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POLYAMINE METABOLISM IN STRESSED WHEAT
AND INHIBITION OF POLYAMINE BIOSYNTHESIS
IN FUNGAL PATHOGENS

by

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A thesis submitted for the degree of
Doctor of Philosophy at the University of Glasgow

January, 1990

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ADDENDUM

Nuclear Fractions

The nuclear fraction referred to in the text would consist of nuclear material, non-nuclear macromolecules and cell wall fragments.

Statistics

The significant differences presented in Tables 20-27 and Figures 13-38 represent differences from the control only and not differences between treatments.

Cell Length and Width of *P. avenae*

Ten cells of *P. avenae* were measured for each treatment to obtain the mean lengths and diameters. The cells were measured as described in Section 4.221.

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ABSTRACT

Polyamine metabolism in wheat responding to biotic and abiotic stress was examined.

Putrescine, spermidine and spermine concentrations increased in *Puccinia graminis* f.sp. *tritici* - infected first leaves of wheat (*Triticum aestivum* L. cv. Sappo). However, an examination of ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) revealed that, with the exception of day 3 after inoculation, no increase in activity occurred. ADC activity in both healthy and *P. graminis*-infected plants was very low. On isolation of pustule and inter-pustule regions of infected first leaves, polyamine concentrations were found to be highest in the pustule areas although inter-pustule regions also exhibited increased polyamine concentrations compared with healthy controls. The activity of ODC was higher in both pustule and inter-pustule regions. The effects of infection by *P. graminis* on polyamine metabolism in second leaves and stems of wheat were variable.

Following a brief study of ADC activity in mannitol-treated wheat leaf segments, a detailed examination was made of polyamine metabolism in osmotic and water-stressed wheat plants. In general, the initial studies revealed an increase in ADC activity during osmotic stress. This increase was prevented by the addition of α -difluoromethylarginine (DFMA) to the incubation mixture. On examination of ADC and ODC activities in whole wheat plants watered with solutions of mannitol, the second

leaves exhibited the greatest increase in enzyme activity. Putrescine, spermine and cadaverine concentrations also increased in the second leaves of treated plants. The concentration of polyamines in the first leaves and roots of treated plants varied throughout the experimental period. On examination of the distribution of ^{14}C -mannitol in wheat plants treated with the radioisotope, greatest accumulation was observed in the roots. The first leaves of treated plants, however, contained a higher concentration of ^{14}C -mannitol than the second leaves.

Polyamine metabolism in wheat plants water-stressed using polyethylene glycol (PEG) was examined. The activities of ADC and ODC in water-stressed tissue were not significantly different from enzyme activities in control tissues, with the exception of cytosolic ODC activity which decreased in water-stressed roots. The concentrations of putrescine and cadaverine in water-stressed wheat plants were not significantly different when compared with healthy controls. However, reductions in the concentrations of spermidine and spermine in stressed first leaves and spermidine in stressed second leaves were observed, on comparison with control tissue.

A detailed examination was made of the growth of the oat-infecting fungus *Pyrenophora avenae*, grown on solid and liquid media containing the polyamine biosynthesis inhibitors α -difluoromethylornithine (DFMO), methylglyoxal bis(guanylhyazone) (MGBG) and ethylmethylglyoxal bis(guanylhyazone) (EMGBG). All of the compounds

inhibited mycelial growth of the fungus. However, MGBG and EMGBG were more effective than DFMO. The addition of exogenous putrescine and spermidine almost completely prevented inhibition of mycelial growth by DFMO. However, no such effect was observed for inhibition by MGBG or EMGBG. In general, the size of the fungal cells was not significantly affected by either the inhibitors or the exogenous polyamines. DFMO and MGBG, alone and in combination, reduced the activity of ODC in *P. avenae* and, when grown in media containing EMGBG, the fungus showed reduced activity of S-adenosylmethionine decarboxylase (SAMDC). DFMO significantly increased SAMDC activity in *P. avenae*. The concentrations of putrescine and spermidine decreased when *P. avenae* was grown in media containing DFMO or DFMO/MGBG combined, while MGBG reduced spermidine and spermine concentrations. All of the compounds reduced the concentration of cadaverine, which is a significant component of *P. avenae*. The respiration rate of the fungus decreased when grown in media containing MGBG, MGBG/carnitine or DFMO/MGBG combined.

The effects of DFMO and MGBG on infection of wheat leaves by *P. graminis* were also examined. A range of concentrations of DFMO and MGBG, alone and in combination, were applied as pre- and post-inoculation treatments. By the end of the experiment, 17 days after inoculation, pre- and post-inoculation application of the inhibitors had substantially reduced *P. graminis* infection. The addition of putrescine or spermidine to inhibitor sprays substantially reduced the effects of the inhibitors and in

some instances increased the infection above that of the controls. Leaf dry weight was usually reduced in plants exposed to pre- and post-inoculation application of inhibitors, with the greatest reductions normally occurring at the higher inhibitor concentrations. This effect was normally reversed by the addition of polyamines to the inhibitor sprays.

S E C T I O N 1

GENERAL INTRODUCTION

1. General Introduction

Polyamine research in plants, animals and micro-organisms has increased greatly in recent years. Much of this interest has resulted from the discovery of their importance and versatility in a variety of biological processes.

1.1 Structure and Distribution of Polyamines

Crystals of a polyamine were first described by Antoni van Leeuwenhoek in 1678 while studying samples of human semen. However, it was not until 1926 that workers in England and Germany finally elucidated the structure of the crystal, which was given the name spermine. Since then a number of naturally occurring polyamines have been described, although the most widely distributed ones are putrescine (1,4-diaminobutane), spermidine (4 azaoctane-1, 8-diamine) and spermine (4,9 diazadodecane-1, 12-diamine). Structures of some of these simple aliphatic compounds, which are cationic at cellular pHs, are shown in Table 1.

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

Polyamine	Structure
Putrescine	$\text{NH}_2(\text{CH}_2)_4\text{NH}_2$
Cadaverine	$\text{NH}_2(\text{CH}_2)_5\text{NH}_2$
Spermidine	$\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2$
Spermine	$\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2$

It is believed that all animal and plant cells contain certain amounts of putrescine, spermidine and spermine (Bachrach, 1973; Smith, 1975; Tabor and Tabor, 1976) with putrescine usually present in lower concentrations than either spermidine or spermine (Stevens and Winther, 1979). This however, can vary depending on the stage of cell division and differentiation. Polyamines have also been found in viruses (Fukuma and Cohen, 1975; Lanzer and Holowczak, 1975; Cohen and Greenberg, 1981), algae (Maiss *et al*, 1982), protozoa (Poso *et al*, 1976; Bacchi, Lipschik and Nathan, 1977), bacteria (Tabor and Tabor, 1972) and fungi (Viotti *et al*, 1971; Nickerson, Dunkle and van Etten, 1977; Hart, Winther and Stevens, 1978).

In fungi spermidine is usually found to be more abundant than putrescine with spermine present in very low concentrations (e.g. Stevens and Winther, 1979; Garcia *et al*, 1980; Paulus and Davis, 1981). However, in the lower eukaryotic slime moulds (Mitchell and Rusch, 1973; North and Murray, 1980) and in *Blastocladiella emersonii* (Mennucci, Rojas and Plessman Camaro, 1975) putrescine levels are higher than those of spermidine. Bacteria normally only contain putrescine and spermidine (Tabor and Tabor, 1985) although spermine has been found in *Acetobacteria* (Paulin *et al*, 1983) and *Lactobacillus* (Poso *et al*, 1976).

As well as being found in the free state, acetylated derivatives (Dubin and Rosenthal, 1960) and conjugated

forms of polyamines (Tabor and Tabor, 1966) have been described. Hydroxycinnamic acid amides 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). Polyamines conjugated with cinnamic acids have been found in tobacco stem apices, callus tissues and cell cultures (Mizusaki *et al*, 1971; Cabanne *et al*, 1981; Kapneck, 1983).

Problems in studying the intracellular distribution of polyamines have been encountered due to their basic nature which allows them to attach strongly to anions within the cell (Kashiwagi, Kobayashi and Igarashi, 1986) and also due to their high solubility in aqueous solutions.

1.2 Biosynthesis and Degradation of Polyamines

In mammalian (Pegg and Williams-Ashman, 1968; Raina and Janne, 1975) and lower eukaryotic cells including fungi (Stevens and Winther, 1979; Davis *et al*, 1985; Digangi, Seyfzadeh and Davis, 1987), putrescine, the precursor for further polyamine biosynthesis is synthesised via the enzyme ornithine decarboxylase (ODC; EC 4.1.1.17). In plants (e.g. Altman, Friedman and Levin, 1982; Birecka, Bitonti and McCann, 1985 a,b) and many bacteria (Morris and Pardee, 1966; Morris and Fillingame, 1974) putrescine synthesis can occur via two routes. One, directly through ornithine decarboxylation as in mammalian

systems and the other, indirectly, via agmatine the first product of arginine decarboxylation, catalysed by the enzyme arginine decarboxylase (ADC; EC 4.1.1.19).

The polyamine biosynthetic pathway (see Figure 1.) was first described for *Escherichia coli* (Tabor and Tabor, 1964; Morris and Pardee, 1966) and it was later found that this bacterium had two forms of the biosynthetic enzymes ADC and ODC (Morris and Fillingame, 1974). The biodegradative ornithine and arginine decarboxylases, first described in 1940 by Gale, are produced under acidic and semianaerobic conditions. However, in normal growing conditions only the biosynthetic forms of the enzymes are found.

In *Physarum polycephalum* (Mitchell and Carter, 1977; Mitchell, Carter and Rybski, 1978; Mitchell and Kottas, 1979) and cultured mouse fibroblasts (Clark and Fuller, 1976) it would appear that two forms of ODC exist, which differ in their affinities for pyridoxal phosphate. Indeed, both ADC and ODC are pyridoxal phosphate dependent enzymes (McCormick, 1977; Smith, 1979). In plants, ADC has been found only in the cytosol, while, ODC may also be found in the nucleus (Panagiotidis, Georgatsos and Kyriakidis, 1982). In animal systems, ODC occurs mainly in the cytosol (McCormick, 1977). The recent availability of monospecific antibodies to ODC have made it possible to study the enzyme in greater detail (Kameji et al, 1982; Persson, 1982; Isomaa et al, 1983).

Mammalian ornithine which is found in the plasma, can

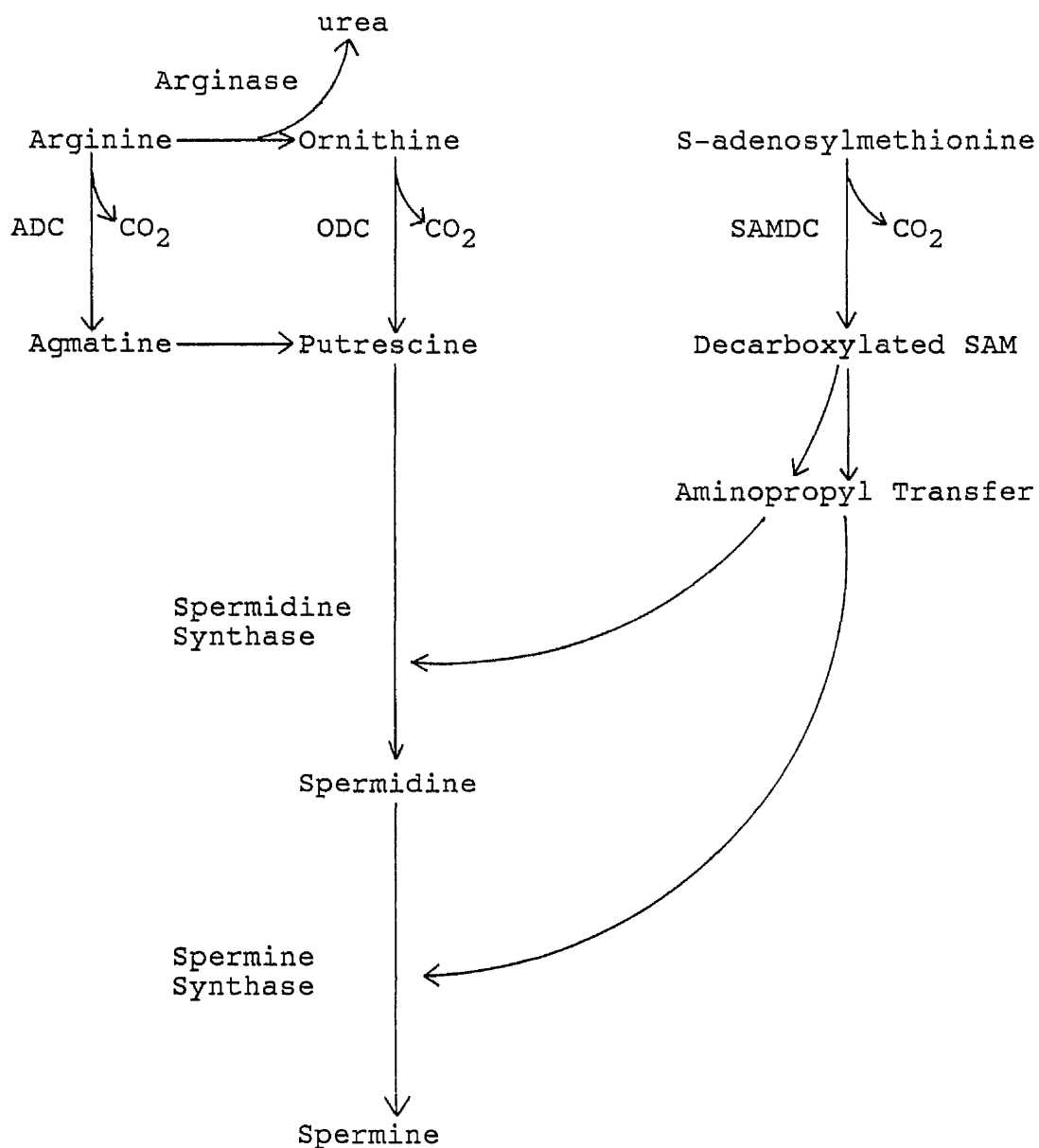


Figure 1: Biosynthesis of the major polyamines putrescine, spermidine and spermine.

also be formed in the cell from arginine, a reaction which is catalysed by the enzyme arginase (EC 3.5.3.1.) (Pegg, 1986). This enzyme is also found in most higher plants (Slocum, Kaur-Sawhney and Galston, 1984) and in the fungi *Aspergillus nidulans*, *Neurospora crassa* and *Saccharomyces cerevisiae* (Stevens and Winther, 1979). In comparison *Escherichia coli* (Morris et al, 1970), *Klebsiella aerogenes* and *Bacillus stearothermophilus* (Stevens et al, 1978) do not contain arginase.

