 1.0 and 5.0 mM BAD on rust infection of broad bean seedlings, when applied three hours pre-inoculation. Results shown are for the final assessment of leaves, seventeen days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of six replicates.


Percentage of leaf area infected
20
10
0
Control
TCHBM
TCCBM
BACP
BDDMC


Percentage of leaf area infected
40
30
20
10
0
Control
5.0 mM
1.0 mM

3.3.3 Effect of BAD and Other Cyclic Compounds on Fungal Pathogens *In Vivo*

In contrast to it's effect on *E. graminis*, BAD gave no control of *U. viciae-fabae* when applied either pre- or post-inoculation (Figures 66 and 67). However BAD did control of *B. fabae* when applied pre-inoculation (Figure 70). BAD also controlled infection by *P. leucotricha*, when applied either pre- or post-inoculation (Table 13).

Similarly, neither trans-BAD or DPH, which both controlled *E. graminis* had any effect on *U. viciae-fabae* when applied post-inoculation (Figures 68 and 69). Likewise, DPH did not significantly reduce *P. leucotricha* infection of apple seedlings (Figure 72). However, trans-BAD at 5.0 mM did reduce infection of potato leaf discs by *P. infestans* (Table 12).

3.3.4 Effect of BAD on Fungi Grown *In Vitro*

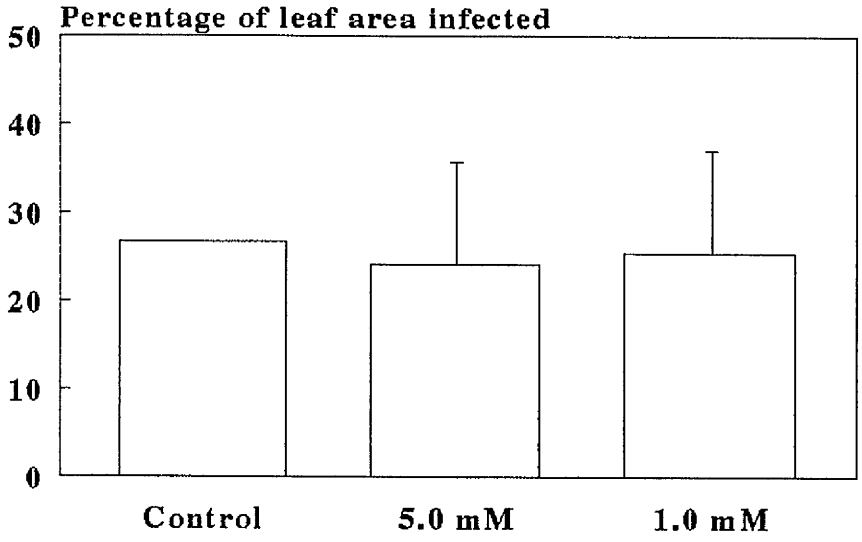
1.0 mM BAD produced a small increase in the growth of *P. oryzae in vitro* (Figure 73). However this, decrease was not significant.

3.4 EFFECT OF MIXTURES OF NOVEL COMPOUNDS ON INFECTION OF BARLEY BY *E. GRAMINIS*

Combinations of novel compounds were examined for their effect on *E. graminis* infection of barley seedlings. All the combinations contained 1.0 mM E-BED. Whilst all of these combinations gave substantial and significant reductions in *E. graminis* infection, greatest control was achieved by treatment with a mixture of 1.0 mM E-BED and 1.0 mM TED-1 (91%; Figure 74)

FIGURE 67. Effect of 1.0 and 5.0 mM BAD on rust infection of broad bean seedlings, when applied three days post-inoculation. Results shown are for the final assessment of leaves, seventeen days post-inoculation. Vertical bars represent the least significant difference from the control at P = 0.05. Figures are the means of six replicates.

FIGURE 68. Effect of 1.0 mM trans BAD on rust infection of broad bean seedlings, when applied three days post-inoculation. Results shown are for the final assessment of leaves, seventeen days post-inoculation. Vertical bars represent the least significant difference from the control at P = 0.05. Figures are the means of six replicates.


Percentage of leaf area infected
50
40
30
20
10
0
Control
5.0 mM
1.0 mM

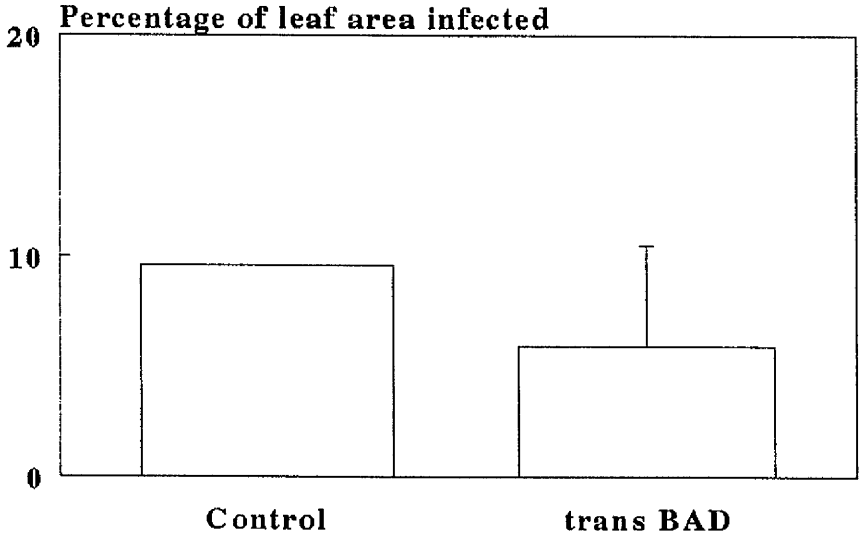
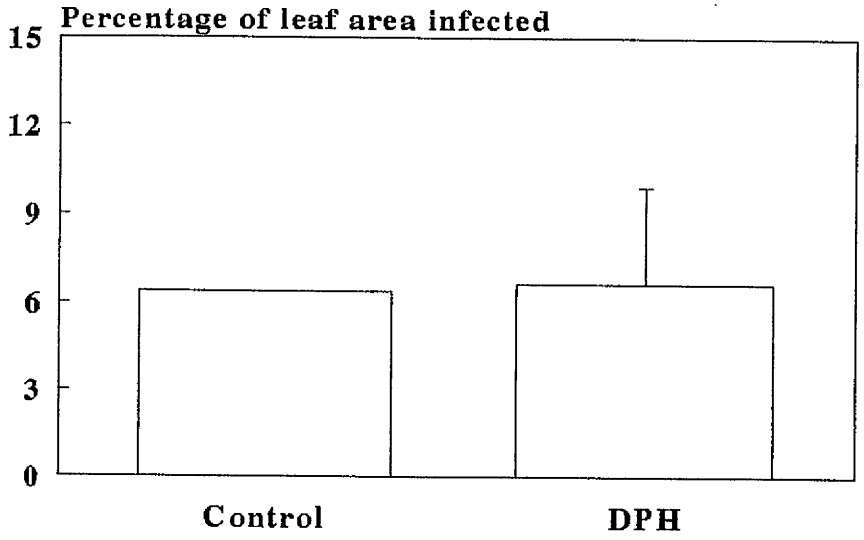

Percentage of leaf area infected
20
10
0
Control
trans BAD

FIGURE 69. Effect of 1.0 mM DPH on rust infection of broad bean seedlings, when applied three days post-inoculation. Results shown are for the final assessment of leaves, seventeen days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of six replicates.

FIGURE 70. Effect of 1.0 and 5.0 mM BAD on chocolate spot infection of broad bean seedlings, when applied three hours pre-inoculation. Results shown are for the final assessment of leaves, seven days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of six replicates.


Percentage of leaf area infected
15
12
9
6
3
0
Control
DPH

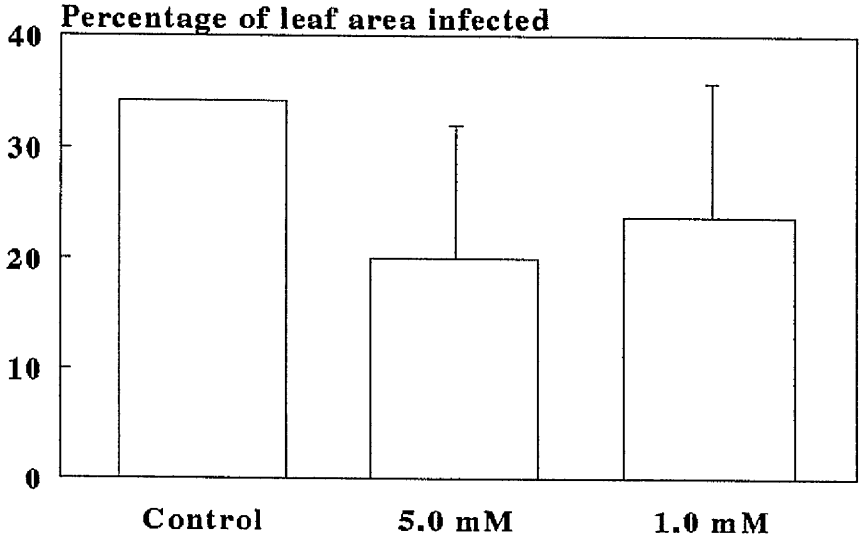
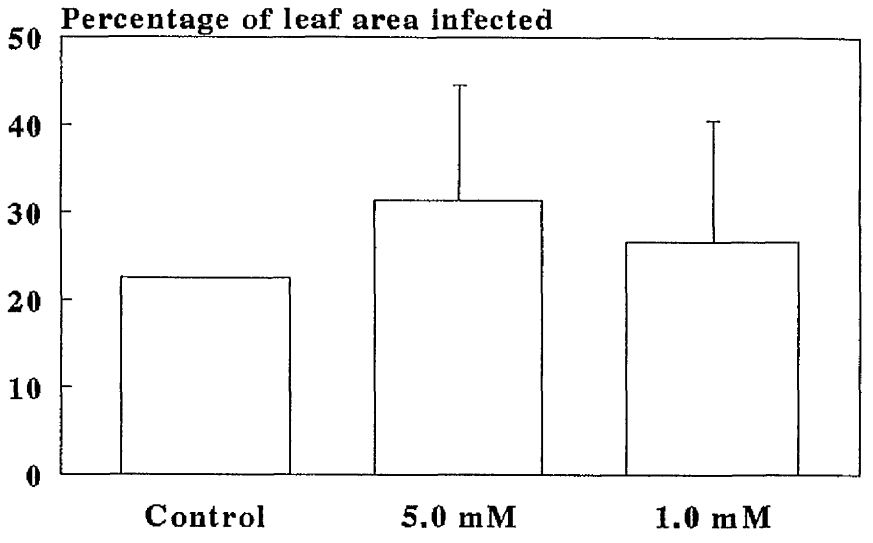

Percentage of leaf area infected
40
30
20
10
0
Control
5.0 mM
1.0 mM

FIGURE 71. Effect of 1.0 and 5.0 mM BAD on chocolate spot infection of broad bean seedlings, when applied three days post-inoculation. Results shown are for the final assessment of leaves, seven days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of six replicates.

FIGURE 72. Effect of 1.0 mM DPH on powdery mildew infection of apple seedlings, when applied three days post-inoculation. Results shown are for the final assessment of leaves, seventeen days post-inoculation. Vertical bars represent the least significant difference form the control at $\underline{P} = 0.05$. Figures are the means of six replicates.


Percentage of leaf area infected
50
40
30
20
10
0
Control
5.0 mM
1.0 mM

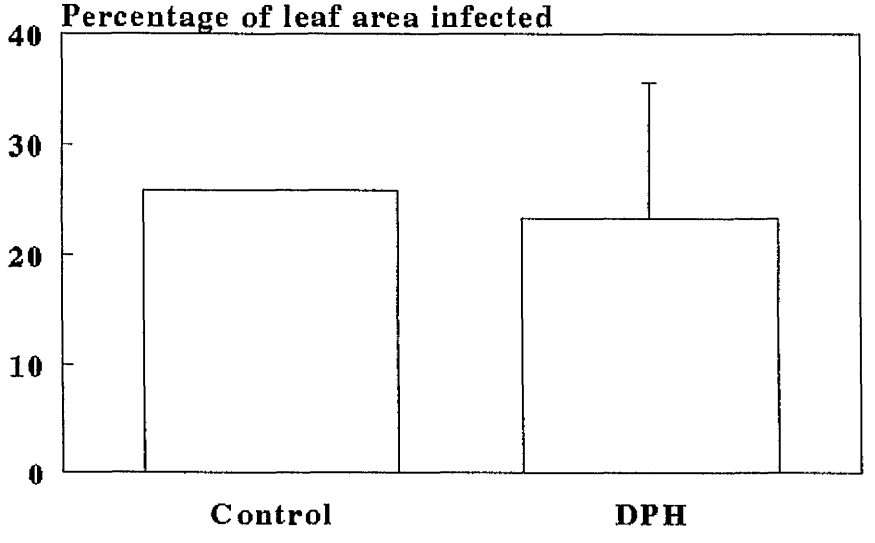

Percentage of leaf area infected
40
30
20
10
0
Control
DPH

TABLE 12. Effect of trans-BAD on infection of potato leaf discs by *Phytophthora infestans.* Potato leaf blight was assessed using the following key; 0 = No growth, up to 5 = Leaf disc entirely covered. Figures are the means of five replicates.

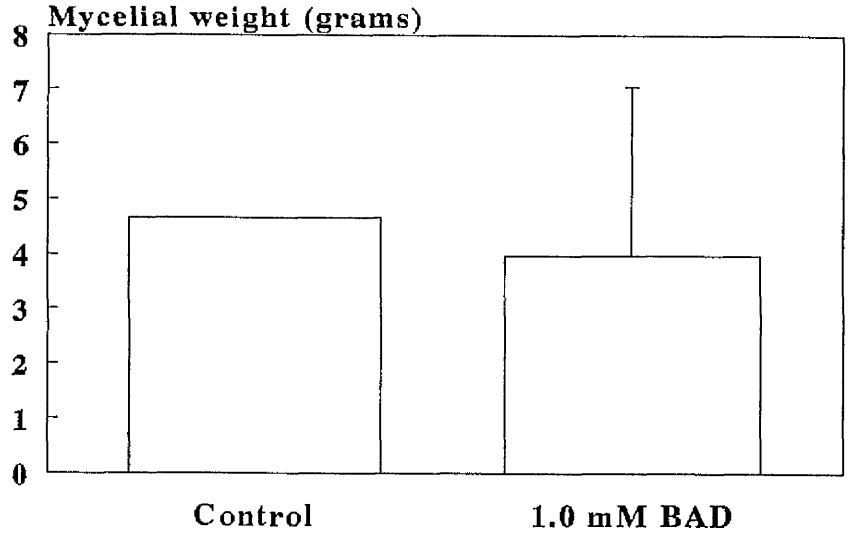
Treatment	Mean Disease Score
Control	3.5
1.0 mM trans-BAD	3.5
5.0 mM trans-BAD	2.5

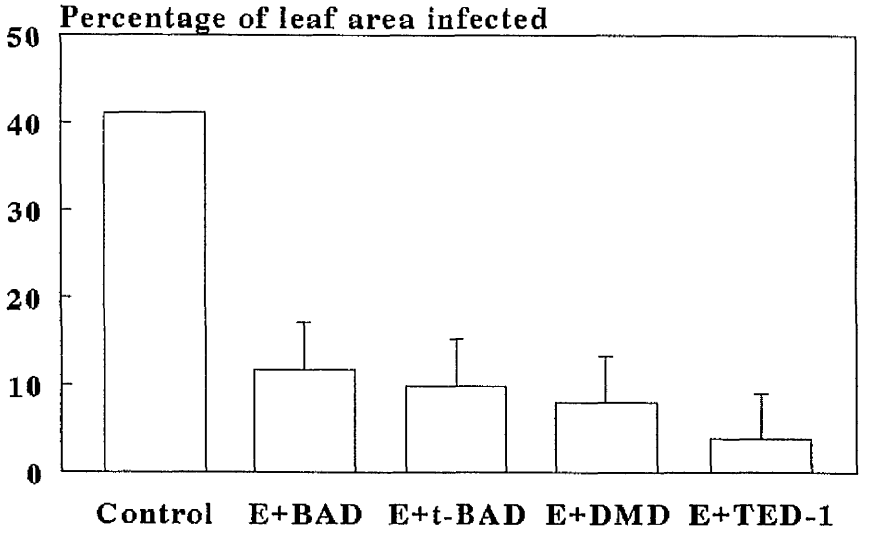
TABLE 13. Effect of BAD on infection of apple seedlings by *Podosphaera leucotricha.* Apple powdery mildew was assessed using the following key; 0 = No infection, 1 = A few isolated spores, 2 = <50% infection, 3 = >50% infection. All figures are the means of six replicates.

Treatment	Mean Disease Score
Control	1.9
Pre-inoculation	
1.0 mM BAD	1.33
5.0 mM BAD	1.17
Post-inoculation	
1.0 mM BAD	1.17
5.0 mM BAD	1.0

FIGURE 73. Effect of 1.0 mM BAD on growth of the fungus, *Pyricularia oryzae* in liquid culture. Results shown are for the fungus harvested after four days. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of four replicates.

FIGURE 74. Effect of application of mixtures of novel compounds on powdery mildew infection of barley seedlings, when applied three days post-inoculation. Mixtures used were; 1.0 mM E-BED (E) + 1.0 mM BAD; 1.0 mM E-BED (E) + 1.0 mM <u>trans</u> BAD; 1.0 mM E-BED (E) + 1.0 mM DMD and 1.0 mM E-BED (E) + 1.0 mM TED-1. Results shown are for the final assessment of leaves, ten days post-inoculation. Vertical bars represent the least significant difference from the control at $\underline{P} = 0.05$. Figures are the means of twenty replicates.