Putrescine is converted into spermidine and spermine by the addition of aminopropyl groups, derived from decarboxylated S-adenosylmethionine (SAM). Decarboxylation of SAM is catalysed by the enzyme S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50). Tabor, Rosenthal and Tabor (1958) first described SAMDC activity in *Escherichia coli*. More recently it has been purified from mammalian cells (e.g. Sakai et al, 1979; Poso and Pegg, 1982; Seyfried et al, 1982), *Escherichia coli* (Wickner, Tabor and Tabor, 1970; Markham, Tabor and Tabor, 1982), *Saccharomyces cerevisiae* (Cohn, Tabor and Tabor, 1977) and *Lathyrus* seedlings (Suresh and Adiga, 1977).

Mammalian SAMDC activity is activated by putrescine (Pegg and Williams-Ashman, 1969; Pegg, 1977; Demetriou et al, 1978) ensuring that increased putrescine, produced by ODC, is converted to spermidine. In mammalian cells, SAMDC has no requirement for magnesium. Unlike the mammalian enzyme, SAMDC from *Escherichia coli*, plants and

some microorganisms is not activated by putrescine but does require magnesium for activity (e.g. Suresh and Adiga, 1977; Markham, Tabor and Tabor, 1982; Pegg and Jacobs, 1983). However, the protozoan *Tetrahymena pyriformis* and the slime moulds *Physarum polycephalum* and *Dictyostelium discoideum* do not appear to require either magnesium or putrescine for maximum SAMDC activity (e.g. Poso et al, 1976; Pegg and Jacobs, 1983).

Aminopropyl transfer from decarboxylated SAM to putrescine is catalysed by the enzyme spermidine synthase (EC 2.5.1.16) and the addition of another aminopropyl group converts spermidine to spermine, in a reaction catalysed by the enzyme spermine synthase (EC 2.5.1.22). Both of these enzymes are specific for their own substrate (Pegg, Shuttleworth and Hibasami, 1981). Spermidine synthase has been purified from *Escherichia coli* (Bowman, Tabor and Tabor, 1973) and Sindhu and Cohen (1983; 1984 a,b) have studied aminopropyl transferases in Chinese cabbage leaves. In mammalian systems both of these enzymes have been purified (Pajula, Raina and Eloranta, 1979; Samejima and Yamanoha, 1982). Although the aminopropyl transferase reactions are irreversible, spermidine and spermine can be converted back into putrescine by the enzymes spermidine and spermine - N¹ - acetyltransferase (these enzymes have not yet been given an IUCN number) and polyamine oxidase (PAO; EC 1.4.3.4) (Pegg, 1986). However, a clear understanding of polyamine degradation remains relatively limited. Thus, in plants PAO has been found only in cereals (Suzuki and Yanagisawa,

1980; Kaur-Sawhney, Flores and Galston, 1981; Smith, 1985), although in legumes a copper-containing diamine oxidase (DAO; EC 1.4.3.6) has been reported to be widespread (Sindhu and Desai, 1980; Matsuda and Suzuki, 1981; Federico, Dilisi and Angelini, 1985). In mammalian cells, PAO, purified in 1977 by Holtta, is flavine adenine dinucleotide dependent and its substrates are N¹ - acetylated derivatives of spermidine and spermine (Bolkenius and Seiler, 1981). Yamada, Isobe and Tani (1980) have found PAO activity in a variety of fungal species.

The pyridoxal phosphate dependent enzyme, lysine decarboxylase (LDC; EC 4.1.1.18) catalyses the conversion of lysine to the diamine cadaverine, and has been characterised and purified from *Lathyrus sativus* seedlings (Ramakrishna and Adiga, 1976). Cadaverine is widely distributed in higher plants (Smith and Wilshire, 1975) but has also been found in the slime mould *Physarum polycephalum* (Hamana and Matsuzaki, 1982) and mycoplasmas (Alhonen-Hongisto et al, 1982). Investigators found an accumulation of cadaverine in mammalian systems when polyamine biosynthesis was blocked (Alhonen-Hongisto and Janne, 1980; Alhonen-Hongisto et al, 1982) and similarly in *Escherichia coli*, workers reported the ability of cadaverine to act as a putrescine substitute (Hafner, Tabor and Tabor, 1979; Goldemberg, 1980). LDC activity was detected when *Escherichia coli* was grown in acidic, semianaerobic conditions (Sabo and Fischer, 1974; Sabo et al, 1974; Boeker and Fischer, 1983). In 1984, Lin

suggested two routes for lysine utilisation. One, for newly synthesised proteins when polyamines are present in normal levels, and the other for cadaverine synthesis when polyamine levels are low. Icekson, Bakhanashvili and Apelbaum (1986) have suggested that increased LDC activity and putrescine accumulation, found in the presence of ethylene, are possible compensatory mechanisms activated in response to ADC and SAMDC inhibition caused by ethylene. Cadaverine has also been shown to serve as a precursor for some alkaloids (Smith and Wilshire, 1975).

1.3 Function of Polyamines

Since all cells contain certain amounts of polyamines it is important to understand their possible physiological functions and involvement in growth regulation.

1.31 Nucleic Acids

Associations between nucleic acids and polyamines are well known (Stevens, 1970; Tabor and Tabor, 1976). The protonated nature of the polyamines allows them to interact with the negatively charged phosphate groups of nucleic acids. Using X-ray techniques Liquori *et al* (1967) suggested possible site-specific models in which the polyamines might stabilise the DNA double helix. More recently Bloomfield and Wilson (1981) worked on a model based on non site-specific interactions. However, Marton and Morris (1987) suggest the possible involvement of both specific and non specific interactions between DNA and

polyamines. The stability which this interaction provides has been shown to protect the nucleic acids from mechanical shearing and to increase the melting point of the double-stranded helix (Mandel, 1962; Tabor, 1962; Mahler and Mehrotra, 1963).

Previously it was difficult to assess the importance of polyamines in protein synthesis as cell-free systems were used, however the availability of polyamine-deficient mutants of *Escherichia coli* has helped to overcome this problem. Using such polyamine deficient mutants of *Escherichia coli*, Young and Srinivasan (1972) described an increase in protein synthesis brought about by the addition of putrescine. Working on the same system, Morris and colleagues found a proportional decrease in the rate of protein synthesis and growth when the mutants were grown in the absence of polyamines (Morris and Hansen, 1973; Morris and Jorstad, 1973).

1.311 DNA

Dion and Cohen (1972) suggested the dependence of *Escherichia coli* on polyamines for optimum DNA replication. When studying polyamine deficient cells Geiger and Morris (1978; 1980) found a decrease in the rate of movement of the DNA replication fork, with no effect of polyamines on initiation of DNA synthesis. With this evidence they suggested that in bacterial systems polyamines might be acting as cofactors for DNA replication rather than as regulatory molecules.

In mammalian cells, polyamine biosynthesis inhibitors have been used to study the role of polyamines in DNA replication. Workers found that a certain level of polyamines was essential for DNA replication but not for entry into the S phase of cell division (Morris, Jorstad and Seyfried, 1977; Seyfried and Morris, 1979). Similarly, Porter (1983) described a requirement of spermidine for DNA synthesis in eukaryotes.

1.312 RNA

Previous studies have shown a relationship between increased ODC activity, polyamine levels and RNA synthesis (Henningsson, Persson and Rosengren, 1978; Kuehn et al, 1979; Atmar et al, 1980), suggesting a possible regulatory role of polyamines in transcriptional events (Russell, 1983). However, Tabor and Tabor (1984) are doubtful of this transcriptional regulatory role although, they do agree that a definite correlation does exist. Several groups of workers have found that polyamines can bind to specific sites on the tRNA molecule (Quigley, Teeter and Rich, 1978; McMahon and Erdmann, 1982) and Tropp and Redfield (1983) described a stabilising effect of spermidine on the secondary and tertiary structures of tRNA.

1.313 Ribosomes

Cohen and Lichtenstein (1960) were the first to observe a relationship between polyamines and ribosomes.

Since then, polyamines have been found to be associated with ribosomes from bacterial and animal cells (e.g. Raina and Talaranta, 1967; Stevens and Pascoe, 1972; Turnock and Birch, 1973).

1.32 Polyamine Involvement in Growth and Development

Since the discovery by Herbst and Snell (1948) that putrescine was essential for the growth of the bacterium *Haemophilus parainfluenza*, much interest has been focused on the involvement of polyamines in growth and development.

Oka et al (1981) described an increase in polyamine synthesis during mammary cell growth and a similar increase in early embryonic development of rodents (Fozard et al, 1980) and chicks (Caldarera, Barbirolli and Moruzzi, 1965) was reported. Use of polyamine biosynthesis inhibitors blocked the development of the chick embryo (Heby and Emanuelsson, 1981) and of the rat ventral prostate (Danzin et al, 1979) suggesting the necessity of polyamines for normal growth. Work by Bethel and Pegg (1981) on cultured cells provides further evidence of a relationship between polyamine synthesis and cell growth.

In general, polyamine biosynthesis and concentration is usually highest in the more active areas of plant cell division (Bagni and Serafini-Fracassini, 1979) although, some workers have found that this is not always the case (Lin and Li, 1983; Mukhopadhyay et al, 1983; Palavan,

Goren and Galston, 1984). In *Phaseolus vulgaris* (Palavan and Galston, 1982), soybean (Lin, Egli and Ciembor, 1983) and mung bean (Chatterjee, Choudhuri and Ghosh, 1983) increased polyamine biosynthesis was correlated with high growth activity. Similar correlations were reported during fruit formation of tomato (Cohen et al, 1982), apple (Biasi, Bagni and Costa, 1988) and mandarin (Nathan, Altman and Monselise, 1984). Increased polyamine synthesis has also been found in plant tumour tissues (Audisio, Bagni and Serafini-Fracassini, 1976; Speranza and Bagni, 1977) and during the breaking of dormancy in Jerusalem artichoke tubers (Bagni, Malucelli and Torrigiani, 1980) and potato tubers (Kaur-Sawhney, Shih and Galston, 1982). During embryogenesis of carrot cell cultures (Montague, Armstrong and Jaworski, 1979 a,b) and in dividing tobacco suspension cultures (Heimer, Mizrahi and Bachrach, 1979), elevated levels of polyamine biosynthesis were noted. As well as cell division, polyamines are also involved in processes such as seed formation (Bagni, Caldarera and Moruzzi, 1967) and germination (Ramakrishna and Adiga, 1975; Smith and Best, 1977).

Certain mutants of the fungi *Neurospora crassa* (Deters, Miskimen and McDougall, 1974) and *Aspergillus nidulans* (Sneath, 1955) demonstrate an absolute requirement of polyamines for growth. Further, since Trinci (1969) described a high growth rate during germination, results obtained by Stevens, McKinnon and Winther (1976) stating an increase in ODC and SAMDC

activities during conidial germination of *Aspergillus nidulans*, suggests a correlation between increased polyamine synthesis and growth. Increased ODC activities have also been observed during germination of *Neurospora crassa* (Sikora and McDougall, 1978), *Blastocladiella emersonii* (Mennucci, Rojas and Plessman Camaro, 1975), *Physarum polycephalum* (Mitchell and Rusch, 1973) and *Saccharomyces cerevisiae* (Brawley and Ferro, 1979).

Although it has been shown that *Escherichia coli* (Hafner, Tabor and Tabor, 1979) and *Saccharomyces cerevisiae* (Cohn, Tabor and Tabor, 1978; 1980; Whitney and Morris, 1978) can grow in the absence of polyamines their growth is abnormally slow. This information together with earlier results describing a correlation between exponentially growing *Escherichia coli* and increased levels of polyamine synthesis (Raina and Cohen, 1966), indicate an involvement of polyamines in growth. Indeed, Setlow (1974 a,b) demonstrated a relationship between increased polyamine levels and bacterial spore germination, much as described above for fungal spore germination.

1.33 Membranes

The cationic nature of the polyamines allows them to interact with the phospholipid groups of membranes. Evidence that polyamines might interact with membranes came from the discovery that when placed in weak salt solutions, polyamines could stabilise *Escherichia coli*

spheroplasts (Mager, 1959a; Grossowicz and Ariel, 1963; Tabor and Tabor, 1972) and halophilic organisms (Mager, 1955; 1959b). In 1960, Tabor discovered the ability of polyamines to stabilise isolated mitochondria and later Huunan-Seppala (1971) described the neutralising effect of polyamines on the negative charges of the mitochondrial membrane. Tabor and Tabor (1964) attempted to explain the process involved which prevented protoplast lysis and suggested that rather than acting through an osmotic mechanism, the polyamines stabilised the membrane by forming complexes with the acidic groups in the cell membrane, thus reducing repulsive forces.

Little (1962) demonstrated the importance of spermine when used to prevent haemolysis in mammalian cells and similarly Wirtz and Becker (1961) found that diamines prevented haemolysis of red blood cells. Schindler, Koppel and Sheetz (1980) and Ballas et al (1983) further described the stabilisation of mammalian membranes by polyamines, while other workers have suggested the importance of intracellular membrane fusion (Harada, Porter and Morris, 1981; Hong, Schuber and Papahadjopoulos, 1983; Kirschbaum, 1984).

Evidence also exists for the interaction between polyamines and membranes in plants. The leakage of ions from some plant material suspension cultures (Srivastava and Smith, 1982), anthocyanin from rose petals (Parups, 1984) and betacyanin from wounded beet storage tissues (Naik and Srivastava, 1978; Altman, 1982), was significantly reduced by spermine. When working with

isolated oat protoplasts, Altman, Kaur-Sawhney and Galston (1977) discovered the ability of polyamines to stabilise against lysis of the membrane. Polyamines are thought to interfere with plant hormone responses at the membrane either by competing for binding sites or by counteracting hormone-induced changes in permeability (Naik, Sharma and Srivastava, 1980; Kyriakidis, 1983).