Mycelial weight (grams)
8
7
6
5
4
3
2
1
0
Control
1.0 mM BAD


Percentage of leaf area infected
50
40
30
20
10
0
Control
E+BAD
E+t-BAD
E+DMD
E+TED-1

3.5 SUMMARY OF RESULTS

The following table provides a summary of the fungicidal effects of the three major, novel compounds synthesized: E-BED, TED-1 and BAD.

TABLE 14. Fungicidal activity of three major compounds. Disease control was defined using the following key, *** = Very Good Control, ** = Good Control, * = Poor Control and 0 = No Control.

A			
Plant pathogen interaction	**E-BED**	**TED-1**	**BAD**
E. graminis/ barley	***	***	***
U. viciae-fabae/ broad bean	**	**	0
B. fabae/ broad bean	***	**	*
P. infestans/ potato	**	**	*
P. leucotricha/ apples	**	**	**
B			
***In Vitro* fungal growth**			
B. cinerea	***	-	-
P. oryzae	*	0	0
P. avenae	**	***	-

SECTION FOUR

DISCUSSION

4.1 EFFECT OF E-BED AND IT'S SALTS AND DERIVATIVES ON FUNGAL PATHOGENS

Product based analogues have been shown previously to be efficient inhibitors of polyamine biosynthesis. In particular, Bitonti *et al* (1987) examined the effect of three novel product analogues on arginine decarboxylase activity in *E. coli* and two plant species (oat and barley). These analogues were found to be more potent than two new substrate analogues tested at the same time and 100 times more potent than α-difluoromethylarginine (DFMA) against ADC activity in *E. coli* extracts.

E-BED and Z-BED, two putrescine analogues, have been shown to possess substantial fungicidal activity against a range of fungal pathogens (Table 2). E-BED, the trans-isomer, always gave better control of the pathogens than Z-BED, the cis-isomer. This phenomenon has been described previously for fungicidal compounds. N- [3-(4-tert-butylphenyl) -2-methylpropyl] -2, 6-dimethyl-morpholine is a 'second generation' morpholine with substantial fungicidal activity. The cis-isomer of this compound was shown to be clearly more active than the trans-isomer against mildew and rust diseases of wheat. The cis-isomer was also significantly more active than a cis, trans-isomer mixture. The cis-isomer is produced industrially and has been given the ISO recommended name of fenpropimorph (Fuchs, 1988).

The % disease control achieved by E-BED (Table 2) was very similar to that produced by the commercial putrescine analogue, keto-putrescine (Foster and Walters, 1993). Interestingly, in both cases, greatest disease control was achieved against the necrotrophic pathogen *Botrytis fabae*. E-BED and Z-BED also gave greater control of *E. graminis* infection of barley than infection of broad bean by the rust fungus *U. viciae-fabae*. These results show a significant

difference to those found for the ODC irreversible inhibitor, DFMO. Galston and Weinstein (1988) found that rust fungi were more sensitive to this inhibitor than powdery mildew fungi.

In general, post-inoculation treatments of E-BED were most effective, while this trend was not observed for Z-BED. A timing experiment was undertaken to further elucidate the nature of E-BED's fungicidal activity against *E. graminis* on barley. Here, a range of application times were used. Treatments applied two days pre-inoculation tested for protective activity, treatments applied two days post-inoculation examined curative activity and treatments applied five days post-inoculation examined eradicative activity (Arnold *et al.,* 1992). E-BED exhibited greatest activity against *E. graminis* infection of barley when applied as a curative treatment, although all the treatments examined gave significant reductions (Figure 7). This curative control may be related to a perturbation of polyamine biosynthesis in the germinating conidia on the leaf surface. Rajam *et al.* (1989) showed that the ODC inhibitor DFMO, can inhibit the germination of uredospores and germ tube growth in *Uromyces phaseoli* L. DFMO was also shown to delay the timing of spore germination. Moreover, Stevens *et al.* (1977) showed that the putrescine analogue, keto-putrescine delayed the germination of conidia of *Aspergillus nidulans.* Keto-putrescine also produced greater disease control against *E. graminis* post-inoculation (Foster and Walters, 1993), indicating a similar effect on germinating mildew conidia.

A series of more detailed experiments was undertaken with E-BED against *E. graminis* infection of barley, in order to establish any possible systemic activity for this compound. Results from the post-inoculation application of E-BED appear to show evidence of xylem transport of this compound (Figure 10). The highly effective systemic fungicides discovered up till now are mainly xylem-

systemic (Jacob and Neumann, 1987). However, the increased infection observed when E-BED was applied pre-inoculation is difficult to explain (Figure 9). It may be that E-BED was metabolised, either in the compost or in the plant, and the metabolite which reaches the leaves is being utilised by the pathogen. Diaminobutene (or the cis-isomer, Z-BED) has been synthesised previously by Inoue *et al.* (1975). Kameji *et al.* (1979) showed that in animal tissues this compound was metabolised to the unsaturated spermidine analogue and could fulfil natural polyamine roles. It is possible that this could be the fate of E-BED in the compost or within the plant.

Application of E-BED to lower leaves one day pre-inoculation gave a significant reduction in *E. graminis* infection of upper leaves (Figure 8). This control indicates the possibility of phloem transport of E-BED within the plant. However, it is difficult to make clear conclusions based on these results, as this was the only treatment to give significant control of the pathogen.

E-BED was originally synthesised as the dihydrochloride salt. After the initial promising results with E-BED, five alternative salts were synthesised. Each of these salts gave reasonable control of *E. graminis* infection of barley seedlings (ranging from 8% to 62%; Figures 11 and 12). In order to build up an activity series for E-BED, various derivatives were synthesised and their fungicidal activities against *E. graminis* infection of barley examined. These derivatives showed variable activity (Figures 13 and 14). Best control was achieved by Dichex-E-BED dihydrobromide (42%, Figure 13). None of these salts or derivatives gave better control of *E. graminis* than E-BED dihydrochloride.

E-BED was compared against active ingredients used in commercially available fungicides, in a study of the control of *E. graminis* infection of barley in the glasshouse (Figure 15). Although 1.0 mM E-BED gave only 70% disease

control, it still outperformed 0.1% flutriafol. Greatest control was achieved with a 0.1% solution of fenpropidin. This difference may reflect the higher concentration of active ingredients used (ranging from 125 to 750 mg litre^{-1} for the commercial compounds, compared to 163 mg litre^{-1} for E-BED). However, it must be remembered that these active ingredients will all have superior formulation to that of E-BED and hence superior efficacy. In most of the screening work, E-BED was applied in a solution of 0.01% Tween 20 as described by Shephard (1987). In this glasshouse comparison, the commercial surfactant Agral 90 was used at the same concentration as Tween 20.

In the field trial experiment, E-BED was compared to the ODC inhibitor, DFMO and the commercial fungicide 'Early Impact', against *E. graminis* infection of barley. E-BED and DFMO were used at two different concentrations (1.0 and 5.0 mM). All treatments were applied either once (Growth Stage 25) or twice (Growth Stages 25 and 59). E-BED at 1.0 and 5.0 mM gave substantial control of *E. graminis* early in the growing season. This control was comparable to that achieved by the commercial fungicide Early Impact (Table 3). Early in the growing season, 5.0 mM DFMO gave substantial control of *E. graminis* infection. Later in the growing season, DFMO did not reduce *E. graminis*, either at 1.0 or 5.0 mM. These results are significantly different to those found for DFMO in pot experiments, where it was shown to give substantial control of rust and mildew infections (Rajam *et al.*, 1985; West and Walters, 1988). The reasons for this apparent difference are not known, although they may be related to environmental conditions and inoculum pressure. In the late season, the only significant control was achieved by two sprays of 5.0 mM E-BED or Early Impact (Table 3).

In the field experiment, the percentage green leaf area was estimated at the same time as the percentage leaf area mildew infected (Table 4). In general, these

figures mirrored the figures for percentage leaf area mildew infected. Green leaf area remained greatest in those plots sprayed with E-BED (at 1.0 and 5.0 mM) and Early Impact. This is important, since the increased photosynthetic area will affect plant height, plant dry weight and grain weight (Table 5). In the late growing season, high green leaf areas are important in the grain filling process in barley.

At the end of the field trial, when the crop was ripe, ten plants were harvested from each plot and measurements made of plant height, plant dry weight and grain weight. The number of replicates chosen was relatively low due to the time consuming nature of the analyses. Therefore, while the results may appear statistically significant, further extrapolation of these figures towards grain yields in terms of kg ha^{-1} has been avoided.

A number of treatments exhibited small increases in plant height compared to the untreated plots, although only two sprays of 5.0 mM E-BED and Early Impact gave significant increases (Table 5). Interestingly, 5.0 mM E-BED applied either once or twice gave a significant increase in plant dry weight over untreated plots. The only other significant increase was produced by 5.0 mM DFMO applied twice.

The main reason for any fungicide application is to produce an economic benefit, in terms of yield, for the farmer. All the treatments examined produced increases in grain yield compared to the untreated plots, but only two treatments, 5.0 mM E-BED and Early Impact (both sprayed twice), gave significant increases. However, the sample size for these results is very small and more detailed experiments on grain yield would be required before drawing definite conclusions about effects of E-BED on grain yield. Despite this, it is

worth noting that 5.0 mM E-BED applied twice, was the only treatment to significantly increase plant height, plant dry weight and grain weight.

E-BED and Z-BED both gave significant reductions in mycelial growth of *B. cinerea*, *P. oryzae* and *P. avenae* (Table 5). E-BED in particular, reduced growth of *B. cinerea* by 87% and *P. avenae* by 58%. These results compare favourably with those obtained for keto-putrescine, where growth of *B. cinerea* was reduced by only 27% (Smith *et al.,* 1992) and *P. avenae* by only 25% (Foster and Walters, 1993). In the case of *P. avenae* however, growth was on solid media and hence results may not be directly comparable.

When *P. avenae* was grown in the presence of 0.5 mM Z-BED and the polyamine biosynthetic enzymes examined, a small decrease in soluble ODC activity and an increase in soluble AdoMetDC activity was observed (Table 7). Interestingly, when the effect of Z-BED on enzyme activities from *P. avenae* was examined *in vitro*, a different pattern emerged. A small, insignificant decrease in ODC activity was observed, but there was no increase in AdoMetDC activity at either 0.5 or 1.0 mM concentrations (Figures 16 and 17). Putrescine is known to be an obligatory activator of AdoMetDC in both mammals and fungi (Davis *et al.,* 1992). It may be that in the fungus, Z-BED (a putrescine analogue) is fulfilling some of the roles of the natural polyamine and stimulating AdoMetDC activity. Fungal tissue grown in Z-BED amended media contained 41% less spermidine than the control, while spermine levels were increased slightly, although this was not significant (Table 7). Putrescine levels were unaltered. Moreover, Z-BED at 0.5 mM had no significant effect on growth of *P. avenae* in liquid culture. Since spermidine is known to be important for fungal growth and in some cases is an absolute requirement e.g. in *Neurospora crassa* (Davis, 1990), it may be that the spermidine pool present in the fungus (116.8 μmol g^{-1} f.wt) was sufficient to support fungal growth.

In contrast, E-BED produced different results on enzyme activities and polyamine concentrations in *P. avenae*. Thus, *in vivo* soluble ODC and AdoMetDC activities were reduced by 87% and 82% respectively (Table 7). When the *in vitro* activities of these enzymes from *P. avenae* were examined, the addition of either 0.5 or 1.0 mM E-BED did not produce a significant decrease in the activity of either enzyme (Figures 18 and 20). Substantial decreases in *in vitro* ODC activity were observed at higher concentrations of E-BED (Figure 19). It may be that an accumulation of E-BED in the cell is responsible for the significant reductions in *in vivo* activities of ODC and AdoMetDC. Interestingly, these *in vivo* reductions were accompanied by a very significant accumulation of putrescine within the cells and a 33% reduction in spermidine levels. Spermine levels were increased slightly over control values although this was not significant. The reduction in the spermidine pool could be accounted for by the reduced AdoMetDC activity. The very large putrescine pool could be the result of an E-BED induced reduction in putrescine catabolism and/or reducing putrescine efflux from fungal cells. Z-BED has been shown to be a potent inhibitor of diamine oxidase (Relyea and Rando, 1975). *P. avenae* grown in the presence of E-BED was also shown to have reduced diamine oxidase activity (Figure 21).

Nevertheless, the inhibition of fungal growth produced by E-BED may be due to high putrescine levels which accumulated in the fungus. Davis and Ristow (1991) have shown that in a mutant of *Neurospora crassa* which concentrates polyamines, addition of 5.0 mM putrescine to the medium led to an intracellular concentration of greater than 200 ηmol putrescine mg^{-1} d.wt (nominally 80 mmol). Under these circumstances *N. crassa* grew at half the normal rate. In

P. avenae treated with E-BED, the putrescine pool increased from 62.1 μmol g^{-1} f.wt to 583.3 μmol g^{-1} f.wt (ca. 65 mmol). It is entirely possible that this greatly elevated internal putrescine concentration was responsible, in part, for the fungicidal effects of E-BED. Here, putrescine could be acted upon by diamine oxidase to yield free radicals and hydrogen peroxide, both of which could damage cell membranes. Such a mechanism has been shown to be responsible for putrescine-induced wounding in maize roots (DiTomaso *et al.*, 1989). High intracellular putrescine levels have also been shown to be toxic in the cyanobacterium *Anabena*, where the lethal effect of putrescine was believed to be the result of conjugation with cellular ribosomes (Davis, 1990).

4.2 EFFECT OF TED-1 AND IT'S SALTS AND DERIVATIVES ON FUNGAL PATHOGENS

TED-1 was shown to possess substantial fungicidal activity. Thus, 1.0 mM TED-1 gave substantial control of *E. graminis*, *U. viciae-fabae, B. fabae, P leucotricha* and *P. infestans* (Table 8). Control of *E. graminis* was comparable to that achieved by keto-putrescine (Foster and Walters, 1993), although control of *U. viciae-fabae* and *B. fabae* infections was slightly less pronounced. In contrast to the results achieved with keto-putrescine and E-BED, TED-1 was found to be most effective in controlling *E. graminis* infection of barley. As a result, a series of experiments were undertaken to further examine the systemic effects of this compound on this pathogen.

In a timing experiment, all pre-inoculation treatments gave significant reductions in *E. graminis* infection (Figure 22). However, greatest control was achieved with a two day post-inoculation treatment (57%; Figure 23). This

result would appear to indicate curative activity for TED-1. Interestingly, a similar effect was found with E-BED. This curative activity could be related to a perturbation in polyamine biosynthesis in the germinating conidia on the leaf surface.

TED-1 applied as root drench to barley plants, gave only small reductions in *E. graminis* infection of upper leaves (Figure 24). This would appear to indicate that this compound possesses only limited xylem mobility. The reasons for this are not known. Again, metabolism of the putrescine analogue could have occurred either in the compost or in the plant.

Application of TED-1 to lower leaves two days pre-inoculation led to significant control of *E. graminis* infection on upper leaves (Figure 25). Small reductions were produced by all the pre-inoculation treatments and by the one day post-inoculation treatment. Thus, it appears that TED-1 may possess some phloem mobility and also some protective activity.

TED-1 was originally synthesised as the dihydrobromide salt. Four alternative salts were synthesised after the initial promising results and although each showed disease control against *E. graminis* infection of barley (ranging from 15% to 58%), none was shown to be superior to the dihydrobromide salt (Figure 27). As with E-BED, the fumarate salt was found to possess the most activity. TED-1 was compared with the commercial active ingredient, propiconazole, for control of *E. graminis* infection of barley in the glasshouse (Figure 26). TED-1 was used at 5.0 mM (1,800 mg litre^{-1}) and propiconazole as a 1% solution (2,500 mg litre $^{-1}$). The novel putrescine analogue reduced mildew infection by 90% compared to a 94% reduction obtained with propiconazole. The slightly greater control achieved with propiconazole might reflect the higher concentration or the superior formulation of propiconazole, since TED-1 was applied in a 0.01% Agral 90 solution.

A derivative of TED-1, DMD was synthesised. This compound also exhibited fungicidal properties, giving a 72% reduction in *E. graminis* infection on barley, when applied post-inoculation (Figure 31). Only a small reduction was produced in *E. graminis* infection when DMD was applied pre-inoculation. A series of experiments to determine the systemic activity of DMD was performed. In a timing experiment, none of the pre-inoculation treatments produced a significant reduction in *E. graminis* infection of barley (Figure 32). Best control of *E. graminis* infection of barley was produced when DMD was applied one day post-inoculation (Figure 33). Thus, DMD appears to possess mainly curative activity.

When DMD was applied as a root drench, almost all the treatments reduced *E. graminis* infection on upper leaves. Most significant control was achieved by the five days post-inoculation treatment (Figure 34). It would appear from these results that DMD possesses xylem mobility. Application of DMD to lower leaves produced variable results on *E. graminis* infection of upper leaves (Figure 35). Only the five days pre-inoculation treatment produced a significant reduction in *E. graminis* infection, other treatments produced an increase in infection of upper leaves. These results are difficult to interpret. The increases in infection produced by some treatments may be due to the transport in the phloem of a metabolite which was subsequently utilised by the fungus. DMD is a novel compound and it's fate in the plant is unknown. However, another putrescine analogue, 1,4-Diaminobutene, has been shown to be metabolised to the unsaturated spermidine analogue and can substitute for natural polyamine functions (Kameji *et al.*, 1979; see Page 99).