1.34 Senescence

While studying the regeneration of oats and other cereals from protoplasts, Brenneman and Galston (1975) discussed how polyamines could be related to plant senescence. Since then, ADC activity and polyamine levels have been found to decrease in ageing and senescing leaves (Altman and Bachrach, 1981; Fuhrer et al, 1982; Kaur-Sawhney, Shih and Galston, 1982). The application of exogenous polyamines has been found to inhibit processes involved in senescence of excised plant leaves, such as loss of chlorophyll, and RNase and protease activities (e.g. Kaur-Sawhney et al, 1982 a,b; Shih et al, 1982). A similar effect has been found in cell suspension cultures (Muhitch, Edwards and Fletcher, 1983; Muhitch and Fletcher, 1985) and isolated protoplasts (Altman, Kaur-Sawhney and Galston, 1977; Galston, Altman and Kaur-Sawhney, 1978). Thus it has been suggested that polyamines can act as antisenescence agents. However, when studying apical senescence in peas investigators found that polyamine concentrations did not decline until late stages of senescence (Smith and Davies, 1985 a,b) and

concluded from this that polyamines were not important in the initiation of senescence.

It is the opinion of some workers that the ability of individual polyamines to inhibit senescence is not equal; rather, spermine is more active than spermidine, which is more active than either putrescine or cadaverine (Kaur-Sawhney and Galston, 1979).

Since ethylene was reported to inhibit growth of etiolated pea seedlings (Stewart, Lieberman and Kunishi, 1974) and polyamines have been associated with growth, much interest has focused on the relationship between ethylene and polyamines. SAM is a common precursor to both ethylene and polyamine biosynthesis and results obtained by Even-Chen, Mattoo and Goren (1982) suggest that the ethylene and polyamine pathways are competing for SAM and may thus be regulating senescence. Indeed, workers found that polyamines inhibit ethylene activity (Apelbaum et al, 1981; Suttle, 1981; Fuhrer et al, 1982) and conversely, ethylene has been shown to inhibit polyamine biosynthesis (Apelbaum, Goldlust and Icekson, 1985; Icekson, Goldlust and Apelbaum, 1985). In contrast to this, some workers found that senescence and ethylene production were not inhibited in the presence of increased polyamine levels (Roberts et al, 1984; Downs and Lovell, 1986).

Various mechanisms have been proposed for the association between polyamines and senescence. It is thought that the application of exogenous polyamines may

inhibit senescence by preventing ethylene synthesis (Apelbaum et al, 1981; Apelbaum, Goldlust and Icekson, 1985) and stabilising membranes (Altman, Kaur-Sawhney and Galston, 1977; Grimes, Slocum and Boss, 1986). In support of this theory Kaur-Sawhney and Galston (1979) and Srivastava and Smith (1982) found that calcium ions inhibited polyamine mediated stabilisation of chlorophyll levels in senescing leaves. Working with peeled oat leaves Fuhrer et al (1982) discovered that ethylene synthesis was inhibited in the presence of exogenous polyamines and suggested that polyamines attach to membranes first and then inhibit ethylene synthesis, thus retarding senescence. Drolet et al (1986) agree with this and, finding that free radical scavenging of polyamines was associated with a number of amino groups, they suggested this to be part of the membrane stabilisation and senescence inhibition. However, Roberts, Dumbroff and Thompson (1986) caution that exogenous polyamines are associated with membrane lipids and thus increase the rigidity of the membrane which could result in misinterpretation of the physiological effects.

Since polyamines have been shown to prevent chlorophyll loss in dark incubated leaves, but not when transferred to the light (Kaur-Sawhney and Galston, 1979), it has been suggested that they are not directly affecting chlorophyll but are more likely to be preventing dark-induced degradative changes. Indeed, Kaur-Sawhney and Galston (1979) found that polyamines increase rather than inhibit chlorophyll loss in light incubated leaves.

Interestingly, results obtained by Dibble, Davies and Mutschler (1988) indicate that exogenous polyamines have a similar effect on senescence as endogenous polyamines. This is in contrast with earlier results obtained by Birecka et al (1984) who suggested that there was no correlation between effects of exogenous and endogenous polyamines.

1.35 Stress

An increase in putrescine accumulation in potassium deficient plants was first reported in 1952 by Richards and Coleman. Since then, similar responses have been documented in a variety of plants experiencing various environmental stress conditions. In agreement with Richards and Coleman (1952) other workers found large increases in putrescine during potassium deficiency in plants (Smith, 1970; Klein, Priebe and Jager, 1979). A similar effect has been shown in barley as a result of magnesium deficiencies (Smith, 1973). In addition, low pH (Smith and Sinclair, 1967; Young and Galston, 1983), excess ammonium (Breteler, 1973; Raven and Smith, 1976; Klein, Priebe and Jager, 1979), atmospheric pollutants (Priebe, Klein and Jager, 1978; Weinstein et al, 1986) and high salt levels (Shevyakova, Stroganov and Kiryan, 1985) are all conditions which lead to an increase in putrescine.

Intact plants subjected to water stress (Flores and Galston, 1982; Kandpal and Rao, 1985; Turner and

Stewart, 1986) and cereal leaf segments exposed to osmotic stress (e.g. Flores and Galston, 1984 a,b; Turner and Stewart, 1988) have been reported to accumulate putrescine. However, the magnitude of this putrescine increase varies depending upon the plant variety used and the experimental conditions. The rise in putrescine in stressed tissue has been attributed to increased ADC activity and also a decreased rate of conversion of putrescine to the higher polyamines spermidine and spermine (Flores and Galston, 1982; 1984a,b; Galston, 1983).

Attempts at regeneration of plants from cereal mesophyll protoplasts have proved difficult (Flores, Kaur-Sawhney and Galston, 1981). In an effort to understand this Flores and Galston (1982; 1984a,b) examined the effects on cereal leaves exposed to osmotic concentrations usually used in protoplast isolation and found phenomenally high putrescine concentrations and ADC activity. In contrast to this, Tiburcio et al (1986) found that in dicotyledons which easily regenerate plants from mesophyll protoplasts (Kao and Michayluk, 1980; Shekhawat and Galston, 1983 a,b) the response to osmotic stress was the opposite to that found in cereal leaves. Since high concentrations of putrescine have been found toxic in some cells (Guarino and Cohen, 1979) and since spermidine and spermine are necessary for cell division (Heby, 1981; Slocum, Kaur-Sawhney and Galston, 1984), Tiburcio et al (1986) suggested that these factors may contribute to the inability of cereal protoplasts to

divide. Tiburcio, Kaur-Sawhney and Galston (1986) found that pretreatment of protoplasts with the ADC inhibitor, α -difluoromethylarginine (DFMA), reduced ADC activity thus preventing putrescine accumulation and allowing normal production of spermidine and spermine. Under these conditions the viability of cereal mesophyll protoplasts increased.

During mineral deficiencies, acid stress and excess ammonium conditions, putrescine may accumulate to balance the ionic environment within the cell (Smith, 1971; Raven and Smith, 1976; Young and Galston, 1983). However, putrescine accumulation associated with osmotic and water stress may have a homoeostatic function similar to proline and glycine betaine (Stewart and Lee, 1974; Storey and Wyn Jones, 1975). However, since proline and glycine betaine are normally found at concentrations much greater than putrescine and there is a specific association of putrescine with nucleic acids, proteins and membranes, Young and Galston (1983) suggested a possible association of the diamine with cellular structures and micro-environments around macromolecules rather than a total cytoplasmic effect. In addition, Smith (1984) theorised that putrescine may be accumulating as a storage product or in an attempt to preserve unstable proteins.

Many tropical crops grown in temperate climates are often exposed to chilling stress, as are stored fruits. Low temperature stress can often result in various physiological and biochemical changes (Lyons, 1973; Wang,

1982). Guye, Vigh and Wilson (1986) discussed the possibility of polyamine involvement in stabilisation of bean membranes during chilling stress, with reference to the hypothesis by Lyons (1973) and Lyons, Raison and Steponkus (1979) that chilling stress altered the physical state of the membrane. Guye, Vigh and Wilson (1986) further suggested that putrescine may be accumulating to prevent loss of membrane integrity, and thus maintaining the response characteristics of the stomata to abscisic acid by preventing a "locking-open" effect (Eamus and Wilson, 1983). Nadeau, Delaney and Chouinard (1987) found a vast increase in putrescine accumulation during low temperature stress in wheat and alfalfa as did McDonald and Kushad (1986) in stored fruits. Nadeau, Delaney and Chouinard (1987) described the effects as reversible when returned to normal growing conditions. In 1987 Wang reported elevated levels of spermidine and ethylene in chilled cucumbers which were subsequently warmed. Since ethylene and polyamine biosynthesis use SAM as a precursor (Cohen, 1971; Adams and Yang, 1977) and both produce 5¹-methylthioadenosine (MTA) as a by-product (Wang, Adams and Lieberman, 1982; Yang and Hoffman, 1984), it would be expected that under stress conditions they might compete with each other. Results presented by Roberts *et al* (1984) support this theory, although Wang (1987) found no competition between the two pathways.

Unlike the other stresses mentioned whose polyamine response seems to be limited to putrescine, increases in spermine in water-stressed plants (Turner and Stewart,

1986), and spermidine in barley leaves infected with rust (Greenland and Lewis, 1984) and chill-stressed plants (Nadeau, Delaney and Chouinard, 1987; Wang, 1987) have been reported.

In addition to their involvement in plant stress, polyamines are known to be important in the control of stress reactions in prokaryotes and animals (Cohen, 1971; Bachrach, 1973; Tabor and Tabor, 1976). Polyamines have been shown to stabilise bacterial cells and protoplasts against osmotic shock and other stress-inducing factors such as heat and lysozyme activity (Mager, 1959 a; Tabor and Tabor, 1976; Peter, Ahlers and Gunther, 1978), as described in Section 1.33.

1.36 Hormones

Polyamine metabolism has been associated with growth and development in mammalian cells, plants and micro-organisms (see Section 1.32). Since hormones are important in growth processes, correlations between polyamines and hormones have been studied.

There is much evidence to suggest that in animal systems, polyamine biosynthesis is sensitive to regulation by hormones (Seely, Poso and Pegg, 1982) and similar molecules (Veldhuis, 1982; Perrella, Takigawa and Boutwell, 1983).

In plants, it has been suggested that polyamines may act as "second messengers" for plant growth regulators

(Galston, 1983), since growth stimuli often have an effect on polyamine biosynthesis (Dai, Kaur-Sawhney and Galston, 1982; Mizrahi and Heimer, 1982). Bagni, Malucelli and Torrigiani (1980) found that the application of auxin activated growth in dormant tuber tissue as did polyamines (Bagni, Calzoni and Speranza, 1978), further supporting the association between polyamines and growth regulators. Similarly, Kyriakidis (1983) observed high increases in ODC activity during the germination of barley seeds in the presence of gibberellic acid and indole acetic acid. Serafini-Fracassini and Alessandri (1983) suggested that, since morphogenesis associated with auxin-induced growth stimulation is similar to growth stimulated by polyamines, they share a common mechanism. Other plant growth regulators also influence polyamine metabolism. Thus, cytokinins were found to increase putrescine content in lettuce (Cho, 1983), barley (Hemantaranjan and Garg, 1984) and cucumber (Suresh, Ramakrishna and Adiga, 1978; Hemantaranjan and Garg, 1984).

In plants, polyamine metabolism also appears to be subject to phytochrome control (Dai and Galston, 1981; Goren *et al*, 1982). Thus, in studies of etiolated pea seedlings workers stated that changes in polyamine biosynthesis were not only the result of changes in growth rates as they separated ADC activity from growth changes brought about by hormones (Palavan, Goren and Galston, 1984) and light (Goren, Palavan and Galston, 1982).

The association between the plant hormone, ethylene

and polyamines has been widely studied (see Section 1.34).

1.4 Polyamines and Plant Disease

In addition to changes in polyamine metabolism brought about by environmental stress, plant pathogens have been shown to have a similar effect on polyamine biosynthesis in the host tissues. Increased polyamine synthesis has been reported in plant tissues infected with pathogens, such as in crown galls (Bagni, Serafini-Fracassini and Corsini, 1972; Speranza and Bagni, 1977) and club root galls (Walters and Shuttleton, 1985). However, such increases in polyamine content may not be a direct result of infection by the pathogens but instead may reflect their association with cell division.

Greenland and Lewis (1984) described an increase in polyamine levels in barley leaves infected with the brown rust, *Puccinia hordei*. Similarly, Walters, Wilson and Shuttleton (1985) reported increased polyamine levels and enzyme activities in barley leaves infected with the powdery mildew fungus *Erysiphe graminis*. These workers suggested that the increase in polyamines may be due to the formation of "green-islands", a characteristic feature of rust and mildew infections resulting from localised retention of chlorophyll around the infection sites (Bushnell, 1967; Scott, 1972).

Tobacco plants infected with tobacco mosaic virus exhibit increased ODC activity and an accumulation of putrescine (Negrel, Vallee and Martin, 1984). In this

type of work correlations between virus multiplication and hydroxycinnamic acid amides of putrescine have been reported. Thus, it has been suggested that these compounds play a role in virus resistance since, infected plants react by increasing levels of polyamine conjugates, which subsequently retard virus multiplication (Martin-Tanguy *et al*, 1987). In a similar vein, Samborski and Rohringer (1970) discovered the occurrence of hydroxycinnamic acid amides of putrescine in infected wheat and suggest their possible role as phytoalexins and hordatines found in barley seedlings increase in infected leaves and exhibit mild antifungal activity (Stoesll, 1967; Stoesll and Unwin, 1970; Smith and Best, 1977).

The infection of tomato fruits by the fungus *Rhizopus stolonifer* resulted in reduced putrescine concentration and ODC activity. However, since increased ethylene synthesis also results in this interaction, it was suggested that elevated ethylene production inhibited polyamine biosynthesis (Bakanashvili *et al*, 1987). (see Section 1.34).

The association between polyamines and diseased plants will be discussed in greater detail in Section 2.

1.5 Pathogens

1.51 *Puccinia graminis*

Puccinia graminis Pers. f.sp. *tritici* Erikss. and Henn. (black stem rust) is an obligate biotroph, which

belongs to the order Uredinales and makes up one of the largest groups in Basidiomycetes. Black stem rust, like many rust fungi, is heteroecious, completing the asexual phase of its life on wheat and the sexual life cycle on common barberry (*Berberis vulgaris*).

Rust fungi are unique in their life cycle, as they have up to six functionally and morphologically different spore states. The overwintering spore form of the rusts, the teliospores, germinate in the spring to produce basidiospores, which infect the barberry plant. The mycelium formed gives rise to pycnia and thus pycniospores, which are distributed by insects. The aecium, which is also formed on the barberry leaf, is found embedded in the lower epidermis. When mature, the aecium bursts releasing a mass of orange aeciospores. The aeciospores, dispersed by wind, can only infect the cereal host. The mycelium in the host produces urediospores and as the plant matures, teliospores, thus completing the cycle (Manners, 1971).