TED-1 was originally synthesised as the trans-isomer. In order to build up an activity series for this compound the cis-isomer was synthesised at a later date. Cis TED-1 exhibited significant control of *E. graminis* infection of barley, when applied both pre- and post-inoculation (Figures 36 and 37). Greatest control

was achieved using a post-inoculation treatment (62%; Figure 37). Both DMD and cis TED-1 exhibited fungicidal activity against other fungal pathogens *in vivo*. 1.0 mM cis TED-1 reduced the infection of broad bean seedlings by the rust fungus, *U. viciae-fabae,* when applied as a post-inoculation treatment (71%; Figure 39). DMD produced a smaller, insignificant reduction in *U. viciae-fabae*, when applied as a post-inoculation treatment (38%; Figure 38). DMD, applied as a post-inoculation treatment, also reduced infection of broad bean seedlings by *B. fabae* (65%; Figure 40), infection of potato leaf discs by *P. infestans* and infection of apple seedlings by *P. leucotricha* (Table 9). 1.0 mM cis TED-1 reduced infection of apple seedlings by *P. leucotricha* by 29% (Figure 41). Interestingly, although E-BED possessed greater fungicidal activity than Z-BED against all the fungi examined, cis TED-1 proved more effective than the trans-isomer, TED-1, against *U. viciae-fabae*. Fuchs (1988) concluded that the modes of action of various stereo-isomers can be identical, though quantitatively different, or dissimilar.

TED-1 was found to have only a small, insignificant effect on growth of *P. avenae* and *P. oryzae* on solid agar (Figures 44 and 45). Keto-putrescine, in contrast, produced 25% and 15% reductions in the growth of these two fungi respectively (Foster and Walters, 1993). Interestingly, when *P. avenae* was grown in the presence of 1.0 mM TED-1 in liquid culture, growth of the fungus was completely inhibited. TED-1 produced significant reductions in the growth of *P. avenae* at lower concentrations (Figure 46). These reductions were similar to the growth inhibition of *P. avenae* produced by E-BED. However, these results appear to show that growing *P. avenae* in amended liquid culture gives a better indication of the susceptibility of this fungus to novel compounds.

Exposure of *P. avenae* to TED-1 at 0.5 mM led to significant reductions in putrescine and spermidine concentrations and unaltered spermine concentration, while exposure to 0.1 mM TED-1 led to increased ODC activity and decreased AdoMetDC activity (Tables 10 and 11). Thus, although the reduced spermidine concentration could be accounted for by the lowered AdoMetDC activity, the greatly increased ODC activity should have led to elevated putrescine levels. Since the activity of diamine oxidase was not significantly altered by treatment with TED-1 (Figure 50), the reduction in putrescine cannot be due to enhanced catabolism. It is possible that there was a greater efflux of putrescine from *P. avenae* grown in the presence of TED-1, compared to the controls, or that putrescine was acetylated to form N-acetylputrescine, thus reducing the pool of free putrescine in TED-1 treated cells. Interestingly, increases in ODC activity have also been observed in *P. avenae* and *Aspergillus nidulans* grown in the presence of keto-putrescine (Foster and Walters, 1993; Stevens *et al.*, 1977). It is thought that this increase in ODC could be the result of enzyme stabilisation.

It is tempting to suggest that the reduction in spermidine (35%) was responsible for the reduced growth of *P. avenae* in the presence of TED-1, and for the fungicidal effects of TED-1, especially since spermidine is known to be important for fungal growth and in some fungi e.g. *Neurospora crassa*, there is an absolute requirement for spermidine (Davis, 1990). However, *P. avenae* grown in the presence of 0.5 mM TED-1 still contained 10.78 μmol spermidine g^{-1} f.wt and substantial amounts of putrescine and spermine. It seems likely therefore, that the antifungal effects of TED-1 against *P. avenae* can only be partly attributed to perturbation of polyamine biosynthesis and catabolism.

In an attempt to elucidate other possible modes of action for TED-1 two experiments were carried out. In the first experiment, growth inhibition of *P. avenae* was increased by the presence of Ascorbic acid (Figure 48). This

compound mops up membrane damaging free radicals produced by diamine oxidase. This would appear to indicate that TED-1 does not substitute for putrescine as a substrate for diamine oxidase. However, the addition of Calcium chloride , as a source of Ca^{2+} to compete for intracellular binding sites, did produce a small decrease in growth inhibition compared to the TED-1 treatment alone (Figure 48). It is possible therefore, that TED-1 exerts it's antifungal effects by binding to the cell membranes. Calcium ions have been shown to be a strong inhibitor of putrescine uptake in the fungus *Neurospora crassa* (Davis and Ristow, 1988).

In the second experiment, *P. avenae* grown previously in 0.5 mM TED-1, was then exposed to labelled putrescine. Results showed that there was a greater efflux of ^{14}C-putrescine back into the medium with fungus from control flasks. These results would appear to indicate that TED-1 does not replace putrescine from intracellular binding sites and may not affect membrane integrity. Porter and Sufrin (1986) suggested that polyamine analogues may inhibit cell growth by competing for polyamine binding sites involved in cell proliferation and by binding at polyamine sites critical for macromolecular structure and function.

4.3 EFFECT OF BAD AND OTHER CYCLIC COMPOUNDS ON FUNGAL PATHOGENS

Bitonti *et al.* (1989) showed that a number of bis(benzyl) polyamine analogs were potent inhibitors of two strains of the human malaria parasite *Plasmodium falciparum in vitro.* They found that these analogs were at least 1000 times more potent than the free-amine analogs. These authors suggested that although these compounds may repress polyamine biosynthesis, their major cytotoxic

effect may be due to direct binding to DNA with subsequent disruption of macromolecular biosynthesis and cell death.

BAD, a cyclic putrescine analogue, was shown to possess substantial fungicidal activity against *E. graminis* infection of barley, when applied pre- or post-inoculation (Figures 51-53; Figure 59). In a detailed timing experiment, BAD gave best control of *E. graminis* infection when applied two days post-inoculation, indicating curative properties for this compound (Figure 54). This is in line with the other putrescine analogues examined, E-BED and TED-1. This observed effect could be related to a perturbation of polyamine biosynthesis in the germinating conidia on the leaf surface. The ODC inhibitor DFMO, has already been shown to inhibit the germination of rust uredospores (Rajam *et al.,* 1989) and unpublished results from this laboratory have shown that a range of polyamine biosynthesis inhibitors reduce conidial germination, germ tube growth and haustorial development (Hannif and Walters, unpublished results).

In a series of experiments to determine any possible systemic properties of BAD, only post-inoculation root drench treatments gave significant control of *E. graminis* infection on barley leaves (Figures 55 and 56). This would suggest that BAD possesses some xylem mobility. When BAD was applied to lower leaves there was no control of *E.graminis* on upper leaves (Figure 57). Indeed, the pre-inoculation treatments all produced an increase in *E. graminis* infection on upper leaves. This could have resulted from the transport of a metabolite within the phloem, which was subsequently utilised by the fungus. Danzin and Mamont (1987) reported that several putrescine analogues are subject to rapid metabolism and Hölttä *et al.* (1981) found that several can fulfil the requirement for endogenous polyamines in polyamine deprived systems. In a glasshouse comparison with four commercial active ingredients, 1.0 mM BAD compared favourably with all of them, giving 75% control of mildew infection and

outperforming a 1% flutriafol solution, which only gave 44% control of mildew (Figure 58).

Other cyclic compounds were synthesised and tested for their ability to control *E. graminis* infection of barley. Two isomers, trans BAD and cis BAD, were found to possess differing fungicidal activity against *E. graminis* infection of barley. Thus, the compound trans BAD gave reasonable control of infection (69%, Figure 61), whilst the cis-isomer, cis BAD, gave only 21% control of mildew (Figure 62). As with E-BED and Z-BED, greatest fungicidal activity of this analogue was found with the trans-isomer. The compound DPH gave a significant reduction in *E. graminis* infection, when applied either pre- or post-inoculation (Figures 63 and 64); control was greatest when DPH was applied three days post-inoculation. Greatest control of *E. graminis* infection of barley was achieved using the compound TCCBM (87%, Figure 65). However, the limited supply of this compound precluded any further examination of it's fungicidal properties.

Unlike the other major analogues examined, E-BED and TED-1, BAD failed to give any control of the rust fungus, *U. viciae-fabae* on broad beans, when applied either pre- or post-inoculation (Figures 66 and 67). It has been shown previously that fungal species vary in their susceptibility to polyamine biosynthesis inhibitors (West and Walters, 1989), although this was the first compound examined which gave good control of *E. graminis* infection and no control of *U. viciae-fabae.* Interestingly, BAD controlled *Botrytis fabae* infection of broad beans, when applied as a pre-inoculation spray only (Figures 70 and 71) and reasonable control of *Podosphaera leucotricha* infection of apple seedlings. BAD did not reduce growth of the fungus, *Pyricularia oryzae,* in liquid culture Figure 73), and there was insufficient compound available to test against *P. avenae* in liquid culture. As a result, it is difficult to establish a mode of action for BAD. In particular, it would be interesting to discover the

effect of BAD on polyamine biosynthetic enzymes and polyamines in the powdery mildew fungi, as these seem particularly susceptible to this compound. Unfortunately, powdery mildew cannot be grown axenically.

Only one of the other cyclic compounds, trans BAD, gave control of other fungal pathogens other than *E. graminis*. It was shown to give some control of *U. viciae-fabae* infection on broad beans (38%, Figure 68), and growth of *Phytophthora infestans* on potato leaf discs. In general, the cyclic compounds exhibited their greatest activity against *E. graminis* infection of barley.

4.4 EFFECT OF MIXTURES OF NOVEL COMPOUNDS ON *E. GRAMINIS* INFECTION OF BARLEY

Mixtures of novel compounds were applied to barley plants three days after inoculation with conidia of *E. graminis*. Best control was achieved with a mixture of 1.0 mM E-BED and 1.0 mM TED-1 (91%, Figure 74), although all the mixtures examined gave substantial reductions in *E. graminis* infection. The disease control achieved by this combination was greater than that achieved when the compounds were used separately against the same pathogen (80% respectively, Table 2 and Table 8). Synergistic reactions against *E. graminis* infection have been demonstrated previously with DFMO + MGBG (West and Walters, 1988) and DFMO + film forming polymers (Walters, 1992). Interestingly, these two compounds, E-BED and TED-1, would appear to possess different modes of action and this may be responsible for their increased control of *E. graminis* infection. Both compounds were also shown to deplete spermidine levels in the oat stripe fungus, *Pyrenophora avenae,* and this

combined effect could be responsible for the reduced fungal growth. The effects of a mixture of E-BED and TED-1 on polyamine concentrations in *P. avenae* is worthy of further examination.

4.5 CONCLUSIONS

Porter and Sufrin (1986) discussed the possibility of using polyamine analogues as anticancer agents and suggested possible modes of actions for these compounds, as well as possible advantages this approach may have over specific enzyme inhibition. The mechanisms they proposed included : inhibiting biosynthetic enzymes, competing for binding sites associated with cell proliferation, and binding at polyamine sites and thus disrupting critical macromolecular structure and function. E-BED and TED-1 both inhibited AdoMetDC activity but produced different effects on ODC activity (Table 15). TED-1 was also shown to bind at polyamine binding sites.

Porter and Sufrin (1986) also suggested that these analogues would provide certain advantages over specific enzyme inhibitors. These included : utilisation of polyamine transport and cell penetration at low concentrations, inhibition of more than one enzyme at a time, no compensatory increases in secondary enzymes, and the chance to decrease the pools of all polyamines, including spermine. The compounds examined in this thesis have not demonstrated all of these advantages, although some have been achieved. Most notably, E-BED decreased both ODC and AdoMetDC activity, but could not deplete pools of all the polyamines in fungal cells.

Most of the analogues examined exhibited good control of *E. graminis* infection of barley seedlings, and systemic experiments indicated xylem mobility for

TABLE 15. Cellular consequences in *Pyrenophora avenae* of treatment with several modulators of polyamine biosynthesis.

Parameter	DFMO	MGBG	Keto-putrescine	E-BED	TED-1
Soluble ODC Activity	depleted	decreased	decreased	depleted	increased
Soluble AdoMetDC Activity	increased	decreased	decreased	depleted	decreased
Putrescine	decreased	unchanged	unchanged	greatly increased	decreased
Spermidine	decreased	decreased	decreased	decreased	decreased
Spermine	unchanged	decreased	increased	increased	unchanged

many of these compounds. The ODC inhibitor, DFMO, has been shown to move in the transpiration stream of barley seedlings grown in nutrient solution, without undergoing metabolism (Walters and Kingham, 1990). When DFMO was applied to the roots, it was detected in the fungus on infected leaves. However, results in this thesis suggest that putrescine analogues may be subject to metabolism in plants.

Despite this, a variety of putrescine analogues have been shown here to exhibit fungicidal activity against a range of pathogens, both *in vivo* and *in vitro*. However, species dependant responses were observed. Analogues were shown to produce significant effects on enzyme activities and polyamine levels in *P. avenae*. Interestingly, each of the compounds examined produced different effects, suggesting different modes of action. E-BED was shown to give good early season control of *E. graminis* in field conditions, but only weaker control later on in the season. Preliminary investigations suggest that 5.0 mM E-BED, applied twice, could produce increases in plant height, plant dry weight and grain weight. These results, taken together with those obtained for keto-putrescine (Foster and Walters, 1993), show that perturbation of polyamine biosynthesis using polyamine analogues might provide a useful alternative mode of action for the development of new fungicides.

4.6 FUTURE CONSIDERATIONS

Despite the work described in this thesis, the exact mode of action of these analogues has not been determined and further work is required in this area. Experiments could be carried out to determine the nature of the binding of E-BED and TED-1 in *P. avenae*. It would also be useful to determine the

competitive nature of their uptake in the presence of natural polyamines. However, a prerequisite to these experiments is the establishment of a reliable method of quantifying the levels of these analogues in fungal tissues. To date, this has not been possible. Such a method could also be used to measure levels of analogue in plant tissues and could help determine the fate of the analogue within plant cells.

Many of the analogues examined exhibited curative activity against *E. graminis* infection of barley seedlings, and a closer examination of the effect of these analogues on conidial germination, germ tube growth and haustorial development would be worthwhile.

Recently in this laboratory, a method has been developed for growing large quantities of the hemibiotrophic fungus, *P. infestans,* in liquid culture. Although this has been possible in the past, yields of fungus were small and growth was very slow. Both E-BED and TED-1 were shown to inhibit the growth of this pathogen on leaf discs. It would be interesting to grow this fungus in the presence of reduced concentrations of these two compounds and examine enzyme activities and polyamine levels. In this way it would be possible to determine whether these compounds produce identical effects on all the fungi they inhibit.

More detailed examination of the performance of these compounds in field scale experiments is also merited. In particular, a detailed examination of the effect of E-BED on yield of spring barley is required, in order to determine whether the results obtained from small samples in the 1991 field trial are truly representative of the effect of the compound on grain yield. Field performance of novel fungicides is often evaluated over a number of years, in order to obtain consistent results.

The experiment in which mixtures of novel compounds were tested against *E. graminis* infection of barley yielded promising results. Synergistic interactions between fungicides are well known and many commercial fungicides contain a combination of active ingredients. New fungicides often have gaps in their fungicidal spectrum filled by combinations with other fungicides (Ammermann *et al.,* 1992). It would be interesting to compare mixtures of active ingredients and novel compounds to determine the recurence of any synergistic reactions.

As part of the screening process for any new fungicide, a number of stabilised formulations are produced and tested against fungal pathogens (Shephard, 1987). In this thesis, only simple formulations have been used i.e. addition of Tween 20 or Agral 90. It is known that formulation and addition of wetting agents can greatly enhance the fungicidal activity of compounds. Formulated versions of E-BED, TED-1 and BAD would produce a more representative comparison with commercial active ingredients. Indeed, in very recent work, two formulated versions of E-BED have been shown to give very significant control of *E. graminis* infection of barley seedlings in the glasshouse. These formulated compounds outperformed an unformulated solution of E-BED and two commercial fungicides (Walters and Havis, unpublished confidential results).

In this thesis, the polyamine analogues examined have been based on the diamine putrescine. In their paper on the use of polyamine analogues as anticancer agents, Porter and Sufrin (1986) demonstrated that spermidine analogues could also exhibit powerful antiproliferative effects. Indeed, certain spermidine analogues have been shown to suppress ODC and AdoMetDC levels, deplete intracellular polyamine pools and inhibit cell growth in human melanoma cell lines (Porter *et al.,* 1991). It may be that, in the future, examination of spermidine analogues will yield even more useful fungicidal compounds.

SECTION FIVE

BIBLIOGRAPHY

ABELES, R.H. and MAYCOCK, A.L. (1976). Suicide enzyme inactivators. *Accounts of Chemical Research 9*, 313-319.

ABELOFF, M.D., SLAVIK, M., LUK., G.D., GRIFFIN, C.A., HERMANN, J., BLANC, O., SJOERDSMA, A. and BAYLIN, S.B. (1984). Phase 1 trial and pharmacokinetic studies of alpha-difluoromethylornithine - an inhibitor of polyamine biosynthesis. *Journal of Clinical Oncology 2*, 124-130.

ABELOFF, M.D., ROSEN, S.T., LUK, G.D., GRIFFIN, C.A., HERMANN, J., BLANC, O., SJOERDSMA, A. and BAYLIN, S.B. (1986). Phase II trials of alpha-difluoromethylornithine, an inhibitor of polyamine synthesis in advanced small cell lung cancer and colon cancer. *Cancer Treatments Reports 70*, 843-845.