Apart from the roots and the seeds all parts of the host are attacked. On wheat, black stem rust appears as orange-brown longitudinally arranged pustules, containing urediospores, which occur mainly on the stems and leaf sheaths. As the host ripens, black pustules containing teliospores appear and give a black appearance to the infected part of the plant. The dry, torn epidermis which forms a frill around each pustule remains conspicuous. On the barberry plant, the disease manifests itself as bright

orange coloured aecia which are arranged in distinct groups.

The expanding cultivation of wheat has led to the world wide distribution of the fungus but in recent years, in Europe, wheat has been at greatest risk in the southwest and southeast (Zadoks, 1988).

The epidemiology of black stem rust has been looked at extensively in North America where it is a serious disease of wheat. Mild winters in the south and moist southerly winds are important factors prior to an epidemic. In eastern United States, the rust overwinters as teliospores in barberry regions (Manners, 1971). In Britain, Ogilvie and Thorpe (1961) stated that when epidemics occur they are caused by urediospores blown in from Europe and are not due to the rust overwintering on barberry.

The control of black stem rust has been effective in countries where the growth of barberry was prohibited. In America, breeding of resistant cereal varieties has been the main method of control. Chemical control is still at an experimental stage and in Europe systemic fungicides have not been used. Resistant varieties and early sowings are the methods of control most in use (Zadoks, 1988).

1.52 *Pyrenophora avenae*

Pyrenophora avenae Ito and Kuribayashi apud Ito (conidial state *Drechslera avenae* (Eidam) Sharif) is an

ascomycete fungus.

Pyrenophora avenae, a plant parasitic fungus, is generally confined to the genus *Avena* although it has been reported on other Gramineae. Data available suggests that there are differences in susceptibility between different oat cultivars (Smedegaard-Petersen, 1988).

Ascospores, pale yellow in colour are formed in globose or semi-globose pseudothecia which develop a short beak when mature. Conidiophores are found on the pseudothecial walls and can be found either singly or in groups of two - four. Light, cylindrical olive-brown conidia are formed at the tips of the conidiophores. The mycelium produced is grey and produces conspicuous white tufts (Smedegaard-Petersen, 1988).

Pyrenophora avenae, a seed borne pathogen, is known as leaf stripe, leaf spot or seedling blight depending on the symptoms produced. There are two distinct phases of the disease. The primary phase is connected with seedling disease and appears in plants arising from infected seed. Seedlings may be discoloured and killed before, or soon after, they emerge from the soil. Irregular, light green spots with reddish-brown centres appear on the first leaf and merge to form a stripe. Severe striping of the lower leaves often results in death of the seedling, although some may grow on to produce weak plants. If the striping is not particularly severe, the plant may continue to develop normally until secondary symptoms develop (Colhoun, 1971). The secondary phase commences when the

fungus begins to sporulate on the primary lesions. Conidia infect the upper leaves and sheaths, producing dark red secondary lesions. These lesions produce conidia which may result in further spread of the disease in the crop and also seed infection (Smedegaard-Petersen, 1988).

Muskett (1937) found that low soil temperatures at the time of sowing favoured primary infection of seedlings. Turner and Millard (1931) described how air humidity influenced the secondary phase of the disease, since greatest spore production occurred in humid conditions. The amount of inoculum, periods of high humidity, temperature and host susceptibility are all factors which influence major outbreaks of the disease. *Pyrenophora avenae* is distributed world-wide. In Britain it poses a serious threat in Scotland, Wales, North England and Ireland where colder and wetter conditions dominate during the early seedling stage. The primary phase of the disease can be controlled by treating the seed with non-mercurial fungicides. Previously, the seeds were treated with organomercury compounds but the fungus developed a natural resistance to the compounds. The secondary phase of the disease can be controlled by spraying the foliage with systemic fungicides such as triadimefon and propiconazole (Smedegaard-Petersen, 1988).

1.6 Host

Triticum aestivum L.

Wheat belongs to the genus *Triticum* of the family

Gramineae. *Triticum* is one of approximately 600 genera belonging to this family, which itself consists of over 5,000 species.

Wheat is one of the most important cultivated plants with respect to human consumption. *Triticum aestivum* is the most highly developed and widely grown of all wheats and where the climate is suitable, it is universally distributed (Peterson, 1965).

Triticum aestivum L. cv Sappo is a spring wheat which is highly susceptible to infection by the black stem rust *Puccinia graminis* f.sp. *tritici*.

S E C T I O N 2

POLYAMINE METABOLISM IN WHEAT INFECTED WITH
THE RUST, *PUCCINIA GRAMINIS* f.sp. *TRITICI*

2.1 Introduction

Infection by plant pathogenic fungi results in multifarious changes in the host plant including alterations in host metabolism and deprivation of host nutrients.

Since parasitism is essentially a nutritional relationship, much work has involved the study of carbohydrate metabolism in plant cells infected with mildews and rusts (Scott, 1972). In the early 1900s it was shown that fungal development was retarded when infected plants were placed in the dark or in areas deficient in atmospheric carbon dioxide. On studying mildewed tissues, Yarwood (1934) found an increase in respiration rate in infected leaves compared with non-infected leaves. Allen and Goddard (1938) were in agreement with these findings and isolated the increase to mesophyll cells of the host. On finding that nutrient accumulation at sites of infection was prevented by inhibiting respiration, Samborski and Shaw (1956) suggested that the pathogen was dependent on photosynthesis or respiration products for growth. However, it was later found that increased respiration rates were not restricted to infections by biotrophs (Millerd and Scott, 1962). Further, increased respiration rates were not always observed during biotrophic infections of cereal leaves (Millerd and Scott, 1963). In addition, infection by fungal pathogens has been reported to induce a change in the rate of photosynthesis in whole

plants or intact leaves (Mignucci and Boyer, 1979; Spotts and Ferre, 1979). In general the effect observed, especially in rust and powdery mildew infections, was a decrease in photosynthetic activity (Montalbini and Buchanan, 1974; Magyarosy, Schurmann and Buchanan, 1976).

In biotroph infections, carbohydrates appear to be the major constituents in the translocation of solutes from host to fungus (Lewis, 1976; Bushnell and Gay, 1978). These substances, however, appear to undergo rapid interconversions after uptake (Manners and Gay, 1982; Manners, Maclean and Scott, 1982). However, major fluxes of other compounds may occur from host to fungus. Thus, an increase in amino acid composition was observed in rust-infected leaves (Shaw and Colotelo, 1961; Raggi, 1974), and in mildew-infected sugar beet a change from sucrose to amino acids was reported (Magyarosy, Schurmann and Buchanan, 1976). Reisener, Ziegler and Prinzing (1970) found that in rust-infected wheat, amino acids and hexoses moved from the host to the fungus. Similarly, Burrell and Lewis (1977) described the movement of amino acids from the host to the fungus in rust-infected coltsfoot.

In view of the above discussion, it is not surprising therefore, that biotrophic fungi have been shown to accumulate host nutrients at infection sites, thus ensuring an adequate supply of nutrients for the development of the fungus (Thrower, 1965). In 1946, Gottlieb and Garner described an accumulation of

phosphorus in wheat leaves infected with black stem rust, particularly at infection sites. Similar accumulations were found in barley infected with powdery mildew, wheat-infected with black stem rust (Shaw and Samborski, 1956) and rust-infected bean (Yarwood and Jacobsen, 1955). Nutrient accumulation at infection sites appears to be the result of either an increase in nutrient flow towards the pustule areas or a decrease in nutrient transport away from the infected area. Wheat leaves infected with yellow rust (Doodson, Manners and Myers, 1965) and barley leaves infected with powdery mildew (Edwards, 1971) exhibited reduced translocation of photosynthate to other parts of the plant, whereas in bean plants nutrients were translocated from young, uninfected leaves to rust-infected leaves (Livne and Daly, 1966; Pozsar and Kiraly, 1966). Work by Ahmad *et al* (1982) describes how reduced phloem export in diseased leaves may result in accumulation of nutrients. It is generally considered that sucrose concentration gradients control the direction and velocity of phloem transport (Moorby, 1977). Thus, reduced supply of sucrose to the phloem, due to fungal infection, would result in reduced phloem transport (Farrar, 1984). Evidence is lacking, however, to support the increased flow of nutrients from the phloem to infection sites (Farrar, 1984). Work on brown rust-infected barley leaves (Farrar, 1984) and rust-infected leeks (Roberts, 1987) has shown that there is very little, if any, assimilate movement into infected leaves. Evidence exists for physical damage by biotrophs to phloem

tissue (Al Khesraji, Losel and Gay, 1980), although it is important to remember that impaired phloem transport would reduce transport into leaves, thus disrupting nutrient supply to the fungus. Nutrient movement, within organs, towards infection sites has been demonstrated for calcium (Durbin, 1967) and nitrogen (Bushnell, 1970; Bushnell and Gay, 1978).

Given that nutrients accumulate at infection sites, it seems important to know if these nutrients are available to the fungus and how the pathogen might acquire them. In fact, biotrophic pathogens have developed efficient ways to obtain nutrients from the host. Specialised structures, such as haustoria, ensure an immediate access to host nutrients. Gay and his colleagues suggested that haustoria are the main organs involved in carbohydrate uptake by the powdery mildew fungus as, unlike the superficial mycelium, haustoria are closely associated with host cells (Gay and Manners, 1981; Spencer-Phillips and Gay, 1981; Manners and Gay, 1982). In the haustorial region structural and functional modifications of the host plasmalemma and the formation of an isolated apoplast, allows the powdery mildew to control the flow of nutrients. Indeed, no transfer of nutrients occurs until the haustoria are fully developed (Mount and Ellingboe, 1969; Slesinski and Ellingboe, 1971). It has been difficult to identify the uptake of nutrients in plants infected with rust fungi as the haustoria are difficult to isolate and study and moreover intercellular hyphae may be involved in nutrient uptake (Mendgen, 1981).

Downy mildew fungi grow within the host like rusts, with intercellular hyphae growing between mesophyll cells and haustoria penetrating these cells. In downy mildew infected plants, some solutes were shown to be taken up by the fungus before haustoria were formed, whereas others were not incorporated until the haustoria were fully developed (e.g. Andrews, 1975). Kneale and Farrar (1985) calculated the surface area of haustoria and intercellular hyphae in brown rust-infected barley and described how all of the hexose needed for growth of the fungus could be taken up through either structure. However, haustoria may be necessary for uptake of specific substances. Indeed, Mendgen (1979) found that in rust-infected bean, maximum lysine uptake occurred through the mature haustorium and only a minimal amount occurred through intercellular hyphae. Nevertheless, the concentration of nutrients found in intercellular spaces is thought to be sufficient to support growth of biotrophic pathogens (Hancock and Huisman, 1981).

The mechanisms underlying nutrient mobilisation have been of interest to workers over the years. In 1965, Thrower suggested that the fungal mycelium was acting as a sink for the diversion of nutrients from other parts of the plant. Likewise, plant growth substances with the ability to create sink effects have been associated with mobilisation of nutrients towards infection sites (Pozsar and Kiraly, 1966; Brian, 1967). This accumulation of nutrients together with retention of chlorophyll at infection sites, are correlated with the formation of

green-islands. Green-islands, described as areas of healthy tissue occurring around sites of infection, when the remainder of the leaf is chlorotic and senescent, were first described by Cornu in 1881. Some workers attributed the green-island effect to re-greening of chlorotic tissues of infected leaves (Allen, 1942; Sziraki et al, 1984), whereas others suggested that chlorophyll retention was responsible for the effect (Bushnell, 1967). In an attempt to elucidate these opposing views, Scholes and Farrar (1987) suggested that retention and re-greening may result from changes in the biosynthetic and degradative pathways for chlorophyll, depending on the host-pathogen combination. Chlorophyll breakdown may occur initially followed by synthesis during fungal development, thus resulting in the re-greening effect. In other systems a constant level of chlorophyll turnover may be observed, resulting in the retention effect.

Green-islands formed from biotroph infections have been compared with those formed on leaves treated with cytokinins (Bushnell and Allen, 1962; Harding, Williams and McNabola, 1968). In rust-infected bean, Dekhuijzen and Staples (1968) suggested that the increased cytokinin activity was of host origin, although rust and powdery mildew spores have been shown to contain high levels of cytokinins (Kiraly, El-Hammady and Pozsar, 1967). Kiraly, Pozsar and El-Hammady (1966) suggested that the release of cytokinins by the biotroph would delay senescence at infection sites. Interestingly, recent work by Roberts (1987) described an increase in cytokinins in rust-

infected leek leaves, with the largest increase occurring at and around infection sites.

Green-island formation has been associated with starch deposition. Scholes and Farrar (1987) observed starch accumulation in chloroplasts surrounded by fungal hyphae, although the starch disappeared during sporulation (Bushnell, 1967; Sziraki et al, 1984). It has been suggested that the accumulation of starch may be related to increased concentration of cytokinins, which are known to cause starch deposition (Wang, 1961) or, alternatively, to reduced phosphate concentration (MacDonald and Strobel, 1970; Herold, 1984). However, in barley leaves infected with rust, starch accumulation was not found to be associated with a reduction in phosphate concentration (Ahmad, Farrar and Whitbread, 1984; Scholes and Farrar, 1986).

From the above discussion it is clear that although green-islands were first reported over 100 years ago, information about them is sketchy and contradictory. Moreover, there is as yet no firm evidence to support a role for cytokinins in the formation of green-islands. Perhaps some effort should be directed towards a study of other compounds which could influence senescence in infected leaves. Thus, polyamines were found to be essential for growth in most organisms (see Section 1.32) and, like cytokinins, were capable of delaying senescence and producing green-islands (see Section 1.34). As limited information exists concerning polyamine levels

during biotrophic infections (see Section 1.4) and in view of the importance of polyamines in growth and development, a detailed study of polyamine metabolism in wheat infected with black stem rust, *P. graminis*, was undertaken.