ADAS (1982). Cereal mildew. Leaflet number 579. Ministry of Agriculture, Fisheries and Food.

ADAS (1984). Broad beans. Booklet 2450. Ministry of Agriculture, Fisheries and Food.

ADAS (1985). Potato and Tomato Blight. Leaflet number 271. Ministry of Agriculture, Fisheries and Food.

ALHONEN-HONGISTO, L. (1980). Regulation of S-adenosylmethionine decarboxylase by polyamines in Ehrlich ascites-carcinoma cells grown in culture. *Biochemical Journal 190*, 747-754.

ALHONEN-HONGISTO, L. and JÄNNE, J. (1980). Polyamine depletion induces enhanced synthesis and accumulation of cadaverine in cultured Ehrlich ascites-carcinoma cells. *Biochemical and Biophysical Research Communications 93*, 1005-1013.

ALHONEN-HONGISTO, L., SEPPÄNEN, P., HÖLTTÄ, E. and JÄNNE, J. (1982). Replacement of natural polyamines by cadaverine and its aminopropyl derivatives in Ehrlich ascites carcinoma cells. *Biochemical and Biophysical Research Communications 106*, 291-297.

ALHONEN-HONGISTO, L., KALLIO, A., SINERVIRTA, R., JÄNNE, O.A., GAHMBERG, C.G. and JÄNNE, J. (1985). Tumourgenicity, cell-surface glycoptotein changes and ornithine decarboxylase gene pattern in Ehrlich ascites-carcinoma cells. *Biochemical Journal 229*, 711-715.

ALTMAN, A. (1982). Polyamines and wounded storage tissues - inhibition of RNase activity and solute leakage. *Physiologia Plantarum 54*, 194-198.

ALTMAN, A., KAUR-SAWHNEY, R. and GALSTON, A.W. (1977). Stabilisation of oat leaf protoplasts through polyamine mediated inhibition of senescence. *Plant Physiology 60*, 570-574.

AMMERMANN, E., LORENZ, G., WENDEROTH, B. and SAUTER, H. (1992). BAS 490F-A broad spectrum fungicide with a new mode of action. *Proceedings of the Brighton Crop Protection Conference: Pests and Diseases 1992,* 403-410.

ARNOLD, W., JOHNSON, D., DANIAU, P. and LONGHURST, C. (1992). XRD-563-A novel foliar applied fungicide for the control of powdery mildew in cereals. *Proceedings of the Brighton Crop Protection Conference: Pests and Diseases 1992*, 443-450.

APPLEBAUM, A. (1986). Polyamine involvement in the development and ripening of avocado fruit. *Acta Horticulturae 179*, 779-785.

ATKINS, J.F. LEWIS, J.B., ANDERSON, C.W. and GESTELAND, R.F. (1975). Enhanced differential synthesis of proteins in a mammalian cell-free system by addition of polyamines. *Journal of Biological Chemistry 250*, 5688-5695.

BACCHI, C.J., VERGARA, C., GAROFALO, J., LIPSCHIK, G.Y and HUNTER, S.H. (1979). Synthesis and content of polyamines in bloodstream *Trypanosoma brucei. Journal of Protozoology 16*, 484-488.

BACCHI, C.J., NATHAN, H.C., CLARKSON, A.B., BIENEN, E.J., BITONTI, A.J., McCANN, P.P. and SJOERDSMA, A. (1987). Effects of the ornithine decarboxylase inhibitors DL-α-difluoromethylornithine and α-monofluoromethyldehydroornithine methyl ester alone and in combination with suramin against *Trypanosoma brucei brucei* central nervous system. *American Journal of Tropical Medicine and Hygiene 36*, 46-52.

BACCHI, C.J., GAROFALO, J., MOCKENHAUPT, D., McCANN, P.P., DIEKEMA, K.A., PEGG, A.E., NATHAN, H.C., MULLANEY, E.A., CHUNOSOFF, L., SJOERDSMA, A. and HUNTER, S.H. (1983). *In vivo* effects of alpha-difluoromethylornithine on the metabolism and morphology of *Trypanosoma brucei-brucei*. *Molecular and Biochemical Parasitology 7*, 209-225.

BACHRACH, U. (1973). *Function of Naturally occurring Polyamines.* Academic Press, New York.

BAGGA, S., DHARMA, A., PHILLIPS, G.C. and KEUHN, G.D. (1991). Evidence for the occurrence of polyamine oxidase in the dicotyledenous plant *Medicago sativa* L. (alfalfa). *Plant Cell Reports 10*, 550-554.

BAGNI, N. and SERAFINI-FRACASSINI, D. (1979). Polyamines and plant tumours. *Italian Journal of Biochemistry 28*, 392-394.

BAILLON, J.G., KOLB, M. and MAMONT, P.S. (1989). Inhibition of mammalian spermine synthase by N-alkylated-1,3-diaminopropane derivatives *in vitro* and in cultured rat hepatoma cells. *European Journal of Biochemistry 179*, 17-21.

BAKANASHVILI, M., BARKAI-GOLAN, R., KOPELIOVITCH, E. and APPLEBAUM, A. (1987). Polyamine biosynthesis in *Rhizopus*-infected tomato fruits: possible interaction with ethylene. *Physiological and Molecular Plant Pathology 31*, 41-50.

BALLAS, S.K., MOHANDAS, N., MARTON, L.J. and SHOHET, S.B. (1983). Stabilisation of erythrocyte membranes by polyamines. *Proceedings of the National Academy of Sciences of the United States of America - Biological Sciences 80*, 1942-1946.

BARTHOLEYNS, J. and KOCH-WESER, J. (1981). Effects of alpha-difluoromethylornithine alone or in combined with adriamycin or vindesine on L1210 leukaemia in mice, EMT6 solid tumours in mice and solid tumours induced by injection of hepatoma tissue-culture cells in rats. *Cancer Research 41*, 5158-5161.

BARTHOLEYNS, J., MAMONT, P. and CASARA, P. (1984). Involvement of histamine in growth of mouse and rat tumours-antitumoural properties of monofluoromethylhistidine, an enzyme activated irreversible inhibitor of histidine decarboxylase. *Cancer Research 44*, 4972-4977.

BERLIN, J. and FOCHE, E. (1981). DL-α-difluoromethylornithine causes enlargement of cultured tobacco cells. *Zeitschrift fur Pflanzenphysiologie 101*, 272-282.

BEY, P. (1978). *In* Enzyme-Activated Irreversible Inhibitors. (N. Seiler, M.J. Jung, and J. Koch-Weser, eds.), pp 27-41. Elsevier, New York.

BEY, P., DANZIN, C., VANDORSSELAER, V., MAMONT, P., JUNG, M. and TARDIF, C. (1978). Analogs of ornithine as inhibitors of ornithine decarboxylase - New deductions concerning topography of enzymes active site. *Journal of Medical Chemistry 21*, 50-55.

BEY, P., GERHART, F., VAN DORSSELAR, V. and DANZIN, C. (1983). Alpha(fluoromethyl)dehydroornithine and alpha(fluoromethyl)dehydroputrescine analogs as irreversible inhibitors of ornithine decarboxylase. *Journal of Medicinal Chemistry 26,* 1551-1556.

BEY, P., DANZIN, C. and JUNG, M. (1987). Inhibition of basic amino acid decarboxylases involved in polyamine biosynthesis. *In:* Inhibition of Polyamine Metabolism. Eds P.P. McCann, A.E. Pegg and A. Sjoerdsma, pp 1-31. Academic Press.

BIASI, R., BAGNI, N. and COSTA, G. (1988). Endogenous polyamines in apple and their relationship to fruit set and fruit growth. *Physiologia Plantarum 73*, 201-205.

BIASI, R., COSTA, G. and BAGNI, N. (1991). Polyamine metabolism as related to fruit set and growth. *Plant Physiology and Biochemistry 29*, 497-506.

BIONDI, S., POLGROSSI, I. and BAGNI, N. (1993). Effect of polyamine biosynthesis inhibitors on mycelial growth and concentrations of polyamines in *Ophiostoma ulmi* (Buism.) Nannf. *New Phytologist 123*, 415-419.

BIRECKA, H., GARRAWAY, M.O., BAUMANN, R.J. and McCANN, P.P. (1986). Inhibition of ornithine decarboxylase and growth of the fungus *Helminthosporium maydis*. *Plant Physiology 80*, 798-800.

BITONTI, A.J., CASARA, P.J., McCANN, P.P. and BEY, P. (1987). Catalytic irreversible inhibition of bacterial and plant arginine decarboxylase activities by novel substrate and product analogues. *Biochemical Journal 242,* 69-74.

BITONTI, A.J., BUSH, T.L. and McCANN, P.P. (1989). Regulation of polyamine biosynthesis in rat hepatoma (HTC) cells by a bisbenzyl polyamine analogue. *Biochemical Journal 257,* 769-774.

BITONTI, A.J., DUMONT, J.A., BUSH, T.L., EDWARDS, M.L., STEMERICK, D.M., McCANN, P.P. and SJOERDSMA, A. (1989). Bis(benzyl)polyamine analogs inhibit the growth of chloroquine-resistant human malaria parasites (*Plasmodium falciparum) in vitro* and in combination with α-difluoromethylornithine cure murine malaria. *Proceedings of the National Academy of Sciences of the United States of America - Medical Sciences 86*, 651-655.

BLOOMFIELD, V.A. and WILSON, R.W. (1981). Interactions of polyamines with polynucleotides. In *Polyamines in Biology and Medicine.* Eds. D.R. Morris and L.J. Marton, pp 183-206. Dekker, New York.

BOWMAN, W.H., TABOR, C.W. and TABOR, H. (1973). Spermidine biosynthesis, purification and properties of propylamine transferase from *Escherichia coli. Journal of Biological Chemistry 248*, 2480-2486.

BRAWLEY, J.V. and FERRO, A.J. (1979). Polyamine biosynthesis during germination of yeast ascospores. *Journal of Bacteriology 140*, 649-654.

BURCHILL, R.T. (1978). Powdery mildews of tree crops. *In* 'The Powdery Mildews' Ed. D.M. Spencer, pp 473-493. Academic Press, London.

BURTIN, D., MARTIN-TANGUY, J., PAYNOT, M. and ROSSIN, N. (1989). Effects of the suicide inhibitors of arginine and ornithine decarboxylase activities on organogenesis, growth, free polyamine and hydroxyycinnamoyl putrescine levels in leaf explants of *Nicotiana Xanthi* n.c. cultivated *in vitro* in a medium producing callus formation. *Plant Physiology 89*, 104-110.

BURTIN, D., MARTIN-TANGUY, J., PAYNOT, M., CARRÉ, M. and ROSSIN, N. (1990). Polyamines, Hydroxycinnamoylputrescines, and root formation in leaf explants of tobacco cultivated *in vitro*. *Plant Physiology 93*, 1398-1404.

BUSHNELL, M.E. and BULL, A.T. (1974). Polyamine, magnesium and ribo-nucleic acid levels in steady-state cultures of the mould *Aspergillus nidulans. Journal of General Microbiology 81*, 271-273.

BYERS, T.L. BUSH, T.L. McCANN, P.P. and BITONTI, A.J. (1991). Antitrypanosomal effects of polyamine biosynthesis inhibitors correlate with increases in *Trypanosoma brucei brucei* S-adenosyl-L-methionine. *Biochemical Journal 274*, 527-533.

CABANNE, F., DALEBROUX, M.A., MARTIN-TANGUY, J. and MARTIN, C. (1981). Hydroxycinnamic acid amides and ripening to flower of *Nicotinia tabacum* var. Xanthi n.c. *Physiologia Plantarum 53*, 399-404.

CALDARERA, C.M., BARBIROLI, B. and MORUZZI, G. (1965). Polyamines and nucleic acids during the development of the chick embryo. *Biochemical Journal 97,* 94-98.

CANELLAKIS, E.S., VICEPS-MADONE, D., KYRIAKIDIS, D.A. and HELLER, J.S. (1979). Regulation and function of ornithine decarboxylase and of the polyamines. *Current Topics in Cell Regulation 15,* 155-202.

CASARA, P., DANZIN, C., METCALF, B. and JUNG, M. (1985). Stereospecific synthesis of (2R, 5R) -hept-6-yne-2, 5-diamine - a potent and selective enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC). *Journal of the Chemical Society - Perkin Transactions 1,* 2201-2207.

CHATTERJEE, S., CHOUDHURI, M.M. and GHOSH, B. (1983). Changes in polyamine contents during root and nodule growth of *Phaseolus mungo. Phytochemistry 22,* 1533-1556.

CHRIQUI, D., D'ORAZI, D. and BAGNI, N. (1986). Ornithine and arginine decarboxylases and polyamine involvement during in vivo differentiation and in vitro differentiation of *Dattura innoxia* leaf explant. *Physiologia Plantarum,* 589-596.

CHUN, P.W., RENNERT, O.M., SAFFERN, E.E. and TAYLOR, W.J. (1976). Effect of polyamines on electrokinetic properties of red blood cells. *Biochemical and Biophysical Research Communications 69,* 1095-1101.

CLARK, J.L. and FULLER, J.L. (1975). Regulation of ornithine decarboxylase in 3T3 cells by putrescine and spermidine: Indirect evidence for translational control. *Biochemistry 14,* 4403-4409.

CLARKSON, A.B. Jr., BACCHI, C.J., MELLOW, G.H., NATHAN, H.C., McCANN, P.P. and SJOERDSMA, A. (1983). Efficacy of combinations of difluoromethylornithine and bleomycin in a mouse model of central nervous system African trypanosomiasis. *Proceedings of the National Academy of Sciences of the United States of America - Biological Sciences 80,* 5729-5733.

C.M.I (1965). *Uromyces viciae-fabae.* Description number 60. Commonwealth Mycological Institute.

C.M.I. (1967). *Erysiphe graminis.* Description number 153. Commonwealth Mycological Institute.

C.M.I. (1967). *Podosphaera leucotricha.* Description number 158. Commonwealth Mycological Institute.

C.M.I. (1968). *Pyricularia oryzae.* Description number 169. Commonwealth Mycological Institute.

C.M.I. (1973). *Pyrenophora avenae.* Description number 389. Commonwealth Mycological Institute.

C.M.I. (1974). *Sclerotinia fuckeliana* (conidial state : *Botrytis cinerea*). Description number 431. Commonwealth Mycological Institute.

C.M.I. (1974). *Botrytis fabae.* Description number 432. Commonwealth Mycological Institute.

C.M.I. (1983). *Plant Pathologists Pocketbook.* Eds. A. Johnston and C. Booth. 2nd Edition. 439 p.

COHEN, S.S. (1971). *Introduction to the Polyamines.* Prentice-Hall, New York.

COHEN, S.S. and LICHTENSTEIN, J. (1960). Polyamines and ribosome structure. *Journal of Biological Chemistry 235*, 2112-2116.

COHEN, S.S. and GREENBERG, M.L. (1981). Spermidine, an intrinsic component of turnip yellow mosaic-virus. *Proceedings of the National Academy of Sciences of the United States of America - Biological Sciences 78*, 5470-5474.

COHEN, E., ARAD, S., HEIMER, Y. and MIZRAHI, Y. (1982). Participation of ornithine decarboxylase in early stages of tomato fruit development. *Plant Physiology 70*, 540-543.

CORTI, A., DAVE, C., WILLIAMS-ASHMAN, H.G., MIHICH, E. and SCHENONE, A. (1974). Specific inhibition of enzymatic decarboxylation of S-adenosylmethionine by methylglyoxal bis(guanylhydrazone) and related substances. *Biochemical Journal 139*, 351-357.

COSTA, G. and BAGNI, N. (1983). Effects of polyamines on fruit-set of apple. *Hortscience 18*, 59-61.

COWARD, J.K. (1982). Polyamine metabolism-recent developments and implications for the design of new chemotherapeutic agents. *Annual review of Medicinal Chemistry 17*, 253-259.

DANZIN, C., JUNG, M.J., METCALF, B.W., GROVE, J. and CASARA, P. (1979a). Decrease of polyamine levels in rat-tissues by 5-Hexyne-1,4-diamine, an enzyme activated irreversible inhibitor of ornithine decarboxylase. *Biochemical Pharmacology 28*, 627-631.

DANZIN, C., JUNG, M.J., SEILER, N. and METCALF, B.W. (1979b). Effect of 5-Hexyne-1,4-diamine on brain J-aminobutyric acid metabolism in rats and mice. *Biochemical Pharmacology 28*, 633-639.

DANZIN, C., CASARA, P., CLAVERIE, N. and METCALF, B.W. (1981). Alpha-etynyl and alpha-vinyl analogues of ornithine as enzyme activated inhibitors of mammalian ornithine decarboxylase. *Journal of Medicinal Chemistry 24*, 16-20.

DANZIN, C., BEY, P., SCHIRLIN, D. and CLAVERIE, N. (1982) Alpha-monofluoromethyl and alpha-difluoromethyl putrescine as ornithine decarboxylase inhibitors *in vitro* and *in vivo* biochemical properties. *Biochemical Pharmacology 31*, 3871-3878.

DANZIN, C. and MAMONT, P.S. (1987). Polyamine inhibition *in vivo* and in organ growth and repairs. *In* Inhibition of Polyamine Metabolism. Eds P.P. McCann, A.E. Pegg and A. Sjoerdsma, pp 141-164. Academic Press.

DAVIS, R.H. (1990). Management of polyamine pools and the regulation of ornithine decarboxylase. *Journal of Cellular Biochemistry 44*, 199-205.

DAVIS, R.H. and RISTOW, J.L. (1988). Polyamine transport in *Neurospora crassa*. *Archives of Biochemistry and Biophysics 267*, 479-489.