2.2 Materials and Methods

2.21 Maintenance of pathogen

14 day old wheat plants (*Triticum aestivum* L. cv. Sappo) grown in 15 cm pots, approximately eight plants per pot, were infected with a spore suspension of *Puccinia graminis* f. sp. *tritici* using a soft camel-hair brush. The plants were covered with clear polythene bags for 48 hours to maintain the high humidity required for infection. Approximately every 14 days after inoculation spores were collected using a dry camel-hair brush, suspended in a small volume of distilled water and painted on to uninfected plants. Plants were grown in a ventilated glasshouse under natural daylight supplemented to a 16 hour photoperiod with 400 W Mercury vapour lamps. Maximum daylight temperature was 24°C falling to a minimum of 9°C at night.

2.22 Growth and inoculation of plant material

Seeds of wheat were sown in Fisons Levington compost in 23 x 18 cm trays. Approximately 25 seeds were sown per tray. 8 days after sowing, when seedlings were at the first leaf stage, they were sprayed to run off with a spore suspension of 25 mg spores per 100 ml distilled

water using a Preval spray unit. Tween 20 was added to the spore suspension, as a surfactant, to obtain a 0.01 % (v/v) solution. Fresh spores were obtained from infected stock plants using a dry camel-hair brush. Control plants were sprayed with distilled water only. Each tray of seedlings was covered with a polythene bag and grown in conditions as described in Section 2.21. By days nine and twelve after inoculation of whole leaves the fungus would be sporulating, while on days three and six after inoculation flecking on the leaves was the only visible sign of infection. Inoculated first leaves usually exhibited 40-50 % infection. However, infection of second leaves and stems was always small and variable, since these tissues were not directly inoculated.

First leaves, second leaves and stem were harvested from healthy and infected plants 3, 6, 9 and 12 days after inoculation, for analysis of polyamine concentrations and enzyme activities. Measurements were also made using material excised from discrete regions of infected first leaves 9 days after inoculation. The regions excised from infected leaves were the pustules and the area between the pustules. The tissue was excised on glass Petri dishes which were placed on ice.

2.23 Enzyme assays

Crude enzyme extracts were prepared by grinding 150 mg tissue per ml of buffer, using a pre-chilled pestle and mortar. Enzyme activities were assayed by measuring $^{14}\text{CO}_2$

released after incubation with [U-¹⁴C] arginine and [1-¹⁴C] ornithine for ADC and ODC respectively. Radioisotopes were obtained from Amersham International plc.

2.231 ADC assay

The 0.1 M sodium phosphate buffer used in the extraction procedure was amended with 1.5 mM ethylenediaminetetra-acetic acid (EDTA), 5.5 mM DL-dithiothreitol (DTT) and 50 μ M pyridoxal 5-phosphate (PLP). The buffer was adjusted to pH 7.2. Maximum activity of ADC was obtained at this pH.

The extracts were centrifuged at 14,000 g for 10 minutes at 0°C in a MSE-Europa 24 M centrifuge. Reaction mixtures contained 0.2 ml aliquots of the supernatant obtained after centrifugation, 40 μ l 3.88 mM unlabelled arginine and 1.6 μ l L-[U-¹⁴C] arginine monohydrochloride. In order to ensure that the reaction was not substrate limited, cold arginine was added in excess of the K_m for the enzyme reaction. The calculated K_m was 0.12 mM and since it was decided to use 5 x the K_m (ie. 0.6 mM) in the reaction vessel, 3.88 mM of cold arginine was added to the assay (dilution in the reaction vessel would have yielded a final arginine concentration of 0.6 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 piece of filter paper, 10 mm in diameter, impregnated with 10 μ l 2M KOH

was fitted to each needle to trap $^{14}\text{CO}_2$ 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 injected into each tube and 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. The samples were counted for radioactivity using a LKB 1215 Rackbeta liquid scintillation counter. Values obtained as disintegrations per minute (DPM) were converted and expressed as $\text{pmol } ^{14}\text{CO}_2 (\text{mg protein})^{-1} \text{ hour}^{-1}$. Protein assays were carried out using the method of Lowry et al (1951) with bovine serum albumin (BSA) as standard (see Appendix I). All results are the means of five replicates. Standard errors were calculated and significance was assessed using the Student's *t*-test (see Section 2.25).

2.232 ODC assay

The 0.1 M potassium phosphate buffer used in the extraction procedure was amended with 5 mM EDTA, 10 mM DTT, 1 mM PLP and 20 mM sodium ascorbate. The buffer was adjusted to pH 7.8. The extracts were centrifuged at 27,000 g for 20 minutes at 0°C . The pellet obtained after centrifugation was resuspended in original volume of the extraction buffer and sonicated using a Soniprep 150 for 3 cycles of 30 seconds on/30 seconds off. Test-tubes were kept on ice during sonication. To the supernatant (cytosolic fraction) and resuspended pellet (nuclear

fraction) 366 mg of ammonium sulphate was added per ml of sample at 0°C and stood on ice for 30 minutes. The samples were then centrifuged at 27,000 g for 20 minutes at 0°C.

The pellets obtained from both the cytosolic and nuclear fractions were resuspended in the original volume of 0.1 M potassium phosphate buffer amended with 0.1 mM EDTA, 1 mM DTT, and 50 µM PLP. The pH of the resuspension buffers were adjusted to 7.8 and 8.0 for the cytosolic and nuclear fractions respectively. Maximum ODC activity was obtained at these pH values. The samples were placed in dialysis tubing (molecular weight cut off, 12,000) and dialysed against 30 volumes of resuspension buffer for 24 hours in the dark at 4°C. Reaction mixtures contained 0.2 ml aliquots of the dialysed extract, 40 µl 10 mM unlabelled ornithine and 5 µl DL-[1-¹⁴C] ornithine hydrochloride.

Assays were carried out as described in Section 2.231. All results are the means of five replicates. Standard errors were calculated and differences between means were analysed for significance using the Student's t-test (see Section 2.25).

2.24 Polyamine analysis

This analysis determined the concentration of free polyamines.

500 mg of tissue was macerated with 1 ml 4% (v/v)

perchloric acid in a chilled pestle and mortar, then centrifuged at $12,000g$ for 25 minutes at $0^{\circ}C$. To obtain a pH of approximately 10, 17 mg of sodium carbonate was added to each 0.2 ml aliquot of supernatant. To this, 0.4 ml dansyl chloride (30 mg ml^{-1} in acetone) was added. This mixture was incubated overnight in darkness at $22^{\circ}C$. Excess dansyl chloride was converted to dansyl proline by incubating the samples for 30 minutes with 0.1 ml L-proline (100 mg ml^{-1}). The dansylated polyamines were extracted in 0.25 ml toluene. 25 μ l aliquots of the toluene extract were spotted on to activated (1 hour at $110^{\circ}C$) Whatman LK6D silica-gel thin layer chromatography (TLC) plates. The plates were left to develop in tanks containing chloroform : triethylamine (5:1 (v/v)) until the solvent front reached the top of the plates. The spots were traced using an ultra violet (UV) lamp (Gallenkamp) and identified against the standards. The spots were cut out, scraped into 100 mm glass test-tubes and the dansylated derivatives extracted in 5 ml ethyl acetate. Standards between 0.1 - 8.0 μ g were measured (see Appendix I). Fluorescence was measured in a Perkin-Elmer LS-5 luminescence spectrometer at excitation 365 nm, emission 506 nm. Values obtained as units of fluorescence were converted and expressed as $\text{nmol gram fresh weight}^{-1}$.

All results are the means of four replicates. Standard errors were calculated and significance was assessed using the Student's *t*-test (see Section 2.25).

2.25 Statistical analysis

The standard error was calculated using the formula:

$$\frac{\sigma - 1}{\sqrt{n}}$$

where $\sigma - 1$ = standard deviation of the sample.

n = number of replicates in a sample.

The following formula was used to obtain the t value when comparing the means of two small samples:

$$s_c^2 = \frac{\left[\sum x_1^2 - \frac{(\sum x_1)^2}{N_1} + \sum x_2^2 - \frac{(\sum x_2)^2}{N_2} \right]}{N_1 + N_2 - 2}$$

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s_c \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

where t has $(N_1 + N_2 - 2)$ degrees of freedom (Parker, 1979).

The probability values were obtained using Student's t-tables.

2.3 Results

2.31 ADC activity

Rust-infected tissue exhibited a reduction in ADC

activity, with the exception of the infected second leaves whose ADC activity increased significantly nine days after inoculation (Table 2). In both healthy and rust-infected tissue, ADC activity was lower in the first leaves and second leaves than in the stem. ADC activities from tissue harvested on the third and sixth days after inoculation were too low to be measured. Although ADC activity was detectable in tissue harvested 9 and 12 days after inoculation, activity of the enzyme was very low. Thus, ADC activities were not measured in discrete regions of infected leaves as any changes observed in polyamine levels were unlikely to be as a result of ADC activity.

2.32 ODC activity

Cytosolic and nuclear ODC activity significantly increased in rust-infected first leaves, three days after inoculation although by day six after inoculation a decrease in activity was observed (Tables 3 and 4). No significant change was found in infected second leaves, stems or nine and twelve days after inoculation first leaves. For both cytosolic and nuclear ODC the changes observed were similar, although the nuclear ODC exhibited higher activity than the cytosolic enzyme.

2.33 Polyamine concentrations

An increase in putrescine concentration was observed in rust-infected first leaves nine and twelve days after inoculation. The second leaves and stems exhibited a

Table 2: Arginine decarboxylase activity in healthy and rust-infected first leaves, second leaves and stems of wheat, on the ninth and twelfth days after inoculation. Values are the means of five replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$; (c) $P = 0.1$.

DAI = Days After Inoculation.

Arginine Decarboxylase Activity ($\mu\text{mol CO}_2 \text{ mg protein}^{-1} \text{ hour}^{-1}$)						
DAI	First Leaf		Second Leaf		Stem	
	Healthy	Rusted	Healthy	Rusted	Healthy	Rusted
9	0.15 ± 0.01	0.09 ± 0.01^b	0.17 ± 0.005	0.30 ± 0.03^b	0.57 ± 0.02	0.44 ± 0.02^b
12	0.09 ± 0.005	0.05 ± 0.002^a	0.23 ± 0.02	0.18 ± 0.02^c	0.32 ± 0.02	0.31 ± 0.02

Table 3: Cytosolic ornithine decarboxylase activity in healthy and rust-infected first leaves, second leaves and stems of wheat, on the third, sixth, ninth and twelfth days after inoculation. Values are the means of five replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$.

ND = Not Determined.

DAI = Days After Inoculation.

Ornithine Decarboxylase Activity ($\mu\text{mol CO}_2 \text{ mg protein}^{-1} \text{ hour}^{-1}$)						
DAI	First Leaf		Second Leaf		Stem	
	Healthy	Rusted	Healthy	Rusted	Healthy	Rusted
3	1.14 \pm 0.05	1.65 \pm 0.15 ^b	ND	ND	1.39 \pm 0.18	1.1 \pm 0.19
6	2.48 \pm 0.13	1.35 \pm 0.15 ^a	1.59 \pm 0.18	1.51 \pm 0.22	2.39 \pm 0.17	2.32 \pm 0.42
9	1.61 \pm 0.25	1.53 \pm 0.14	1.52 \pm 0.13	1.32 \pm 0.17	1.38 \pm 0.12	1.18 \pm 0.09
12	1.41 \pm 0.09	1.48 \pm 0.28	1.21 \pm 0.19	1.57 \pm 0.06	1.26 \pm 0.16	1.52 \pm 0.07

Table 4: Nuclear ornithine decarboxylase activity in healthy and rust-infected first leaves, second leaves and stems of wheat on the third, sixth, ninth and twelfth days after inoculation. Values are the means of five replicates \pm standard error. Significant differences are shown as follows: (b) $P = 0.01$; (c) $P = 0.1$.

ND = Not Determined.

DAI = Days After Inoculation.

Ornithine Decarboxylase Activity
($\mu\text{mol CO}_2 \text{ mg protein}^{-1} \text{ hour}^{-1}$)

DAI	First Leaf		Second Leaf		Stem	
	Healthy	Rusted	Healthy	Rusted	Healthy	Rusted
3	1.17 \pm 0.19	2.76 \pm 0.39 ^b	ND	ND	1.75 \pm 0.17	1.38 \pm 0.28
6	3.24 \pm 0.28	2.23 \pm 0.20 ^c	2.25 \pm 0.32	3.16 \pm 0.62	2.31 \pm 0.20	2.52 \pm 0.18
9	2.12 \pm 0.79	1.78 \pm 0.24	1.84 \pm 0.04	1.99 \pm 0.08	2.14 \pm 0.62	1.95 \pm 0.29
12	2.72 \pm 0.37	2.12 \pm 0.05	2.03 \pm 0.26	1.72 \pm 0.19	2.91 \pm 0.70	1.66 \pm 0.24

similar increase on the third and sixth days after inoculation. A decrease in putrescine concentration was observed in rust-infected first leaves, three days after inoculation and rusted stems, nine and twelve days after inoculation. No change in putrescine concentration was found in rusted second leaves, nine and twelve days after inoculation (Table 5).

Spermidine concentrations increased significantly in rust-infected first leaves, nine and twelve days after inoculation, second leaves twelve days after inoculation and stems, three and six days after inoculation (Table 6). A decrease in concentration was found in rusted first leaves three days after inoculation and rusted second leaves nine days after inoculation. No change was observed in rust-infected second leaves days three and six after inoculation or rusted stems on days nine and twelve after inoculation.

Increased concentrations of spermine were observed in rust-infected first leaves nine and twelve days after inoculation (Table 7). Infected second leaves and stems showed increased levels of spermine twelve and three days after inoculation, respectively. In contrast, a reduction in spermine was observed on the other days (Table 7).

Cadaverine concentrations increased significantly in infected first leaves and stems, nine and three days after inoculation respectively (Table 8). A decrease was observed in rust-infected first leaves three days after inoculation and rusted second leaves and stems nine days

Table 5: Putrescine concentration in healthy and rust-infected first leaves, second leaves and stems of wheat, on the third, sixth, ninth and twelfth days after inoculation. Values are the means of four replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$; (c) $P = 0.1$.

ND = Not Determined.

DAI = Days After Inoculation.