DAVIS, R.H. and RISTOW, J.L. (1991). Polyamine toxicity in *Neurospora crassa*: Protective role of the vacuole. *Archives of Biochemistry and Biophysics 285*, 306-311.

DAVIS, R.H., MORRIS, D.R. and COFFINO, P. (1992). Sequestered end-products and enzyme regulation- the case for ornithine decarboxylase. *Microbiological Reviews 56*, 280-290.

DETERS, J. MISKIMEN, J. and MacDOUGALL, K.J. (1974). Putrescine-requiring mutant of *Neurospora crassa. Genetics 77*, 16-17.

DION, A.S. and COHEN, S.S. (1972). Polyamine stimulation of nucleic-acid synthesis in an uninfected and phage-infected polyamine auxotroph of *Escherichia coli* K12. *Proceedings of the National Academy of Sciences of the United States of America 69*, 213-217.

DITOMASO, J.M., SHAFF, J.E. and KOCHLAN, L.V. (1989). Putrescine-induced wounding and it's effects on membrane integrity and ion-transport processes in roots of intact corn seedlings. *Plant Physiology 90,* 988-995.

DOBSON, S.C. and GILTRAP, N.J. (1991). Timing of sprays for control of rust and chocolate spot in spring and winter field beans. *Aspects of Applied Biology 27*, 111-116.

DUMAS, E., PEDRIZET, E. and VALLEE, J. (1981). Évolution quantitative des acides aminés et amines libres au cours du développement de diverses espéces de *Nicotiana. Physiologie Vegétale 19*, 155-165.

DUMAS, E., VALLEE, J. and PEDRIZET, E. (1982). Étude comparée du métabolisme des acides aminés et amines libres chez deux espéces de Tabac. *Physiologie Vegétale 20*, 505-514.

EGEA-CORTINES, M. and MIZRAHI, Y. (1993). The effect of difluoromethylornithine on polyamine levels in pollinated and napthaleneacetic acid-induced young tomato fruits. *Plant Growth Regulation 12*, 287-292.

EL HADRAMI, I. and D'AUZAC, J. (1992). Effects of polyamine biosynthetic inhibitors on somatic embryogenesis and cellular polyamines in *Hevea brasiliensis*. *Journal of Plant Physiology 140*, 33-36.

ELAD, Y. (1991). An inhibitor of polyamine biosynthesis-difluoromethylornithine-and the polyamine spermidine for the control of gray mould (*Botrytis cinerea*). *Phytoparasitica 19*, 201-209.

ELO, H., MUTIKAINEN, I., ALHONEN-HONGISTO, L., LAINE, R., JÄNNE, J. and LUMME, P. (1986). Biochemical properties and crystal structure of ethylmethylglyoxal bis guanyl(hydrazone) sulfate - an extremely powerful novel inhibitor of adenosylmethionine decarboxylase. *Zeitschrift fur Naturforschung 41c*, 851-855.

ERWIN, B.G. and PEGG, A.E. (1982). Uptake of α-difluoromethylornithine by mouse fibroplasts. *Biochemical Pharmacology 31*, 2820-2823.

FAUCI, A.S., MACHER, A.M., LONGO, D.L., LANE, C.H., ROOK, A.H., MASUR, H. and GELMAN, E.P. (1984). Acquired immunodeficiency syndrome - epidemiologic, clinical, immunological and therapeutic considerations. *Annals of Internal Medicine 100*, 92-106.

FEIRER, R., MIGNON, G. and LITVAY, J. (1984). Arginine decarboxylase and polyamines required for embryogenesis in the wild carrot. *Science 223*, 1433-1435.

FELIX, H. and HARR, J. (1987). Association of polyamines to different parts of various plant species. *Physiologia Plantarum 71*, 245-250.

FELIX, H. and HARR, J. (1989). Influence of inhibitors of polyamine biosynthesis on polyamine levels and growth of plants. *Zeitschrift fur Naturforschung 44*, 55-58.

FEVERSTEIN, B., PORTER, C.W. and DAVE, C. (1979). A selective effect of methylgloxal bis (guanylhydrazone) on the synthesis of mitochondrial DNA in cultures of L1210 leukaemia cells. *Cancer Research 39*, 4130-4137.

FOSTER, S.A. and WALTERS, D.R. (1990). The effects of polyamine biosynthesis inhibitors on mycelial growth, enzyme activity and polyamine levels in the oat infecting fungus *Pyrenophora avenae*. *Journal of General Microbiology 136*, 233-239.

FOSTER, S.A. and WALTERS, D.R. (1992). Effects of two polyamine biosynthesis inhibitors on infection of wheat by *Puccinia graminis tritici*. *Tests of Agrochemicals and Cultivars 13*, 32-33.

FOSTER, S.A. and WALTERS, D.R. (1993). Fungicidal activity of the polyamine analogue, keto-putrescine. *Pesticide Science 37*, 267-272.

FOZARD, J.R., PART, M.L., PRAKASH, N.J. GROVE, J., SCHECHTER, P.J., SJOERDSMA, A. and KOCH-WESER, J. (1980). L-Ornithine decarboxylase: An essential role in early mammalian embryogenesis. *Science 208*, 505-508.

FOZARD, J.R. and KOCH-WESER, L. (1982). Pharmacological consequences of inhibition of polyamine biosynthesis with DL-α-difluoromethylornithine. *Trends in Pharmacological Science 3*, 107-110.

FUCHS, A. (1987). Implications of stereoisomerism in agricultural fungicides. *In* Stereoselectivity of Pesticides: Biological and Chemical Problems. eds. E.J. Ariëns, J.J.S. van Rensen and W. Welling. pp 203-262. Elsevier Press.

GALLARDO, M., BUENO, M., ANGOSTO, T., GALLARDO, E. and MATILLA, A.J. (1992). Free polyamines in *Cicer arientum* seeds during the onset of germination. *Phytochemistry 31*, 2283-2287.

GALSTON, A.W., ALTMAN, A. and KAUR-SAWHNEY, R. (1978). Polyamines, ribonuclease and the improvement of oat leaf protoplasts. *Plant Science Letters 11*, 69-79.

GALSTON, A.W. and WEINSTEIN, L.H. (1988). Control of phytopathogens by inhibitors of polyamine biosynthesis. *In* Progress in polyamine research: Novel biochemical, pharmacological and clinical aspects. eds. V. Zappia and A.E.Pegg. pp 589-599. Plenum Press.

GAROFALO, J., BACCHI, C.J., McLAUGHLIN, S.D., MOCKENHAUPT, D., TREUBA, G. and HUNTER, S.H. (1982). Ornithine decarboxylase in *Trypanosoma brucei-brucei*-evidence for selective toxicity of difluoromethylornithine. *Journal of Protozoology 29*, 389-394.

GEIGER, L.E. and MORRIS, D.R. (1978). Polyamine deficiency reduces the rate of DNA replication fork movement in *Escherichia coli. Nature (London) 272*, 730-732.

GEIGER, L.E. and MORRIS, D.R. (1980). Stimulation of deoxyribonucleic acid replication fork movement by spermidine analogs in polyamine deficient *Escherichia coli. Journal of Bacteriology 141*, 1192-1198.

GHODA,L., BASU, H.S., PORTER, C.W., MARTON, L.J. and COFFINO, P. (1992). Role of ornithine decarboxylase suppression and polyamine depletion in the antiproliferative activity of polyamine analogs. *Molecular Pharmacology 42*, 302-306.

GOLDEN, J.A., SJOERDSMA, A. and SANTI, D.V. (1984). *Pneumocystis carinii* pneumonia treated with α-difluoromethylornithine- a prospective study among patients with the acquired immunodeficiency syndrome (clinical investigation). *Western Journal of Medicine 141*, 613-623.

GUYE, M.G., VIGH, L. and WILSON, J.M. (1986). Polyamine titre in relation to chill-sensitivity in *Phaseolus* sp. *Journal of Experimental Botany 37*, 1036-1043.

HALLINE, A.G. DUDEJA, P.K. and BRASITUS, T.A. (1989). Differential stimulation of S-adenosylmethionine decarboxylase by difluoromethylornithine in the rat colon and small intestine. *Biochemical Journal 259*, 513-518.

HANNONEN, P., JÄNNE, J. and RAINA, A. (1972). Partial purification and characterisation of spermine synthase from rat brain. *Biochimica et Biophysica Acta 289*, 225-231.

HARIK, S.I., and SNYDER, S.H. (1974). Ornithine decarboxylase - Inhibition by alpha-hydrazinoornithine. *Biochimica et Biophysica Acta 327*, 501-509.

HARIK, S.I., HOLLENBERG, M.D. and SNYDER, S.H. (1974). Ornithine decarboxylase turnover is slowed by alpha-hydrazinoornithine. *Molecular Pharmacology 10*, 41-47.

HART, D., WINTHER, M and STEVENS, L. (1978). Polyamine distribution and S-adenosyl methionine decarboxylase activity in filamentous fungi. *FEMS Microbiology Letters 3*, 173-175.

HEBY, O. and JÄNNE, J. (1981). *In* "Polyamines in Biology and Medicine" (D.R. Morris and L.J. Marton, eds.), pp 243-310. Dekker, New York.

HEIMER, Y., MIZRAHI, Y. and BACHRACH, U. (1979). Ornithine decarboxylase activity in rapidly proliferating plant cells. *FEBS Letters 104*, 146-149.

HEIMER, Y. and MIZRAHI, Y. (1982). Characterisation of ornithine decarboxylase of tobacco cells and tomato ovaries. *Biochemical Journal 201*, 373-376.

HELLER, J.S., FONG, W.F. and CANNELLAKIS, E.S. (1976). Induction of a protein inhibitor to ornithine decarboxylase by end products of its reaction. *Proceedings of the National Academy of Sciences of the United States of America 73*, 1858-1862.

HERBST, E.J. and SNELL, E.E. (1948). Putrescine as a growth factor for *Haemophilus parainfluenza. Journal of Biological Chemistry 176*, 989-990.

HIBASAMI, H., BORCHARDT, R.T., CHEN, S.Y., COWARD, J.K. and PEGG, A.E. (1980a). Studies of inhibition of rat spermidine synthase and spermine synthase. *Biochemical Journal 187*, 419-428.

HIBASAMI, H., TANAKA, M., NAGAI, J. and IKEDA, T. (1980b). Inhibition of aminopropyltransferases by S-adenosylmethionine-effect of simultaneous administration of S-adenosylmethionine and methylglyoxal bis (guanylhydrazone) on polyamine concentrations in regenerating rat liver. *FEBS Letters 116*, 99-101.

HOLM, I., PERSSON, L., STJERNBORG, L., THORSSON. and HEBY, O. (1989). Feedback control of ornithine decarboxylase expression by polyamines. *Biochemical Journal 258*, 343-350.

HÖLTTÄ, E., HANNONEN, P., PISPA, J. and JÄNNE, J. (1973). Effect of methylglyoxal bis(guanylhydrazone) on polyamine metabolism in normal and regenerating rat-liver and rat thymus. *Biochemical Journal 136*, 669-676.

HÖLTTÄ, E., JÄNNE, J. and HOVI, T. (1979). Suppression of the formation of polyamines and macromolecules by DL-alpha-difluoromethylornithine and methylglyoxal bis (guanylhydrazone) in phytohemagglutin activated human lymphocytes. *Biochemical Journal 178*, 109-117.

HÖLTTÄ, E., KORPELA, H. and HOVI, T. (1981). Several inhibitors of ornithine and adenosylmethionine decarboxylases may also have antiproliferative effects unrelated to polyamine depletion. *Biochimica et Biophysica Acta 677*, 90-102.

HUNTER, K.J., STROBOS, C.A.M. and FAIRLAMB, A.H. (1991). Inhibition of polyamine biosynthesis in *Crithidia fasciculata* by D,L-α-difluoromethylornithine and D,L-α-difluoromethylarginine. *Molecular and Biochemical Parasitology 46*, 35-44.

INOUE, H., KATO, Y., TAKIGAWA, M., ADACHI, H. and TAKEDA, Y. (1975). Effect of DL-alpha-hydrazino-delta-aminovaleric acid, an inhibitor of ornithine decarboxylase, on polyamine metabolism in isoproterenol-stimulated mouse portid glands. *Journal of Biochemistry 77*, 879-893.

IVENS, G.W. (1993). The U.K. Pesticide Guide. CAB International / British Crop Protection Council.

JACOB, F. and NEUMANN, St. (1987). Principles of uptake and systemic transport of fungicides within the plant. *In* Modern Selective Fungicides: Properties, Applications and Mechanisms of Action. eds. H. Lyr. pp 13-30. Longman Scientific Press.

JÄNNE., ALHONEN-HONGISTO, L., SEPPÄNEN, P. and HÖLTTÄ, E. (1981). Cellular compensatory mechanisms during chemically induced polyamine deprivation. *Advances in Polyamine Research 3*, 85-95.

JARVIS, B.C., YASMIN, S. and COLEMAN, M.T. (1985). RNA and protein metabolism during adventitious root formation in stem cuttings of *Phaseolus aureus* cultivar berkin. *Physiologia Plantarum 64*, 53-59.

JOHANSSON, J.G., ALTO, P. and SKINNER, W.A. (1973). U.S.Patent 3754027; *Chemistry Abstracts 79*, 137504t.

KAKKAR, R.K. and RAI, V.R. (1987). Effects of spermine and IAA on carbohydrate metabolism during rhizogenesis in *Phaseolus vulgaris*. 1. Hypocotyl cuttings. *Indian Journal of Experimental Botany 25*, 476-478.

KALLIO, A. and JÄNNE, J. (1983). Role of diamine oxidase during the treatment of tumour-bearing mice with combinations of polyamine anti-metabolites. *Biochemical Journal 212*, 895-898.

KAMEJI, T., MURAKAMI, Y. and HAYASHI, S. (1979). Effect of diaminobutene and diaminopropane on diet-stimulated polyamine synthesis and cell proliferation in rat liver. *Journal of Biochemistry 86*, 191-197.

KARVONEN, E., KAUPPINEN, L., PARTENAN, T. and PÖSÖ, H. (1985). Irreversible inhibition of putrescine-stimulated S-adenosyl-L-methionine decarboxylase by Berenil and pentamidine. *Biochemical Journal 231*, 165-169.

KASHIWAGI, K., KOBAYASHI, H. and IGARASHI, K. (1986). Apparently unidirectional polyamine transport by proton motive force in polyamine-deficient *Escherichia coli*. *Journal of Bacteriology 165*, 972-977.

KATO, Y., NISHIHARI, H., INOUE, H., GHODA, E. and TAKEDA, Y. (1978). Polyamine metabolism and DNA synthesis in regenerating rat liver. *Journal of Biochemistry 84*, 1617-1623.

KAUR-SAWHNEY, R. and GALSTON, A.W. (1979). Interaction of polyamines and light on biochemical processes involved in leaf senescence. *Plant, Cell Environment 2*, 189-196.

KAUR-SAWHNEY, R., FLORES, H.E. and GALSTON, A.W. (1980). Polyamine-induced DNA-synthesis and mitosis in oat leaf protoplasts. *Plant Physiology 65*, 368-371.

KAUR-SAWHNEY, R., FLORES, H.E. and GALSTON, A.W. (1981). Polyamine oxidase in oat leaves : a cell wall localised enzyme. *Plant Physiology 68*, 494-498.

KAUR-SAWHNEY, R., TIBURCIO, A.F. and GALSTON, A.W. (1988). Spermidine and flower-bud differentiation in thin-layer explants of tobacco. *Planta 173*, 282-284.

KHAN, A.J. and MINOCHA, S.C. (1989a). Biosynthetic arginine decarboxylase in phytopathogenic fungi. *Life Sciences 44*, 1215-1222.

KHAN, A.J. and MINOCHA, S.C. (1989b). Polyamine biosynthetic enzymes and the effect of their inhibition on the growth of some phytopathogenic fungi. *Plant Cell Physiology 30*, 655-663.

KHAN, A.J. and MINOCHA, S.C. (1991). Polyamines and somatic embryogenesis in carrot II. The effects of cyclohexylammonium phosphate. *Journal of Plant Physiology 137*, 446-452.

KHAN, N.A., HAVOUIS, R., QUEMENER, V. and MOULINOUX, J.-P. (1992). Protein kinase C inhibitor (H-7) potentiates antiproliferative effects of a polyamine biosynthesis inhibitor. *Anticancer Research 12*, 1223-1226.

KHOMUTOV, R.M., HYVÖNEN, T., KARVONEN, E., PAALANEN, T., PAULIN, L., ELORANTA, T., PAJULA, R. -L., ANDERSON, L.C. and PÖSÖ, H. (1985). 1-aminooxy,3-aminopropane, a new and potent inhibitor of polyamine biosynthesis that inhibits ornithine decarboxylase, adenosylmethionine decarboxylase and spermidine synthase. *Biochemical and Biophysical Research Communications 130*, 596-602.

KOLB, M., DANZIN, C., BARTH, J. and CLAVERIE, N. (1982). Synthesis and biochemical properties of chemically stable product analogs of the reaction catalysed by S-adenosyl-L-methionine decarboxylase. *Journal of Medical Chemistry 25*, 550-556.

KOLLONITSCH, J., PATCHETT, A.A., MARBURG, S., MAYCOCK, A.L., PERKINS, L.M., DOLDURAS, G.A., DUGGAN, D.E. and ASTER, S.D. (1978). Selective inhibitors of biosynthesis on aminergic neurotransmitters. *Nature (London) 274*, 906-908.

KONECKI, D., KRAMER, G., PINPHANICHAKARN, P. and HARDESTY, B. (1975). Polyamines are necessary for maximum *in vitro* synthesis of globin peptides and play a role in chain initiation. *Archives of Biochemistry and Biophysics 169*, 192-198.