Putrescine Concentration (nmol g Fresh Weight ⁻¹)						
DAI	First Leaf		Second Leaf		Stem	
	Healthy	Rusted	Healthy	Rusted	Healthy	Rusted
3	74.5 \pm 2.9	62.1 \pm 2.9 ^c	62.1 \pm 3.5	74.5 \pm 3.1 ^c	24.8 \pm 0.4	62.1 \pm 1.7 ^a
6	50.0 \pm 1.9	ND	62.1 \pm 2.7	74.5 \pm 1.9 ^b	74.5 \pm 2.2	111.7 \pm 1.8 ^a
9	50.0 \pm 8.5	111.7 \pm 5.3 ^a	74.5 \pm 2.3	74.5 \pm 1.2	86.9 \pm 1.9	74.5 \pm 0.8 ^b
12	50.0 \pm 16.1	124.0 \pm 7.7 ^b	74.5 \pm 5.7	74.5 \pm 8.6	86.9 \pm 4.4	74.5 \pm 3.9 ^c

Table 6: Spermidine concentration in healthy and rust-infected first leaves, second leaves and stems of wheat, on the third, sixth, ninth and twelfth days after inoculation. Values are the means of four replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$.

ND = Not Determined.

DAI = Days After Inoculation.

Spermidine Concentration (nmol g Fresh Weight ⁻¹)						
DAI	First Leaf		Second Leaf		Stem	
	Healthy	Rusted	Healthy	Rusted	Healthy	Rusted
3	37.6 \pm 1.3	23.9 \pm 1.1 ^a	49.3 \pm 2.1	49.3 \pm 2.5	8.2 \pm 0.2	34.2 \pm 1.1 ^a
6	21.9 \pm 1.4	ND	27.4 \pm 1.1	27.4 \pm 0.7	21.9 \pm 0.6	41.1 \pm 1.8 ^a
9	37.6 \pm 1.4	123.2 \pm 3.6 ^a	51.3 \pm 1.9	44.5 \pm 1.4 ^b	49.3 \pm 1.5	47.9 \pm 1.0
12	13.7 \pm 0.3	75.3 \pm 4.1 ^a	23.9 \pm 0.6	41.1 \pm 0.7 ^a	41.1 \pm 1.3	44.5 \pm 1.6

Table 7: Spermine concentration in healthy and rust-infected first leaves, second leaves and stems of wheat, on the third, sixth, ninth and twelfth days after inoculation. Values are the means of four replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$; (c) $P = 0.1$.

ND = Not Determined.

DAI = Days After Inoculation.

Spermine Concentration (nmol g Fresh Weight ⁻¹)							
DAI	First Leaf		Second Leaf		Stem		
	Healthy	Rusted	Healthy	Rusted	Healthy	Rusted	
3	88.5 \pm 2.2	82.7 \pm 3.3	63.2 \pm 4.4	48.2 \pm 1.9 ^b	34.5 \pm 1.0	40.2 \pm 2.6 ^c	
6	51.7 \pm 1.3	ND	74.7 \pm 2.9	53.9 \pm 1.8 ^a	45.9 \pm 2.0	34.5 \pm 0.7 ^a	
9	36.7 \pm 1.3	63.2 \pm 1.7 ^a	74.7 \pm 1.6	45.9 \pm 2.2 ^a	45.9 \pm 0.8	34.5 \pm 1.6 ^a	
12	13.8 \pm 0.8	31.0 \pm 1.5 ^a	22.9 \pm 2.9	31.0 \pm 2.7 ^c	23.0 \pm 0.9	17.2 \pm 1.7 ^c	

Table 8: Cadaverine concentration in healthy and rust-infected first leaves, second leaves and stems of wheat, on the third, sixth, ninth and twelfth days after inoculation. Values are the means of four replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$; (c) $P = 0.1$.

ND = Not Determined.

DAI = Days After Inoculation.

Cadaverine Concentration (nmol g Fresh Weight ⁻¹)						
DAI	First Leaf		Second Leaf		Stem	
	Healthy	Rusted	Healthy	Rusted	Healthy	Rusted
3	11.4 \pm 0.2	4.6 \pm 0.2 ^a	11.4 \pm 0.5	11.4 \pm 1.0	4.6 \pm 0.4	15.9 \pm 0.7 ^a
6	11.4 \pm 3.0	ND	11.4 \pm 1.8	11.4 \pm 0.2	16.0 \pm 0.6	15.9 \pm 1.4
9	16.0 \pm 0.5	22.8 \pm 1.9 ^c	27.4 \pm 2.4	11.4 \pm 0.4 ^a	15.9 \pm 0.7	11.4 \pm 0.8 ^b
12	15.9 \pm 5.7	15.9 \pm 1.6	11.4 \pm 2.3	15.9 \pm 4.4	11.4 \pm 3.3	11.4 \pm 1.4

after inoculation. No change was found in the other tissue analysed (Table 8).

2.34 Discrete regions of infected leaves

ODC activity and polyamine concentrations were studied in discrete regions of infected first leaves, nine days after inoculation because of the significant increases of polyamine levels found in infected whole leaves, not accompanied by corresponding increases in ODC activity. By looking at discrete regions, any "dilution" effect occurring as a result of uninfected areas on an infected leaf should be reduced. The discrete regions were examined on first leaves, nine days after inoculation as all polyamine concentrations of whole leaves increased greatly on this day.

In discrete regions of rust-infected first leaves, cytosolic ODC activity was increased (Table 9), although no significant increase was observed for nuclear ODC (Table 10). A substantial increase in putrescine, spermidine, spermine and cadaverine was found in discrete regions of rusted first leaves (Table 11). The pustules contained a higher concentration of the polyamines than the areas between the pustules.

2.4 Discussion

Increased polyamine levels have been found in barley infected with brown rust (Greenland and Lewis, 1984) and powdery mildew (Walters, Wilson and Shuttleton, 1985).

Table 9: Cytosolic ornithine decarboxylase activity in healthy and discrete regions of rust-infected first leaves of wheat on the ninth day after inoculation. Values are the means of five replicates \pm standard error. Significant differences are shown as follows: (c) $P = 0.1$.

Ornithine Decarboxylase Activity ($\mu\text{mol CO}_2 \text{ mg protein}^{-1} \text{ hour}^{-1}$)				
DAI	Healthy		Rusted	
			Area Between Pustules	Pustules
9	2.71 \pm 0.43		4.66 \pm 0.74 ^c	4.0 \pm 0.13 ^c

Table 10: Nuclear ornithine decarboxylase activity in healthy and discrete regions of rust-infected first leaves of wheat on the ninth day after inoculation. Values are the means of five replicates \pm standard error.

Ornithine Decarboxylase Activity (pmol CO ₂ mg protein ⁻¹ hour ⁻¹)			
DAI	Rusted		
	Healthy	Area Between Pustules	Pustules
9	3.66 \pm 0.34	3.87 \pm 0.34	4.18 \pm 0.56

Table 11: Polyamine concentration in healthy and discrete regions of rust-infected first leaves of wheat on the ninth day after inoculation. Values are the means of four replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$; (c) $P = 0.1$.

Polyamine Concentration (nmol g Fresh Weight ⁻¹)				
	Putrescine	Spermidine	Spermine	Cadaverine
Healthy	31.0 \pm 7.8	17.1 \pm 0.2	33.5 \pm 8.3	9.5 \pm 2.9
Area Between Pustules	49.7 \pm 3.6 ^c	47.9 \pm 8.2 ^b	68.9 \pm 2.9 ^b	11.4 \pm 2.5
Pustules	73.0 \pm 3.5 ^b	150.9 \pm 4.5 ^a	304.1 \pm 10.7 ^a	33.6 \pm 1.9 ^a

The observations reported here are consistent with these findings, since increases were reported in the concentrations of putrescine, spermidine, spermine and cadaverine in *P. graminis* - infected first leaves of wheat, nine and twelve days after inoculation. The reductions observed in polyamine concentrations, three days after inoculation may have been due to the high ratio of uninfected tissue to infected tissue in those leaves. Thus, any possible effect due to the presence of the fungus would be "masked" by the rest of the leaf. Discrete regions of infected leaves were analysed in an attempt to overcome this problem. The variation of polyamine concentrations in second leaves and stems of infected plants may reflect the fact that these tissues were not directly infected with the fungus, although some visible infection was usually observed by twelve days after inoculation. It is also important to remember that concentrations of polyamines may vary throughout the plant and also within individual tissues, with the concentration usually highest in young actively growing areas and lower in senescing tissues (Kaur-Sawhney, Shih and Galston, 1982; Kaur-Sawhney *et al*, 1982b).

The origin of the increase in polyamines in infected tissue is still under investigation. It has been suggested that elevated polyamine concentrations in infected tissues are of fungal origin. Indeed, work by Bailey, Bower and Lewis (1987) using autoradiography indicated that the high level of ODC activity found in pustules of rust-infected barley leaves was probably of

fungal origin. A high level of ODC activity would normally result in increased polyamine concentrations. However, in an attempt to determine the origin of increased polyamine concentrations, Walters and Wylie (1986) removed surface growth of the barley powdery mildew fungus and found that very little of the increase was due to fungal growth and sporulation, with most of the increase occurring in the leaf. This method, however, did not remove the haustoria within host epidermal cells. Removing and analysing pustule areas of rust-infected leaves may be considered a crude separation of host and fungus. The results in this thesis describe a large increase in polyamine concentrations in pustule areas and a similar, but lower, increase in inter-pustule areas. Although no visible infection was apparent in the areas between pustules, increased polyamine levels may be due to the presence of haustoria and inter-cellular hyphae of the fungus. Difficulties in removing only the pustule regions, due to their very small size, probably resulted in the incorporation of surrounding host tissue in the pustule samples, in particular the green-island region. Elevated polyamine concentrations in infected leaves may be used in the stabilisation of membranes. The stabilising effects of polyamines on plasma and organelle membranes (see Section 1.33; Birecka *et al*, 1984) and also on the structure of the thylakoid membrane within the chloroplasts (Popovic *et al*, 1979) are well documented. In fact, the hypothesis that polyamines in infected tissues may stabilise membranes is supported by work on

brown rust-infected barley which demonstrated normal membrane functions in diseased leaves (Ahmad, Farrar and Whitbread, 1985), and by recent work from this department (Coghlan and Walters, unpublished results), which show that the activities of both lipoxygenase (which generates free radicals and damages membranes) and catalase (which leads to reduced free radical production and hence reduced membrane damage), were much decreased in mildewed barley.

The formation and establishment of haustorial complexes in host cells during rust and mildew infections results in changes in the host nucleic acid metabolism (Chakravorty and Scott, 1982). These include changes in infected leaf ribonuclease fractions, increased rate of ribonucleic acid synthesis and changes in polysomal m-RNA populations (Manners and Scott, 1983). It has been established that polyamines are involved in the stabilisation of nucleic acids (see Section 1.31). It has been suggested that newly synthesised polyamines bind to new RNA, thus activating it. During stress-induced damage a role of *in vivo* protection by inhibiting wound-induced RNase activity has also been suggested (Serafini-Fracassini, Torrigiani and Branca, 1984). Thus, the increased concentrations of polyamines in infected leaves may be associated with increased RNA synthesis and transcription which occurs in infected plants.

The results presented in this thesis show that in comparison with ODC activity, ADC activity in both rusted and healthy tissue was extremely low. This is the

opposite to results obtained for powdery mildew infection of barley (Walters and Wylie, 1986) and may reflect differences between the plant tissues and the host-pathogen complexes. In fact, it would appear that wheat (Flores and Galston, 1982), and in particular the variety used in these experiments, exhibits very low levels of ADC activity. Increased polyamine concentrations in mildewed leaves have been associated with an increase in the activities of polyamine biosynthetic enzymes (Walters, Wilson and Shuttleton, 1985; Walters and Wylie, 1986). The results presented in this thesis for whole rust-infected leaves, however, are inconsistent with these findings. Indeed, increased cytosolic and nuclear ODC activities in infected first leaves three days after inoculation, occurred at the time of reduced polyamine concentrations. This is possibly a result of increased polyamine catabolism, as has been detected in mildewed barley (Coghlan and Walters, unpublished results). However, the large increases in polyamine concentrations in discrete regions of infected leaves were accompanied with a significant increase in cytosolic ODC activity and a small, although not significant, increase in nuclear ODC activity. As the increase in ODC activity was small in relation to the magnitude of increase in polyamine concentrations other factors affecting polyamine accumulation may be involved.

A reduction in activity of the enzymes involved in polyamine catabolism (although this may be unlikely in view of the increased polyamine oxidase activity detected

in mildewed barley - see above), and a release of polyamines from the conjugated form to the free state are two such possibilities which must be considered further. Indeed, recent work on mildewed barley (Coghlan and Walters, unpublished) has shown that there is a movement from conjugated to free polyamines in infected leaves. This is consistent with earlier suggestions that movement of polyamines from conjugated forms may be used to replenish free polyamine pools (Slocum and Galston, 1985). Very little is known about the turnover of polyamine conjugates and of their relationship with free polyamines. Clearly, in view of the increases in polyamine concentrations in infected tissue which cannot be accounted for in terms of biosynthesis or catabolism, this is an area worthy of further investigation.

S E C T I O N 3

CHANGES IN POLYAMINE METABOLISM
IN OSMOTICALLY-STRESSED WHEAT

3.1 Introduction

Changes in polyamine metabolism occur not only in plants responding to biotic stress (as described in Section 2) but also to abiotic stresses such as acid, salt, chilling, water and osmotic stress (see Section 1.35). Most studies have been carried out on polyamine metabolism during osmotic shock, using non-penetrating osmotica on detached leaves (Flores and Galston, 1984 a b; Tiburcio, Kaur-Sawhney and Galston, 1986; Tiburcio et al, 1986). Since treatment with mannitol, the osmoticum used in these experiments, results in accumulation of the osmoticum in intercellular spaces, entering the cell only very slowly, the cells are exposed to osmotic shock and withdrawal of water from the cells. Thus, various plant responses to water stress will be discussed in this section. This is particularly important since polyamines have been implicated in a variety of processes in plants responding to stress.

Water stress is the situation in which plant water potential and turgor are reduced enough to interfere with normal functioning. However, the water potential at which this occurs varies with the plant and the stage of development (Kramer, 1983). Turgor pressure is important in controlling cell growth and in maintaining structural integrity and gas exchange capacity in leaves (Turner and Jones, 1980). Thus, the inability to maintain turgor by osmotic adjustment would affect many cellular metabolic functions in intact plants.

During water stress, photosynthesis can be reduced by a reduction in leaf area, closure of stomata and a decrease in the efficiency of the carbon fixation process. Thus a reduction in cell enlargement and vegetative growth was observed in many plants as a result of water stress (Hsiao, 1973). Since stomatal opening is important for the flow of oxygen and carbon dioxide in and out of the leaf and since it appears to be controlled by cell turgor, water stress would have indirect effects on photosynthesis and respiration. Indeed, the closure of stomata results in reduced rates of photosynthesis, leading to a decrease in carbon income. As the leaf can only store a small amount of carbon in labile forms, the effects of carbon starvation could spread to the metabolism of other organs (Hanson and Hitz, 1982). In fact, it has been found that carbon transport via the phloem, can continue during severe water stress (Sung and Krieg, 1979). Similarly, as the pool of nitrate is small in relation to nitrogen flux, any changes in flux could have a profound effect on leaf nitrogen metabolism (Tully and Hanson, 1979).

Oat sections incubated in varying concentrations of mannitol exhibited decreased rates of protein synthesis with increased mannitol concentration (Dhindsa and Cleland, 1975). A reduced rate of protein synthesis during water stress may be explained by a decreased level of free energy accompanying decreased respiration and photosynthesis (Barlow, Ching and Boersma, 1976). However, the synthesis of some proteins are enhanced during water stress (Dhindsa and Cleland, 1975) and it has

been suggested that they are specifically synthesised during water stress, with production controlled at the transcription level (Heikkila *et al*, 1984). Similarly, large increases in RNase activity of isolated tobacco leaf protoplasts, osmotically stressed using mannitol, were due to synthesis of the enzyme protein, since cycloheximide completely inhibited the increase (Premecz *et al*, 1977). In general, the activity of hydrolytic enzymes and some oxidases increases as a result of water stress (Levitt, 1980). Indeed, the level of amino acids have been found to increase during exposure to osmotic stress (Barlow, Boersma and Young, 1976). However, many enzymes are inhibited probably as a result of protein loss or conformational changes.

In expanded organs, sugars and amino acids are major constituents during osmotic adjustment (Barlow *et al*, 1977; Acevedo *et al*, 1979; Meyer and Boyer, 1981). During organ expansion the import of solutes is necessary for osmoregulation (Barlow *et al*, 1977), and solute import is dependent on photosynthetic rates. It has been suggested that potassium may be involved in regulation during osmotic stress by possibly balancing the negative charges on amino acids (Jones, Osmond and Turner, 1980). However, changes in sugars and amino acids are not always accompanied by changes in potassium (Cutler and Rains, 1978). As these compounds are found in non-stressed tissue it seems unlikely that major metabolic pathways exist specifically for osmotic adjustment (Hanson and Hitz, 1982). Indeed, solute accumulation may be the

result of unutilised assimilates in mature leaves (Jones, Osmond and Turner, 1980) or an accumulation of imported precursors in expanded leaves when growth is reduced (Munns, Brady and Barlow, 1979). An increase in proline has been described during water stress of both young (Huang and Cavalieri, 1979) and old plants (Jones, Osmond and Turner, 1980). It has been suggested that proline may be accumulating to act as an osmoticum, a non-toxic nitrogen source or as an enzyme or membrane protectant (Wyn Jones, 1979; Stewart and Hanson, 1980). Indeed, proline was found to play an important role in the osmotic adjustment of microorganisms (Rains, Valentine and Hollaender, 1980). It has been suggested that stimulation of glutamate conversion to proline (Boggess et al, 1976), reduced rate of proline oxidation and a reduction in the utilisation of proline for protein synthesis (Stewart et al, 1977) are all possible causes for proline accumulation. Betaine accumulation has also been reported during water stress in some plants (Hanson and Nelson, 1978).

In addition to solute accumulation, abscisic acid (ABA) has also been found to increase during water stress. In some species ABA concentrations increase as leaf turgor approaches zero (Pierce and Raschke, 1980), suggesting a possible involvement of plant growth regulators in maintaining turgor during water stress. Further, the application of exogenous ABA mimics the effects of water stress on leaf growth (Quarrie and Jones, 1977). It is thought that ABA promotes stomatal closure, thus enabling

the plant to regain full turgor (Jones and Mansfield, 1972), inhibits photosynthesis (Cornic and Miginiac, 1983) and increases the movement of water through the roots (Davies, Rodriguez and Fiscus, 1982). It is the opinion of some workers that ABA distribution is probably determined by pH gradients within the cell (Hartung, Gimmler and Heilmann, 1982) with the high pH of chloroplasts acting as traps for ABA. Some workers suggest that ABA is probably formed in leaf mesophyll, moving to other parts of the plant via both xylem and phloem (Davies et al, 1986). ABA accumulation appears to be due to synthesis and not to release from a stored precursor (Milborrow and Robinson, 1973). It has been suggested that the signal for ABA production is a decline in turgor (Pierce and Raschke, 1980) or a shrinkage of the cells (Hartung, Kaiser and Burschka, 1983) rather than a reduction in water potential. However, work by Zhang and Davies (1989) described a consistent level of turgor in the leaves of water-stressed plants. They suggested that increased ABA, produced in dehydrating roots, was transported to the shoots thus providing a measure of the soil water status and possibly acting as a regulator of stomatal movement.

In 1965, Itai and Vaadia suggested that soil drying would result in reduced cytokinin synthesis in the roots and thus transport to the leaves. However, others are doubtful that decreased cytokinin synthesis in the roots would have any effect on the stomata (Aspinall, 1980) and since leaves are able to synthesise their own cytokinins,

transport from roots to leaves as an accurate measure of soil water content is unlikely.

The initial impetus for the experimental work described in this section was the finding (see Section 2) that this variety of wheat possesses very low ADC activity. Since previous workers had shown the importance of ADC in polyamine biosynthesis in osmotically-stressed wheat (Flores and Galston, 1982) it seemed important to determine if a variety exhibiting very low levels of ADC would respond similarly, and more importantly, to determine the whole plant response to osmotic stress. The latter aspect has not been examined previously. These experiments seemed all the more exciting in view of the association of polyamines with membrane integrity, inhibition of hydrolytic enzymes, nucleic acids and plant growth regulators. Thus, an initial examination of ADC activity in osmotically-stressed wheat leaf segments was followed by a more detailed study of polyamine metabolism in whole plants exposed to osmotic and water stress.

3.2 Materials and Methods

3.21 Exposure of wheat segments to osmotic stress

Seeds of wheat (*T. aestivum* L. cv. Sappo) were sown in Fisons Levington compost in 23 x 18 cm trays. Approximately 25 seeds were sown per tray. Plants were grown in a ventilated glasshouse under conditions as

described in Section 2.21. Eight days after sowing the first leaves were harvested and cut to a length of 5 cm. The segments were placed in 9 cm plastic Petri dishes containing 15 ml of 1 mM sodium phosphate buffer, pH 5.8 (Flores and Galston, 1982). The following additions were made to the buffer: (a) 0.4 M mannitol; (b) 0.4 M mannitol and 1 mM difluoromethylornithine (DFMO); (c) 0.4 M mannitol and 1 mM difluoromethylarginine (DFMA).

Four leaf segments were floated in each Petri dish. Controls were placed in phosphate buffer only. Throughout the treatments the Petri dishes were placed in an environment with a temperature of 25°C and a mean irradiance of 110 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Segments were removed from the phosphate buffer and phosphate buffer with mannitol solutions every 15 minutes for the first hour and then at 30 minute intervals for the following 2 hours. Further samples were removed after 4 and 5 hours. Segments floated on solutions of phosphate buffer with mannitol and DFMO or DFMA were removed at 30 minute intervals from 1.5 hours to 3 hours after incubation commenced. Once removed, the segments were rinsed in fresh phosphate buffer and analysed for ADC activity.

3.22 Treatment of wheat segments with transcription or translation inhibitors prior to osmotic stress

Plant material was obtained as described in Section 3.21. The cut ends of 8 day old first leaves of wheat were placed in either 30nM rifamycin or 180nM

cycloheximide for 2 hours. The leaves were then removed, rinsed in distilled water and cut into 5 cm segments before floating on solutions of phosphate buffer with 0.4 M mannitol, as described in Section 3.21. Controls consisted of leaf segments pretreated for 2 hours with distilled water before floating on buffer only or buffer with 0.4 M mannitol. Leaf segments were removed from the solutions after 1, 1.5 and 2 hours of exposure to treatment, rinsed in fresh buffer and analysed for ADC activity.

3.23 Determination of ADC activity

The activity of ADC was assayed in crude leaf extracts of tissue incubated on buffer only, buffer with 0.4 M mannitol and tissue pretreated with rifamycin or cycloheximide prior to incubation on buffer with 0.4 M mannitol, using the method described in Section 2.231. All results are the means of five replicates. Standard errors were calculated and differences between means were analysed for significance using the Student's *t*-test (see Section 2.25). Standard errors and significance values are shown in Appendix II.

3.24 Polyamine metabolism in whole plants treated with mannitol

Wheat seeds (*T. aestivum* L. cv. Sappo) were sown in Fisons Levington compost in 10 cm pots, three seeds per pot, and grown in conditions as described in Section 2.21.

Eight days after sowing (Day 0) and every following day for 5 days, the seedlings were watered with a solution of 0.4 M mannitol. Each pot was watered with 150 ml of solution. Control plants were watered with water only.

The first leaves and roots were sampled every day for 5 days after treatment with mannitol commenced (Day 1 - 5). The second leaves were sampled from the third to the fifth day after treatment began (Day 3 - 5).

ADC and ODC activities were analysed in mannitol treated and healthy first leaves, second leaves and roots as described in Section 2.23. Results are the means of five replicates. Polyamine concentrations were determined in mannitol treated and healthy first leaves, second leaves and roots using the method described in Section 2.24. Results are the means of four replicates. Standard errors were calculated and differences between means were analysed for significance using the Student's *t*-test (see Section 2.25).

3.25 Distribution of mannitol in treated plants

The distribution of mannitol was determined by measuring ^{14}C -mannitol accumulation within the plant after incubation with D-[1- ^{14}C]mannitol. Radioisotope was obtained from Amersham International plc.

Wheat seeds (*T. aestivum* L. cv. Sappo) were placed on wet paper tissues in clear plastic boxes (22 x 12 x 9 cm) for 8 days, to germinate. The boxes were kept in a

controlled environment with a daytime temperature of 18.5°C falling to 16.5°C at night. Artificial light was provided by fluorescent tubes for 16 hours per day, giving a mean irradiance of 251 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

250 ml jars, covered with tin foil were plugged with pieces of foam through which the seedlings were inserted for support. Five seedlings were placed in each jar. Each jar contained 200 ml Letcombe Laboratory Nutrient Solution (see Appendix III) and was gently aerated using an Interpet Second Nature Whisper 900 air pump. When 13 days old the seedlings were placed in jars as before, in fresh solutions of Letcombe Laboratory nutrients containing 5 mM unlabelled mannitol and 40 μl D-[1- ^{14}C]mannitol, and gently aerated for 3 hours. The plants were grown in a controlled environment, as described above. Each plant was removed and divided into first leaves, second leaves and roots. The roots were blotted dry with paper tissue. Each section was weighed, chopped up very finely and placed in 1.5 ml distilled water in a Packard glass scintillation vial. To this, 10 ml of Packard Emulsifier-safe scintillant was added and mixed thoroughly by shaking. The vials were left exposed to daylight for four days to bleach the tissue (Lee, 1980). The samples were then counted for radioactivity in a LKB 1215 Rackbeta liquid scintillation counter. Values obtained as disintegrations per minute were converted and expressed as nmol ^{14}C -mannitol (g dry weight) $^{-1}$.

Dry weight conversion factors were obtained by drying

a known weight of fresh material then weighing the dried material. The conversion factors obtained were used to convert the fresh weight of experimental tissue to dry weight. All results are the means of nine replicates. Standard errors were calculated as described in Section 2.25.

3.26 Polyamine metabolism in water-stressed whole plants

Wheat seeds (*T. aestivum* L. cv. Sappo) were germinated as described in Section 3.25. After 8 days the seedlings were inserted through pieces of foam, for support, and used to plug 250 ml jars covered with tin foil. Five seedlings were placed in each jar. The plants were kept in a controlled environment as described in Section 3.25.

Polyethylene glycol (PEG) was used to obtain water stress effects in the plant without the osmotic effects observed in the presence of mannitol.

Treatment jars contained 200 ml Letcombe Laboratory nutrient solution with 2% PEG 4000. Control jars contained 200 ml Letcombe Laboratory nutrient solution only. The solutions were gently aerated. When 13 days old the plants were removed from the solutions and separated into first leaves, second leaves and roots. The roots were blotted dry with paper tissue.

ADC and ODC activities were analysed in first leaves, second leaves and roots of healthy and PEG-treated plants

as described in Section 2.23. All results are the means of five replicates. Polyamine concentrations were determined in first leaves, second leaves and roots of healthy and PEG-treated plants, as described in Section 2.24. Results are the means of four replicates. Standard errors were calculated and differences between means were analysed for significance using the Student's *t*-test (see Section 2.25).