KOVACS, J.A. and MASUR, H. (1985). *In* "AIDS: A basic guide for clinicians" (P. Ebbeson, R.J. Biggar and M. Melbye, eds.), pp. 84-98. Saunders, Philadelphia.

KRAMER, D.L., KHOMUTOV, R.M., BUKIN, Y.V. KHOMUTOV, A.R. and PORTER, C.W. (1989). Cellular characterisation of a new irreversible inhibitor of S-adenosylmethionine decarboxylase and its use in determining the relative abilities of individual polyamines to sustain growth and viability of L1210 cells. *Biochemical Journal 259*, 325-331.

KRAMER, G.F., WANG, C.Y. and CONWAY, W.S. (1991). Inhibition of softening by polyamine application in 'Golden Delicious' and 'McIntosh' apples. *Journal of American Society of Horticultural Science 116*, 813-817.

KUSHAD, M. and DUMBROFF, E. (1991). Metabolic and physiological relationships between the polyamine and ethylene biosynthetic pathways. *In* R.D. Slocum and H. E. Flores eds. Biochemistry and Physiology of Polyamines in Plants, pp 77-92. Boca Raton : CRC Press.

KYRIAKIDIS, D.A. (1983). Effect of plant growth hormones and polyamines on ornithine decarboxylase activity during the germination of barley seeds. *Physiologia Plantarum 57*, 499-504.

LAFARGE-FRAYSSINET, C., BERTAUX, O., VALENCIA, R. and FRAYSSINET, C. (1978). Evolution of ornithine decarboxylase activity during cell cycle of *Euglena gracilis* Z in synchronous culture-Influence of vitamin B12. *Biochimica et Biophysica Acta 539*, 435-444.

LAST, F.T. and HAMLEY, R.E. (1956). A local-lesion technique for measuring the infectivity of conidia of *Botrytis fabae* Sardina. *Annals of Applied Biology 44*, 410-418.

LAW, D.M., DAVIES, P.J. and MUTSCHLER, M.A. (1991). Polyamine-induced prolongation of storage in tomato fruits. *Plant Growth Regulation 10*, 283-290.

LEVITZ, S.M., DIBENEDETTO, D.J. and DIAMOND, R.D. (1990). Inhibition and killing of fungi by the polyamine oxidase-polyamine system. *Antonie van Leeuwenhoek Institute Journal of Genetic and Molecular Microbiology 58*, 107-114.

LIBBY, P.R. and PORTER, C.W. (1992). Inhibition of enzymes of polyamine back-conversion by pentamidine and berenil. *Biochemical Pharmacology 44*, 830-832.

LIQUORI, A.M., CONSTANTINO, L., CRESCEUZI, V., ELIA, V., GIGLIO, E., PULITI, R., DESANTIS SAVINO, M. and VITAGLIANO, V. (1967). Complexes between DNA and polyamines : a molecular model. *Journal of Molecular Biology 24*, 113-122.

LITTLE, J.R. (1962). Inhibition of reticulocyte lysis by alkyl polyamines. *Proceedings of the Society for Experimental Biology and Medicine 109*, 910-912.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry 193*, 265-272.

LUK, G.D., GOODWIN, G., GAZDAR, A.F. and BAYLIN, S.B. (1983). Successful treatment with DL-alphadifluoromethylornithine in established human small cell variant lung-carcinoma implants in athymic mice. *Cancer Research 42*, 3070-3073.

McCANN, P.P., TARDIFF, C., DUCHESNE, M.C. and MAMONT, P.S. (1977). Regulation of ornithine decarboxylase by ODC-antizyme in HTC cells. *Biochemical and Biophysical Research Communications 76*, 893-899.

McCANN, P.P. (1980). *In* "Polyamines in Biomedical Research" (J.M. Gaugas, ed.), pp. 109-123. Wiley, New York.

McCORMICK, F. (1977). Polyamine metabolism in enucleated mouse L-cells. *Journal of Cell Physiology 93*, 285-292.

MACHATSCHKE, S., KAMROWSKI, C., MOERSCHBACHER, B.M. and REISENER, H.-J. (1990). Polyamine levels in stem rust infected wheat leaves and effects of alpha-difluoromethylornithine on fungal infection. *Physiological and Molecular Plant Pathology 36*, 451-459.

MADHUBULA, R., SECRIST, J.A. and PEGG, A.E. (1988). Effect of inhibitors of S-adenosylmethionine decarboxylase on the contents of ornithine decarboxylase and S-adenosylmethionine decarboxylase in L1210 cells. *Biochemical Journal 254*, 45-50.

MAGER, J. (1962). Common site of action for polyamines and streptomycin. *Biochimica et Biophysica Acta 62*, 202-204.

MAJUMDER, S., WIRTH, J.J., BITONTI, A.J., McCANN, P.P. and KIERSZENBAUM, F. (1992). Biochemical evidence for the presence of arginine decarboxylase activity in *Trypanosoma cruzi*. *Journal of Parasitology 78*, 371-374.

MAMONT, P.S., DUCHESNE, M.C., GROVE, J. and BEY, P. (1978). Antiproliferative properties of DL-alpha-difluoromethylornithine in cultured cells-consequence of irreversible inhibition of ornithine decarboxylase. *Biochemical and Biophysical Research Communications 81*, 58-66.

MAMONT, P.S., DANZIN, C., WAGNER, J., SIAT, M., JODER-OHLENBUSCH, A.M. and CLAVERIE, N. (1982). Accumulation of decarboxylated S-adenosyl-L-methionine in mammalian cells as a consequence of the inhibition of putrescine biosynthesis. *European Journal of Biochemistry 123*, 499-504.

MAMONT, P.S., SIAT, M., JODER-OHLENBUSCH, A.M., BERNHARDT, A. and CASARA, P. (1984). Effects of (2R,5R)-6,heptyne-2,5-diamine a potent inhibitor of L-ornithine decarboxylase in rat hepatoma-cells cultured *in vitro*. *European Journal of Biochemistry 142*, 457-463.

MAMONT, P.S., DANZIN, C., KOLB, M., GERHART, F., BEY, P. and SJOERDSMA, A. (1986). Marked and prolonged inhibition of mammalian ornithine decarboxylase *in vivo* by esters of (E)-2-(fluoromethyl) dehydroornithine. *Biochemical Pharmacology 35*, 159-165.

MARTIN-TANGUY, J. (1985). The occurrence and possible function of hydroxycinnamoyl acid amides in plants. *Plant Growth Regulation 3*, 381-399.

MARTIN-TANGUY, J., CABANNE, F., PERDRIZET, E. and MARTIN, C. (1978). The distribution of hydroxycinnamic acid amides in flowering plants. *Phytochemistry 17*, 1927-1928.

MARTIN-TANGUY, J., NEGREL, J., PAYNOT, M. and MARTIN, C. (1987). Hydroxycinnamic acid amides in hypersensitivity flowering and sexual organogenesis in plants. In *Plant Molecular Biology.* Eds. D. von Wettstein and N.H. Chua, pp. 253-269. Plenum, New York.

MARTON, L.J. and MORRIS, D.R. (1987). Molecular and cellular functions of the polyamines. In *Inhibition of Polyamine Metabolism. Biological significance and basis for new therapies.* Eds. P.P. McCann, A.E. Pegg and A. Sjoerdsma, pp. 79-105. Academic Press.

MEIJER, E.G.M. and SIMMONDS, J. (1988). Polyamine levels in relation to growth and somatic embryogenesis in tissue cultures of *Medicago sativa* L. *Journal of Experimental Botany 203*, 787-794.

MENNUCI, L., ROJAS, S. and PLESSMAN CAMARO, E. (1975). Polyamines and ornithine decarboxylase activity during growth and differentiation in *Blastocladiella emersonii. Biochimica et Biophysica Acta 404*, 249-256.

METCALF, B.W., BEY, P., DANZIN, C., JUNG, M.J., CASARA, P. and VEVERT, J.P. (1978). Catalytic irreversible inhibition of mammalian ornithine decarboxylase (EC 4.1.1.17) by substrate and product analogues. *Journal of the American Chemical Society 100*, 2551-2553.

MELVIN, M.A.L. and KEIR, H.M. (1978). Diminished excretion of polyamine from BHK-21-C13 cells exposed to methylglyoxal bis (guanylhydrazone). *Biochemical Journal 174*, 349-352.

MINOCHA, S.C., MINOCHA, R. and KOMAMINE, A. (1991). Effects of polyamine biosynthesis inhibitors on S-adenosylmethionine synthase and S-adenosylmethionine decarboxylase activities in carrot cell cultures. *Plant Physiology and Biochemistry 29*, 231-237.

MIRZA, J.I. and BAGNI, N. Effects of exogenous polyamines and difluoromethylornithine on seed germination and root growth of *Arabidopsis thaliana*. *Plant Growth Regulation 10*, 163-168.

MITCHELL, J.L.A and RUSCH, H.P. (1973). Regulation of polyamine synthesis in *Physarum polychepalum* during growth and differentiation. *Biochimica et Biophysica Acta 297*, 503-516.

MITCHELL, J.L.A. and CARTER, D.D. (1977). Physical and kinetic distinction of two ornithine decarboxylase forms in *Physarum*. *FEBS Letters 102*, 265-268.

MONTAGUE, M., KOPPENBRINK, J. and JAWORSKI, E. (1978). Polyamine metabolism in embryogenic cells of *Daucus carota*. I. Changes in intracellular content and rates of synthesis. *Plant Physiology 62*, 430-433.

MORRIS, D.R. and PARDEE, A.R. (1966). Multiple pathways of putrescine biosynthesis in *Escherichia coli*. *Journal of Biological Chemistry 241*, 3129-3135.

MORRIS, D.R. and JORSTAD, C.M. (1973). Growth and macromolecular composition of a mutant of *Escherichia coli* during polyamine limitation. *Journal of Bacteriology 113*, 271-277.

MORRIS, D.R. and FILLINGAME, R.H. (1974). Regulation of amino acid decarboxylation. *Annual Review of Biochemistry 43*, 303-325.

MORRIS, D.R., JORSTAD, C.M. and SEYFRIED, C.E. (1977). Inhibition of synthesis of polyamines and DNA in activated lymphocytes by a combination of alpha-methylornithine and methyl bis(guanylhydrazone). *Cancer Research 37*, 3169-3172.

MUSSELL, H., OSMELOSKI, J., WETTLAUFER, S.H. and WEINSTEIN, L. (1987). Suppression of verticillium wilt of tomato by difluoromethylornithine, a suicidal inhibitor of polyamine biosynthesis. *Plant Disease 71*, 313-316.

NAIK, B.I. and SRIVASTAVA, S.K. (1978). Effect of polyamines on tissue permeability. *Phytochemistry 17*, 1885-1887.

NAIK, B.I., SHARMA, V. and SRIVASTAVA, S.K. (1980). Interaction between growth regulator and polyamine effects on membrane permeability. *Phytochemistry 19*, 1321-1322.

NATHAN, R., ALTMAN, A. and MONSELISE, S.P. (1984). Changes in activity of polyamine biosynthetic enzymes and in polyamine contents in developing fruit tissues of "Murcott" mandarin. *Scientia Horticulturae 22*, 359-364.

NICKERSON, K.W., DUNKLE, L.D. and VAN ETTEN, J.L. (1977). Absence of spermine in filamentous fungi. *Journal of Bacteriology 129*, 173-176.

NIKULA, P., ALHONEN-HONGISTO, L., SEPPÄNEN, P. and JÄNNE, J. (1984). Inhibition of long chain fatty acids oxidation by methylgloxal bis (guanylhydrazone). *Biochemical and Biophysical Research Communications 120*, 9-14.

NORTH, M.J. and TURNER, R. (1977). Diamine content of the cellular slime mould *Dictyostelium discoideum* - presence of 1,3-diaminopropane and putrescine. *Microbios Letters 4*, 221-228.

NORTH, M.J. and MURRAY, S. (1980). Cellular slime mould polyamines species specificity of 1,3-diaminopropane. *FEMS Letters 9*, 271-274.

NÖTHIG-LASLO, V., WEYGAUND-DURASAVIC, I. and KUCAN, Z. (1985). Structural changes of yeast transfer RNA TYZ caused by the binding of divalent ions in the presence of spermine. *Journal of Biomolecular Structure and Dynamics 5*, 941-951.

OKA, T., PERRY, J.W., TAKEMOTO, T., SAKAI, T., TERADA, N. and INOUE, H. (1981). The multiple regulatory roles of polyamines in the hormonal induction of mammary gland development. *Advances in Polyamine Research 3*, 309-320.

OKUYAMA, T. and KOBAYASHI, Y. (1961). Determination of diamine oxidase activity by liquid scintillation counting. *Archives of Biochemistry and Biophysics 95*, 242-250.

OREDSSON, S., ANEHUS, S. and HEBY, O. (1980). Irreversible inhibition of the early increase in ornithine decarboxylase activity following growth-stimulation is required to block Ehrlich ascites tumour cells proliferation in culture. *Biochemical and Biophysical Research Communications 94*, 151-158.

OUIMETTE, D.G. and COFFEY, M.D. (1989). Phosphonate levels in avocado (*Persea americana*) seedlings and soil following treatment with Fosetyl-Al or Potassium Phosphate. *Plant Disease 73*, 212-215.

PAJULA, R. -L. (1983). Kinetic properties of spermidine synthases isolated from rat and pig-liver. *Biochemical Journal 215*, 669-676.

PAJULA, R.-L. and RAINA, A. (1979). Methylthioadenosine, a potent inhibitor of spermine synthase from bovine brain. *FEBS Letters 99*, 343-345.

PAJULA, R.-L., RAINA, A. and ELORANTA, T.E. (1979). Polyamine biosynthesis in mammalian tissues - Isolation and characterisation of spermine synthase from bovine brain. *European Journal of Biochemistry 101*, 619.

PALAVAN, N., GOREN, R. and GALSTON, A.W. (1984). Effects of some growth regulators on polyamine biosynthetic enzymes in etiolated pea seedlings. *Plant and Cell Physiology 25*, 541-546.

PALAVAN-UNSAL, N. (1987). Polyamine metabolism in the roots of *Phaseolus vulgaris* - interaction of the inhibitors of polyamine biosynthesis with putrescine and in growth and polyamine biosynthesis. *Plant Cell Physiology 28*, 565-572.

PANS (1970). Manual number 3 Pest Control in Rice. Ministry of Overseas Development.

PARKER, R.E. (1983). Introductory Statistics for Biology. Studies in Biology number 43. Edward Arnold, London.

PARRY, D. (1990). 'Plant Pathology in Agriculture'. Cambridge University Press.

PEGG, A.E. (1984). S-adenosylmethionine decarboxylase - a brief review. *Cell Biochemistry and Function 2*, 11-15.

PEGG, A.E. (1986). Recent advances in the biochemistry of polyamines in eukaryotes. *Biochemical Journal 234*, 249-262.

PEGG, A.E. (1988). Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Research 48*, 759-774.

PEGG, A.E., MATSUI, I., SEELY, J.E., PRITCHARD, M.L. and PÖSÖ, H. (1981). Formation of putrescine in rat liver. *Medical Biology 59*, 327-333.

PEGG, A.E. and McCANN, P.P. (1982). Polyamine metabolism and function. *American Journal of Physiology 243*, 212-221.

PEGG, A.E., TANG, K.-C. and COWARD, J.K. (1982). Effects of S-adenosyl-1,8-diamino-3-thiooctane on polyamine metabolism. *Biochemistry 21*, 5082-5089.

PEGG, A.E., PÖSÖ, H., SHUTTLEWORTH, K. and BENNET, R.A. (1982). Effect of inhibition of polyamine synthesis on the content of decarboxylated S-adenosylmethionine. *Biochemical Journal 202*, 519-526.

PEGG, A.E. and JACOBS, G. (1983). Comparison of inhibitors of S-adenosylmethionine decarboxylase from different species. *Biochemical Journal 213*, 495-502.

PEGG, A.E., BITONTI, A.J., McCANN, P.P and COWARD, J.K. (1983). Inhibition of bacterial aminopropyltransferases by s-adenosylmethionine 1,8-diamino-3-thiooctane and by dicyclohexylamine. *FEBS Letters 155*, 192-196.

PEGG, A.E. and COWARD, J.K. (1985). Growth of mammalian cells in the absence of the accumulation of spermine. *Biochemical and Biophysical Research Communications 133*, 82-89.

PEGG, A.E., COWARD, J.K., TALEKAR, R.R. and SECRIST, J.A. (1986). Acetylation of decarboxylated S-adenosylmethionine by mammalian cells. *Biochemistry 25*, 4091-4097.

PEGG, A.E. and WILLIAMS-ASHMAN, H.G. (1987). Pharmacologic interference with enzymes of polyamine biosynthesis and of 5'-methylthioadenosine metabolism. *In* Inhibition of Polyamine Metabolism. Eds P.P. McCann, A.E. Pegg and A. Sjoerdsma, pp 141-164. Academic Press.

PEGG, A.E., KAMEJI, T., SHIRAHATA, A., STANLEY, B., MADHUBALA, R. and PAJUNEN, A. (1988). Regulation of mammalian S-adenosylmethionine decarboxylase. *Advances in Enzyme Regulation 27*, 43-55.

PERSSON, L., OREDSON, S.M., ANEHUS, S. and HEBY, O. (1985). Ornithine decarboxylase inhibitors increase the cellular content of the enzyme - implications for translational regulation. *Biochemical and Biophysical Research Communications 131*, 239-245.

PERSSON, L., KHOMUTOV, A.R. and KHOMUTOV, R.M. (1989). Feedback regulation of S-adenosylmethionine decarboxylase synthesis. *Biochemical Journal 257*, 929-931.

PFALLER, M.A., GERARDEN, T., and RILEY, J. (1987). Growth inhibition of pathogenic yeast isolates by α-Difluoromethylornithine: An inhibitor of ornithine decarboxylase. *Mycopathologia 98*, 3-8.

PFALLER, M.A., RILEY, J. and GERARDEN, T. (1988). Polyamine depletion and growth inhibition in *Candida albicans* and *Candida tropicalis* by α-difluoromethylornithine and cyclohexylamine. *Journal of Medical and Veterinary Mycology 26*, 119-126.

PFALLER, M.A., RILEY, J. and GERARDEN, T. (1990). Polyamine depletion and growth inhibition of *Cryptococcus neoformans* by α-difluoromethylornithine and cyclohexylamine. *Mycopathologia 112*, 27-32.

PLESHKEWYCH, A., KRAMER, D.L. and PORTER, C.W. (1980). Independance of drug-action on mitochondria and polyamines in L1210 leukaemia cells treated with methylglyoxal bis (guanylhydrazone). *Cancer Research 40*, 4533-4540.

PÖSÖ, H., SINERVIRTA, R. and JÄNNE, J. (1975). S-adenosylmethionine decarboxylase from bakers-yeast. *Biochemical Journal 151*, 67-73.

PORTER, C.W., DAVE, C., and MIHICH, E. (1981). *In* Polyamines in Biology and Medicine" (D.R. Morris and L.J. Marton, eds.), pp. 407-436. Dekker, New York.

PORTER, C.W. (1983). Spermidine requirement for cell proliferation in eukaryotic cells : structural specificity and quantitation. *Science 219*, 1083-1085.

PORTER, C.W. and SUFRIN, J.R. (1986). Interference with polyamine biosynthesis and / or function by analogs of polyamines or methionine as a potential anticancer chemotherapeutic strategy. *Anticancer Research 6*, 525-542.

PORTER, C.W., BERGER, F.G., PEGG, A.E., GANIS, B. and BERGERON, R.J. (1987). Regulation of ornithine decarboxylase activity by spermidine and the spermidine analogue N^1N^8-bis(ethyl)spermidine. *Biochemical Journal 242*, 433-440.

PORTER, C.W., GANIS, B., LIBBY, P.R. and BERGERON, R.J. (1991). Correlations between polyamine analogue-induced increases in spermidine/spermine N^1-acetyltransferase activity, polyamine pool depletion, and growth inhibtion in human melanoma cell lines. *Cancer Research 51,* 3715-3720.

PRAKASH, L., JOHN, P., NAIR, G. M. and PRATHAPASENAN, G. (1988). Effect of spermidine and methylglyoxal-bis(guanylhyydrazone)(MGBG) on *in vitro* pollen germination and tube growth in *Catharanthus roseus*. *Annals of Botany 61*, 373-375.

PRIEBE, A., KLEIN, H. and JAGER, H.-J. (1978). Role of polyamines in SO_2-polluted pea plants. *Journal of Experimental Botany 29*, 1045-1050.

RAHIALA, A.E.-L., KEKOMAKI, M., JÄNNE, J., RAINA, A. and RAIHA, N.C. (1971). Inhibition of pyridoxal enzymes by L-canaline. *Biochimica et Biophysica Acta 227*, 337-343.

RAINA, A. (1963). Studies on the determination of spermidine and spermine and their metabolism in the developing chick embryo. *Acta Physiologica Scandanavica ., Supplement 218*, 1-81.

RAINA, A., HYVONEN, T., ELORANTA,T., VOUTILAINEN, M., SAMEJIM, K. and YAMANOHA, B. (1984). Polyamine synthesis in mammalian tissues - isolation and characterisation of spermidine synthase from bovine brain. *Biochemical Journal 219*, 991-1000.

RAJAM, M.V. (1988). Restriction of pollen germination and tube growth in lily pollen by inhibitors of polyamine metabolism. *Plant Science 59*, 53-56.

RAJAM, M.V. and GALSTON, A.W. (1985). The effects of some polyamine biosynthetic inhibitors on growth and morphology of phytopathogenic fungi. *Plant Cell Physiology 26*, 683-692.

RAJAM, M.V., WEINSTEIN, L.H. and GALSTON, A.W. (1985). Prevention of a plant disease by specific inhibition of fungal polyamine biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America 82*, 6874-6878.

RAJAM, M.V., WEINSTEIN, L.H. and GALSTON, A.W. (1986). Kinetic studies on the control of the bean rust fungus (*Uromyces phaseoli* L.) by an inhibitor of polyamine biosynthesis. *Plant Physiology 82*, 485-487.

RAJAM, M.V., WEINSTEIN, L.H. and GALSTON, A.W. (1989). Inhibition of uredospore germination and germ tube growth by inhibitors of polyamine metabolism in *Uromyces phaseoli* L. *Plant Cell Physiology 30*, 37-41.

RAJAM, M.V., WEINSTEIN, L.H. and GALSTON, A.W. (1990). Effect of polyamine biosynthesis inhibitors on polyamine levels in bean seedlings. *Current Science 60*, 178-180.

RELYEA, N. and RANDO, R.R. (1975). Potent inhibition of ornithine decarboxylase by beta, gamma unsaturated substrate analogs. *Biochemical and Biophysical Research Communications 67*, 392-402.

ROTEM, J., BASHI, E. and KRANZ, J. (1983). Studies on crop loss in potato blight caused by *Phytopthora infestans. Plant Pathology 32,* 117-122.

RUGINI, E. and MENCUCCINI, M. (1985). Increased yield in the olive with putrescine treatment. *Hortscience 20*, 102-103.

RUGINI, E., JACOBINI, A. and LUPPINO, M. (1993). Role of basal shoot darkening and exogenous putrescine treatments on in vitro rooting and on endogenous polyamine changes in difficult to root woody species. *Scientia Horticulturae 53*, 63-72.

RUSSELL, D.H. (1970). Discussion: Putrescine and spermidine biosynthesis in growth and development. *Annals of the New York Academy of Sciences 171*, 772-782.

RUSSELL. D.H. (1973). *In* "Polyamines in Normal and Neoplastic Growth." (D.H. Russell ed.) pp. 1-13. Raven, New York.

SAMEJIMA, K. and NAKAZAWA, Y. (1980). Action of decarboxylated S-adenosylmethionine analogues in the spermidine-synthesising system from rat prostrate. *Archives of Biochemistry and Biophysics 201*, 241-246.

SCHECHTER, P.J., BARLOW, J.L.R. and SJOERDSMA, A. (1987). Clinical aspects of inhibition of ornithine decarboxylase with emphasis on therapeutic trials of eflornithine (DFMO) in cancer and protozoan diseases. *In* Inhibition of polyamine metabolism. eds. P.P McCann, A.E. Pegg and A. Sjoerdsma, pp 345-364. Academic Press.

SCHINDLER, M., KOPPEL, D.E. and SHEETZ, M.P. (1980). Modulation of membrane protein lateral mobility by polyphosphates and polyamines. *Proceedings of the National Academy of Sciences of the United States of America - Biological Sciences 77*, 1457-1461.

SCHWARTZ, M., ALTMAN, A., COHEN, Y. and ARZEE, T. (1986). Localisation of ornithine decarboxylase and changes in polyamine content in root meristems of *Zea mays. Physiologia Plantarum 67*, 485-492.

SEELY, J.E. and PEGG, A.E. (1983). Effect of 1,3 diamino-propane on ornithine decarboxylase enzyme protein in thioacetamide-treated rat liver. *Biochemical Journal 216*, 701-707.

SEYFRIED, C.E. and MORRIS, D.R. (1979). Relationship between inhibition of polyamine biosynthesis and DNA replication in activated lymphocytes. *Cancer Research 39*, 4861-4867.

SHAFF, R.E. and BEAVEN, M.A. (1976). Turnover and synthesis of diamine oxidase (DAO) in rat tissues-studies with Heparin and cycloheximide. *Biochemical Pharmacology 25*, 1057-1062.

SHEPHARD, M.C. (1987). Screening for fungicides. *Annual Review of Plant Pathology 25,* 189-206.

SHIRAHATA, A., MOROHOSHI, T. and SAMEJIMA, K. (1988). *Trans*-4-methylcyclohexylamine, a potent new inhibitor of spermidine synthase. *Chemical Pharmacology Bulletin 36*, 3220-3222.

SHYR, Y. -Y. and KAO, C. -H. (1985). Polyamines and root formation in mung bean hypocotyl cuttings. *Botany Bulletin of Academia Sinica (Taipei) 26*, 179-184.

SIIMES, M., SEPPÄNNEN, P., ALHONEN-HONGISTO, L. and JÄNNE, J. (1981). Synergistic action of 2 polyamine metabolites leads to a rapid therapeutic response in childhood leukaemia. *International Journal of Cancer 28*, 567-570.

SIKORA, L. and McDOUGALL, K.J. (1978). The regulation of ornithine decarboxylase in *Neurospora crassa. Experimental Mycology 2*, 352-358.

SINGH, P.P., SINGH, S., BASRA, A.J. and BEDI, P.S. Inhibition of mycelial growth and sporidial formation in the wheat pathogen *Neovissia indica* by a polyamine biosynthetic inhibitor. *Phytoparasitica 17*, 323-326.

SINGHANIA, S., SATYANARAYANA, T. and RAJAM, M.V. (1991). Polyamines of thermophilic moulds: distribution and effect of polyamine biosynthesis inhibitors on growth. *Mycological Research 95*, 915-917.

SJOERDSMA, A. and SCHECHTER, P.J. (1984). Chemotherapeutic implications of polyamine biosynthesis inhibition. *Clinical Pharmacology Therapies 35*, 287-300.

SJOERDSMA, A., GOLDEN, J.A., SCHECHTER, P.J., BARLOW, J.L.R. and SANTI, D.V. (1984). Successful treatment of lethal protozoal infections with the ornithine decarboxylase inhibitor, alpha-difluoromethylornithine. *Transactions of the Association of American Physicians 97*, 70-79.

SLOCUM, R.D., KAUR-SAWHNEY, R. and GALSTON, A.W. (1984). The physiology and biochemistry of polyamines in plants. *Archives of Biochemistry and Biophysics 235*, 283-303.

SLOCUM, R.D. and GALSTON, A.W. (1985a). *In vivo* inhibition of polyamine biosynthesis and growth in tobacco ovary tissues. *Plant Cell Physiology 26*, 1519-1526.

SLOCUM, R.D. and GALSTON, A.W. (1985b). Changes in polyamine biosynthesis associated with post fertilisation growth and development in tobacco ovary tissues. *Plant Physiology 79*, 336-343.

SMEDEGARD-PETERSON, V. (1988). *Pyrenophora avenae* Ito and Kurabayashi. *In* 'European Handbook of Plant Diseases'. Eds. I.M. Smith, J. Dunez, R.A. Lelliot, D.H. Philips and S.A. Archer, pp. 377-378. Blackwell Scientific Publications.

SMITH, T.A. (1970). The biosynthesis and metabolism in higher plants. *Annals of the New York Academy of Science 171,* 988-1001.

SMITH, T.A. (1972). The physiology of the polyamines and related compounds. *Endeavour 31,* 22-28.

SMITH, T.A. (1979). Arginine decarboxylase of oat seedlings. *Phytochemistry 18,* 7-54

SMITH, T.A. (1980). Plant Amines. *In* EA Bell, BV Charlwood, eds, Encyclopedia of Plant Physiology, Vol. 8. Springer-Verlag, Berlin, pp 433-460.

SMITH, T.A. (1985). Polyamines. *Annual Review of Plant Physiology 36,* 117-143.

SMITH, T.A., BARKER, J.H.A. and JUNG, M. (1990). Growth inhibition of *Botrytis cinerea* by compounds interfering with polyamine metabolism. *Journal of General Microbiology 136,* 985-992.

SMITH, T.A., BARKER, J.H.A. and JUNG, M. (1990). Effect of enzyme-activated inhibitors on ornithine decarboxylase and growth of *Botrytis cinerea. Phytochemistry 29,* 1759-1762.

SMITH, T.A., BARKER, J.H.A. and OWEN, W.J. (1992). Insensitivity of *Septoria tritici* and *Ustilago maydis* to inhibitors of ornithine decarboxylase. *Mycological Research 96,* 395-400.

SNEATH, P.H.A. (1955). Putrescine as an essential growth factor for a mutant of *Aspergillus nidulans. Nature 175,* 818-819.

SNYDER, S.H., KREUZ, D.S., MEDINA, V.J. and RUSSELL, D.H. (1970). Polyamine synthesis and turnover in rapidly growing tissues. *Annals of the New York Academy of Sciences 171,* 749-771.

SRIVASTAVA, S.K. and SMITH, T.A. (1982). The effect of some oligoamines and guanidines on membrane permeability in higher plants. *Phytochemistry 21*, 997-1008.

STEVENS, L. (1970). The biochemical role of naturally occurring polyamines in nucleic acid synthesis. *Biological Reviews 45*, 1-27.

STEVENS, L., McKINNON, I.M. and WINTHER, M. (1976). Polyamine and ornithine metabolism during the germination of conidia of *Aspergillus nidulans*. *Biochemical Journal 158*, 235-241.

STEVENS, L., McKINNON, I.M., and WINTHER, M. (1977). Effects of 1,4-Diaminobutanone on polyamine synthesis in *Aspergillus nidulans*. *FEBS Letters 75,* 180-182.

STEVENS, L., McKINNON, I.M., TURNER, R. and NORTH, M.J. (1978). The effects of 1,4-diaminobutanone on polyamine metabolism in bacteria, a cellular slime mould and rat tissues. *Biochemical Society Transactions 6*, 407-409.

STEVENS, L. and WINTHER, M.D. (1979). Spermine, spermidine and putrescine in fungal development. *Advances in Microbial Physiology 19*, 63-148.

STEVENS, L. and STEVENS, E. (1981). Regulation of ornithine decarboxylase activity during the germination of conidia of *Aspergillus nidulans*. *Biochemical Journal 158*, 235-241.

SUNKARA, P.S., FOWLER, S.K., NISHIOKA, K. and RAO, P.N. (1980). Inhibition of polyamine biosynthesis by alpha-difluoromethylornithine potentiates the cyto-toxic effects of arabinosyl cytosine in HeLa cells. *Biochemical and Biophysical Research Communications 95*, 423-430.

SUNKARA, P.S., PRAKASH, N.J., CHANG, C.C. and SJOERDSMA, A. (1983). Cytotoxicty of methylgloxal bis (guanylhydrazone) in combination with alpha-difluoromethylornithine against helacells and mouse L1210 leukaemia. *Journal of the National Cancer Institute 70*, 505-509.

SUNKARA, P.S. and PRAKASH, N.J. (1984). Inhibitors of Polyamine Biosynthesis as Antitumor and Antimetastatic Agents. *In* Novel Approaches to Cancer Chemotherapy. Academic Press, New York.

SUNKARA, P.S., CHANG, C.C. and PRAKASH, N.J. (1985). Effect of inhibition of polyamine biosynthesis by DL-alpha-difluoromethylornithine on the growth and melanogenesis of B16 melanoma *in vitro* and *in vivo*. *Cancer Research 45*, 4867-4870.

SUNKARA, P.S., BAYLIN, S.B. and LUK, G.D. (1987). Inhibitors of polyamine biosynthesis: Cellular and *in vivo* effects on tumour proliferation. *In* Inhibition of polyamine metabolism. Eds P.P. McCann, A.E. Pegg and A. Sjoerdsma pp 121-140. Academic Press.

SURESH, M.R. and ADIGA, P.R. (1977). Putrescine sensitive (artifactual) and insensitive (biosynthetic) S-adenosyl-L-methionine decarboxylase activities of *Lathyrus sativus* seedlings. *European Journal of Biochemistry 79*, 511-518.

TABOR, H. and TABOR, C.W. (1964). Spermidine, spermine and related amines. *Pharmacological Reviews 16*, 245-300.

TABOR, C.W. and DOBBS, L.G. (1970). Metabolism of 1,4-diaminobutane and spermidine in *Escherichia coli*: the effects of low temperature during storage and harvesting of cultures. *Journal of Biological Chemistry 245*, 2086-2091.

TABOR, H. and TABOR, C.W. (1972). Biosynthesis and metabolism of 1,4-diaminobutane, spermidine, spermine, and related amines. *Advances in Enzymology 36*, 203-211.

TABOR, H. and TABOR, C.W. (1975). Isolation, characterisation and turnover of glutathionylspermidine from *Escherichia coli*. *Journal of Biological Chemistry 250*, 2648-2654.

TABOR, C.W. and TABOR, H. (1976). 1,4-diaminobutane (putrescine), spermidine and spermine. *Annual Review of Biochemistry 45*, 285-306.

TABOR, C.W. and TABOR, H. (1984). Methionine adenosyltransferase (S-adenosylmethionine synthase) and S-adenosylmethionine decarboxylase. Review. *Advances in Enzymology and Related Areas of Molecular Biology 56*, 251-282.

TABOR, C.W. and TABOR, H. (1985). Polyamines in microorganisms. *Microbiological Reviews 49*, 81-99.

TAELMAN, H., SCHECHTER, P.J., MARCELIS, L., SONNET, J., KAZYUMBA, G., DASNOY, J., HAEGELE, K.D., SJOERDSMA, A. and WERY, M. (1987). Difluoromethylornithine, an effective new treatment of Gambian trypanosomiasis. *The American Journal of Medicine 82*, 607-614.

TAKEMOTO, T., NAGAMATSU, Y. and OKA, T. (1983). The study of spermidine stimulated polypeptide synthesis in cell-free translation of messenger RNA from lactating mouse mammary gland. *Biochimica et Biophysica Acta 740*, 73-79.

TEITEL, D.C., COHEN, E., ARAD, S.M., BIRNBAUM, E. and MIZRAHI, Y. (1985). The possible involvement of polyamines in the development of tomato fruits *in vitro. Plant Growth Regulation 3*, 309-317.

TORRIGIANI, P., ALTAMURA, M.M., PASQUA, G., MONACELLI, B., SERAFINI-FRACASSINI, D. and BAGNI, N. (1987). Free and conjugated polyamines during denovo floral and vegetative bud formation in thin cell-layers of tobacco. *Physiologia Plantarum 70*, 453-460.

TORRIGIANI, P., SCOCCIANTI, V. and BAGNI, N. (1988). Polyamine oxidase activity and polyamine content in maize during seed germination. *Physiologia Plantarum 74*, 427-432.

TORRIGIANI, P., SERAFINI-FRACASSINI, D. and FARA, A. (1989). Diamine oxidase activity in different physiological stages of *Helianthus tuberosus* tuber. *Plant Physiology 89*, 69-73.

TRINCI, A.P.J. (1969). A kinetic study of the growth of *Aspergillus nidulans* and other fungi. *Journal of General Microbiology 57*, 11-24.

TRIONE, E.J., STOCKWELL, V.O. and AUSTIN, H.A. (1988). The effects of polyamines on the growth and development of the wheat bunt fungi. *Botanical Gazette 149*, 173-178.

TROPP, J.S. and REDFIELD, A.G. (1983). Proton exchange rates in transfer RNA as a function of spermidine and magnesium. *Nucleic Acids Research 11*, 2121-2134.

UHLEMANN, A. and REINBOTHE, H. (1977). Characterisation of L-arginine decarboxylase of *Panus tigrinus*. *Biochemie und Physiologie der Pflanzen 171*, 85-92.

VOLZ, R.K. and KNIGHT, J.N. (1986). The use of growth regulators to increase precosity in apple trees. *Journal of Horticultural Science 61*, 181-189.

WAGNER, J., DANZIN, C. and MAMONT, P. (1982). Reversed-phase-ion-pair liquid chromatographic procedure for the simultaneous analysis of S-adenosylmethionine, its metabolites and the natural polyamines. *Journal of Chromatography 227*, 349-368.

WALKER, M.A., ROBERTS, D.R., SHIH, C.Y. and DUMBROFF, E.B. (1985). A requirement for polyamines during the cell division phase of radical emergence in seeds of *Acer saccharum*. *Plant Cell Physiology 26*, 967-972.

WALTERS, D.R. (1986). The effects of a polyamine biosynthesis inhibitor on infection of *Vicia faba* L. by the rust fungus, *Uromyces viciae-fabae* (Pers.) Schroet. *New Phytologist 104*, 613-619.

WALTERS, D.R. (1989). Polyamines and plant disease. *Plants Today*, January-February, 22-26.

WALTERS, D.R. (1992). The effects of three film forming polymers, with and without a polyamine biosynthesis inhibitor, on powdery mildew infection of barley seedlings. *Annals of Applied Biology 120*, 41-46.

WALTERS, D.R and KINGHAM, G. (1990). Uptake and translocation of α-difluoromethylornithine, a polyamine biosynthesis inhibtor, by barley seedlings: effects on mildew infection. *New Phytologist 114*, 659-665.

WANG, S.Y. and FAUST, M. (1986). Effect of growth retardants on root formation and polyamine content in apple seedlings. *Journal of American Horticultural Society 111*, 912-917.

WEINSTEIN, L.H., OSMELOSKI, J.F., WETTLAUFER, S.H. and GALSTON, A.W. (1987). Protection of wheat against leaf and stem rust and powdery mildew diseases by inhibition of polyamine metabolism. *Plant Science 51*, 311-316.

WEISS, R.L., KIMES, B.W. and MORRIS, D.R. (1973). Cations and ribosomal structure. Effects on 30s and 50s subunits of replacing bound Mg^{2+} by inorganic cations. *Biochemistry 12*, 450-456.

WEST, H.M. and WALTERS, D.R. (1988). The effects of polyamine biosynthesis inhibitors on infection of *Hordeum vulgare* L. by *Erysiphe graminis* f.sp. *hordei* Marchal. *New Phytologist 110*, 193-200.

WEST, H.M. and WALTERS, D.R. (1989). Effects of polyamine biosynthesis inhibitors on growth of *Pyrenophora teres, Gaeumannomyces graminis, Fusarium culmorum and Septoria nodorum in vitro. Mycological Research 92*, 453-457.

WILLIAMS-ASHMAN, H.G. and PEGG, A.E. (1981). In: *Polyamines in Biology and Medicine*. Eds. D.R. Morris and C.J. Marton, pp 43-73. Dekker, New York.

WILLIAMS-ASHMAN, H.G. and SEIDENFIELD, J. (1986). Aspects of the biochemical pharmacology of methylgloxal bis (guanylhydrazone). *Biochemical Pharmacology 35*, 1217-1225.

YAKUBU, M.A., BASSO, B. and KIERSZENBAUM, F. (1992). DL-α-Difluoromethylornithine inhibits intracellular *Trypanosma cruzi* multiplication by affecting cell division but not trypomastigote-amastigote transformation. *Journal of Parasitology 78*, 414-419.

YAMADA, H., ISOBE, K. and TANI, Y. (1980). Oxidation of polyamines by fungal enzymes. *Agricultural and Biological Chemistry 44*, 2469-2476.

YAMANOHA, B., SAMEJIMA, K., NAKAJIMA, T. and YASHURA, T. (1984). Differences between homogenous spermidine synthases isolated from rat and pig-liver. *Journal of Biochemistry 96*, 1273-1281.

YAMANOHA, B. and COHEN, S.S. (1985). S-adenosylmethionine decarboxylase and spermidine synthase from Chinese cabbage. *Plant Physiology 78*, 784-790.

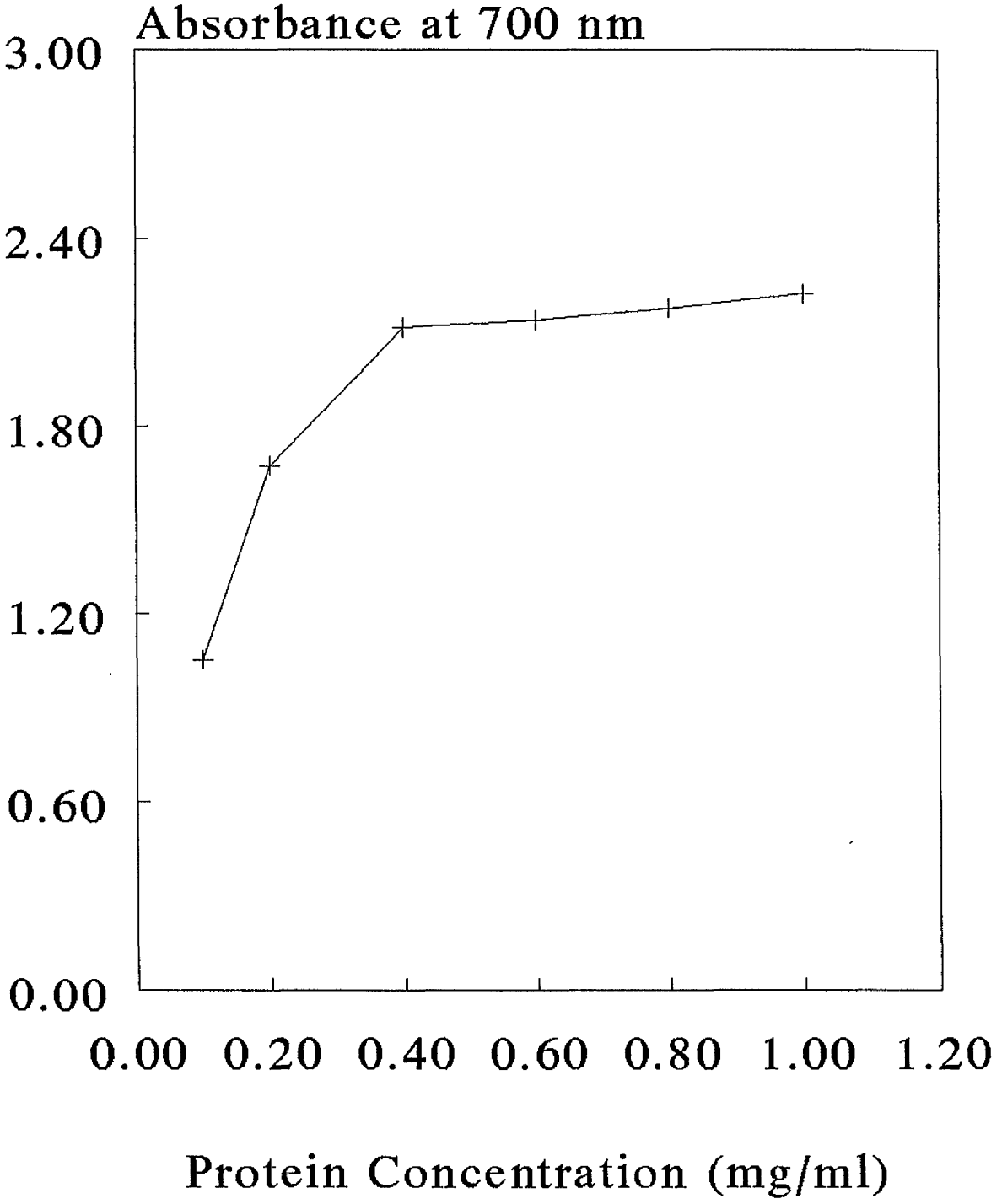
YOUNG, D.V. and SRINIVASAN, P.R. (1972). Regulation of macromolecular synthesis by putrescine in a conditional *Escherichia coli* putrescine auxotroph. *Journal of Bacteriology 112*, 30-39.

YOUNG, N.D. and GALSTON, A.W. (1983). Are polyamines transported in etiolated peas? *Plant Physiology 73*, 912-914.

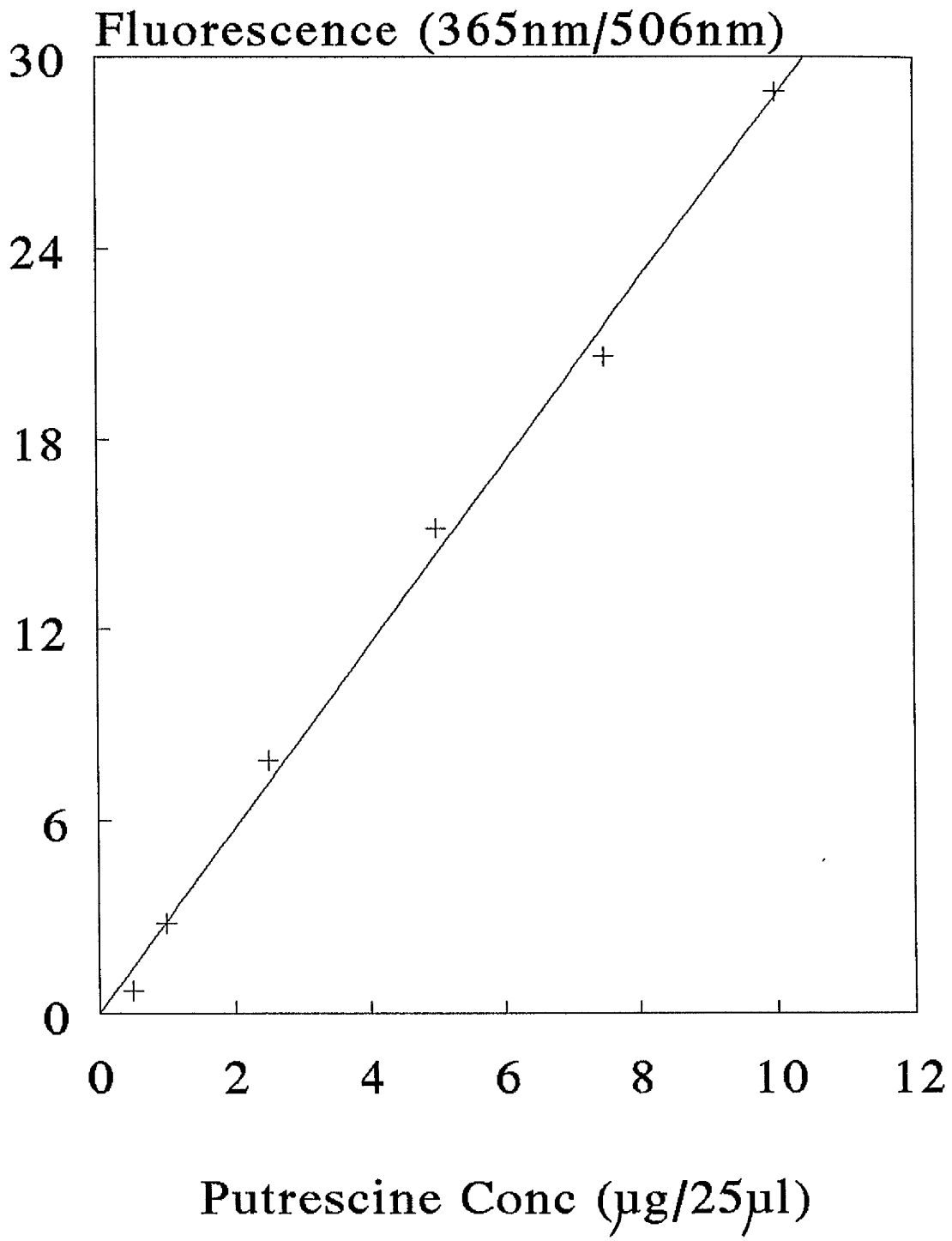
APPENDIX I

CALIBRATION CURVES

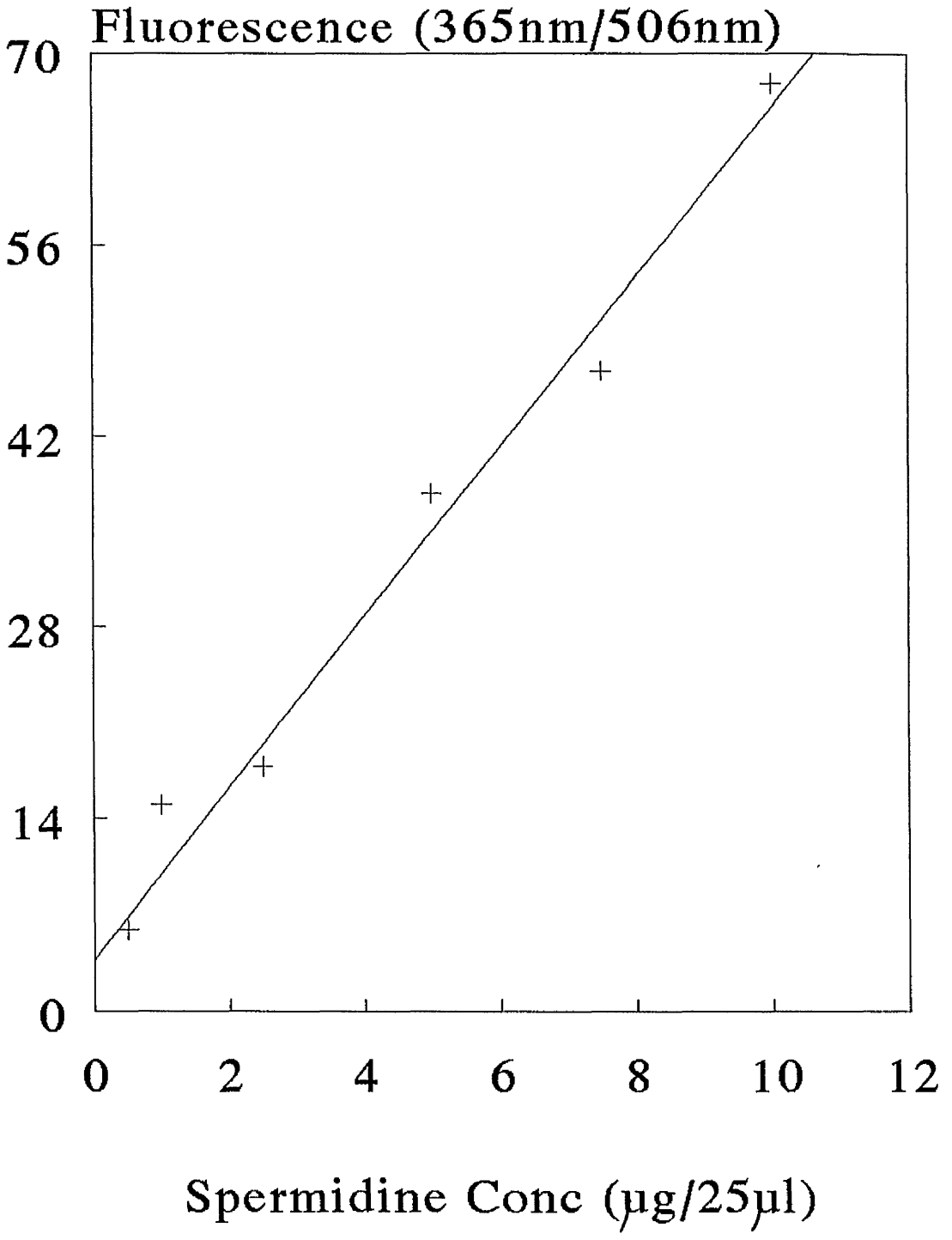
Protein Calibration Curve



Putrescine Standard Curve



Spermidine Standard Curve



Spermine Standard Curve