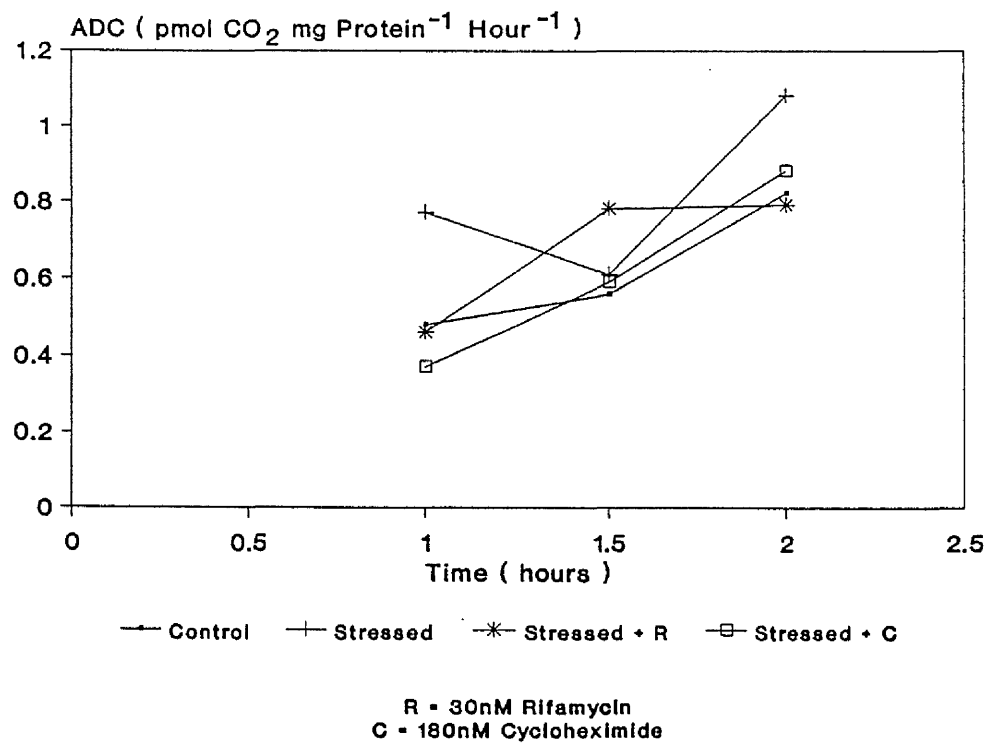
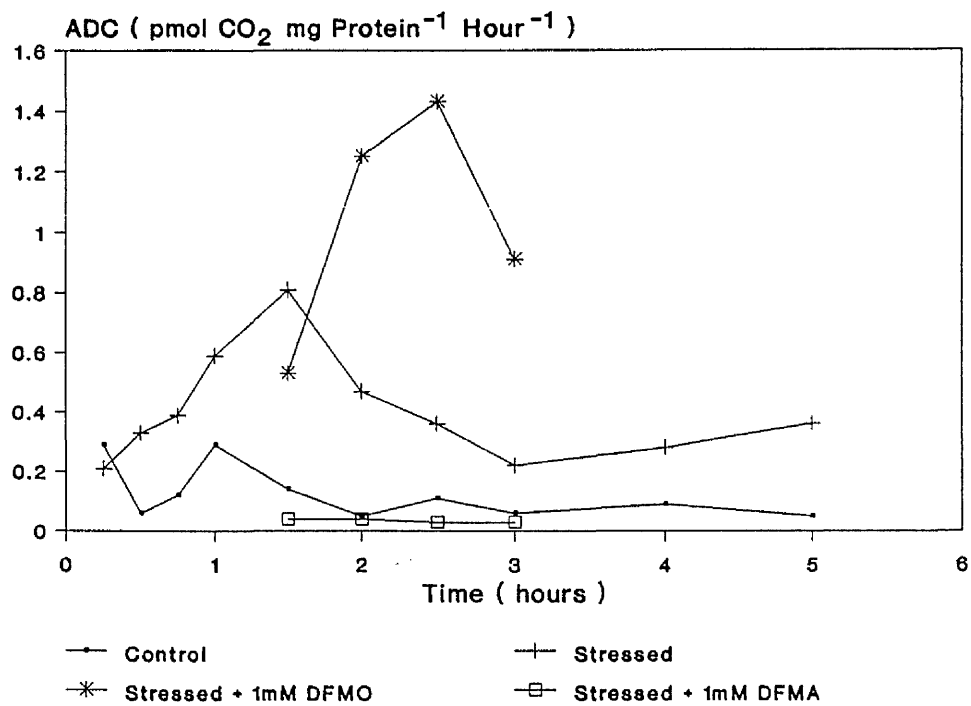
3.3 Results

3.31 ADC activity in wheat segments exposed to mannitol solutions *in vitro*

With the exception of tissue sampled after 15 minutes incubation, ADC activity was significantly greater in tissue exposed to mannitol than in non-mannitol treated tissue (Figure 2). The addition of DFMO to the mannitol incubation solution resulted in significantly greater ADC activity in the treated tissue than in the controls (Figure 2). However, the activity of ADC was significantly lower in leaf segments incubated for 1.5 and 3 hours in the mannitol with added DFMA solution than ADC activity in control tissue (Figure 2). The activity of ADC in leaves incubated in mannitol solutions for 1 and 2 hours was significantly greater than in the controls. However, ADC activity in tissue incubated in mannitol solutions for 1.5 hours was not significantly different from ADC activity in the controls (Figure 3). The activity of ADC in leaf segments pretreated with rifamycin

Figure 2: The effect of osmotic stress alone and in the presence of the inhibitors DFMO or DFMA on arginine decarboxylase (ADC) activity in first leaves of wheat. Values are the means of five replicates.

Figure 3: The effect of treatment with rifamycin or cycloheximide, prior to osmotic stress, on arginine decarboxylase (ADC) activity in first leaves of wheat. Values are the means of five replicates.



or cycloheximide before incubation in mannitol solutions was not significantly altered in comparison with ADC activity in controls, with the exception of rifamycin treated leaves incubated for 1.5 hours in mannitol solutions (Figure 3). Nevertheless, both rifamycin and cycloheximide caused a substantial reduction in the stress-induced rise of ADC activity observed at 1 and 2 hours.

3.32 ADC activity in mannitol treated whole plants

ADC activity in first leaves of mannitol treated plants on the first, second and third days of treatment, was not significantly different from ADC activity in control first leaves (Figure 4). However, ADC activity in first leaves of mannitol treated plants on the fifth day of treatment was significantly greater than in control tissue (Figure 4). ADC activity in the second leaves of mannitol treated plants, on the third, fourth and fifth days of exposure to osmoticum, was significantly greater than in control tissue (Figure 5). In roots of mannitol treated plants on the first, third and fourth days of treatment, no significant difference in ADC activity was observed in comparison with the controls (Figure 6). However, on the second and fifth days of exposure to mannitol, ADC activity was significantly greater in the treated tissue than in the controls (Figure 6).

Figure 4: Arginine decarboxylase (ADC) activity in first leaves of healthy and mannitol treated wheat plants sampled from the first to the fifth day of exposure to osmoticum. Values are the means of five replicates with standard error bar shown. Significant differences are shown as follows: P = 0.001 ***; P = 0.1 *.

Figure 5: Arginine decarboxylase (ADC) activity in second leaves of healthy and mannitol treated wheat plants sampled from the third to the fifth day of exposure to osmoticum. Values are the means of five replicates with standard error bar shown. Significant differences are shown as P = 0.001 ***.

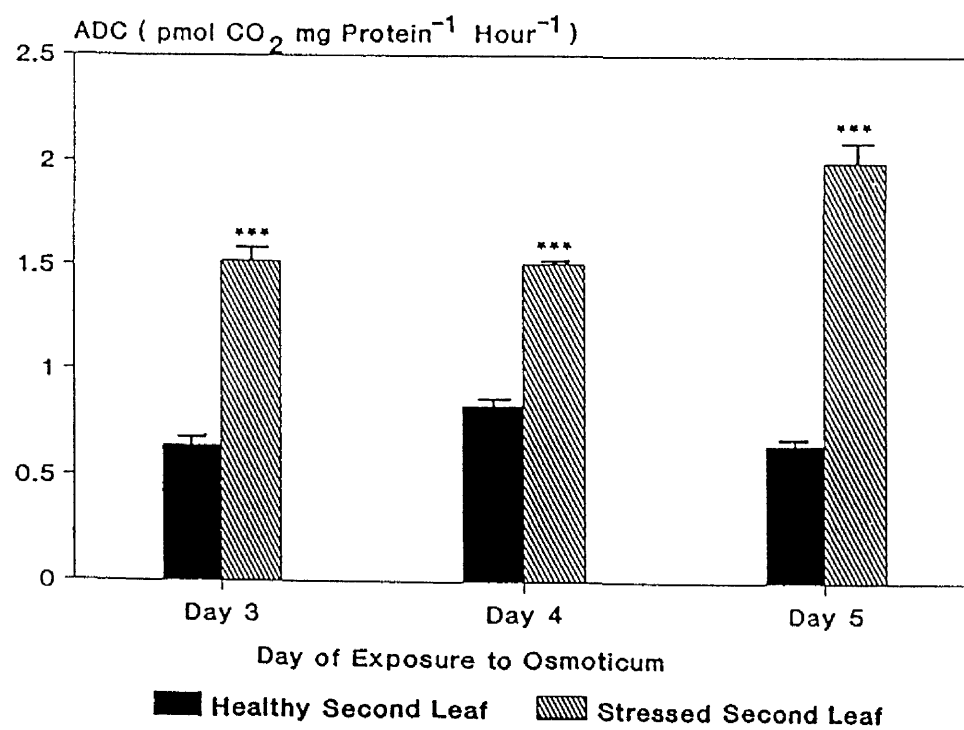
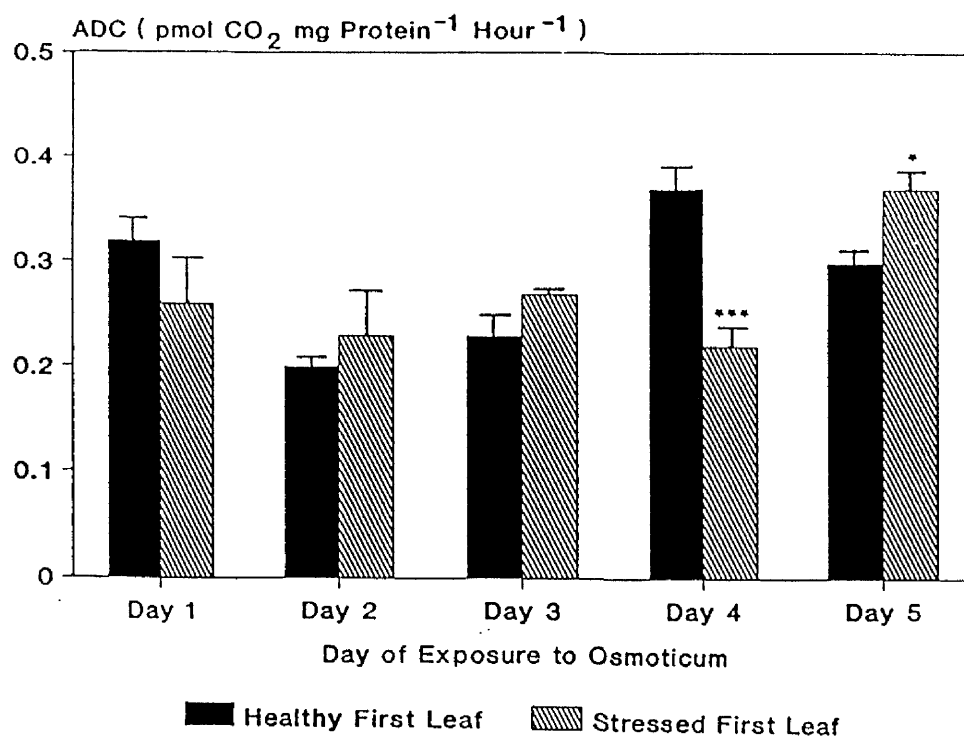
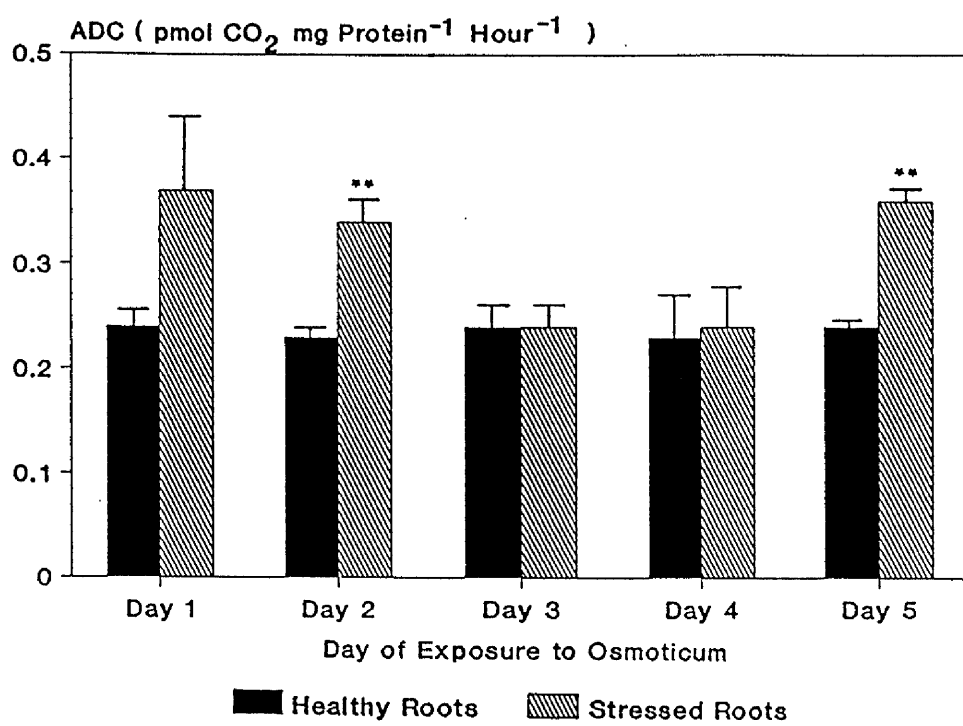


Figure 6: Arginine decarboxylase (ADC) activity in roots of healthy and mannitol treated wheat plants sampled from the first to the fifth day of exposure to osmoticum. Values are the means of five replicates with standard error bar shown. Significant differences are shown as $P = 0.01$ **.



3.33 Cytosolic ODC activity in mannitol treated whole plants

The activity of ODC in first leaves of mannitol treated plants on the third and fourth days of treatment was significantly greater than in control first leaves (Figure 7). However, ODC activity in treated first leaves on the first, second and fifth days of exposure to treatment was not significantly different from ODC activity in control tissue (Figure 7). With the exception of Day 5, the activity of ODC in the second leaves of mannitol treated plants was not significantly different from ODC activity in control second leaves (Figure 8). The activity of ODC in roots of mannitol treated plants on the first, second and third days of treatment was not significantly different from ODC activity in control roots (Figure 11). However in mannitol treated roots on days four and five of treatment, a significant reduction in ODC activity was observed in comparison with ODC activity in control roots (Figure 11).

3.34 Nuclear ODC activity in mannitol treated whole plants

ODC activity in first leaves of mannitol treated plants on the first, second and fourth days of exposure to treatment was not significantly different from ODC activity in control first leaves (Figure 9). On the third day of treatment, however, ODC activity was significantly greater in treated first leaves than in controls (Figure

Figure 7: Cytosolic ornithine decarboxylase (ODC) activity in first leaves of healthy and mannitol treated wheat plants sampled from the first to the fifth day of exposure to osmoticum. Values are the means of five replicates with standard error bar shown. Significant differences are shown as follows: $P = 0.01$ **; $P = 0.1$ *.

Figure 8: Cytosolic ornithine decarboxylase (ODC) activity in second leaves of healthy and mannitol treated wheat plants sampled from the third to the fifth day of exposure to osmoticum. Values are the means of five replicates with standard error bar shown. Significant differences are shown as $P = 0.1$ *.

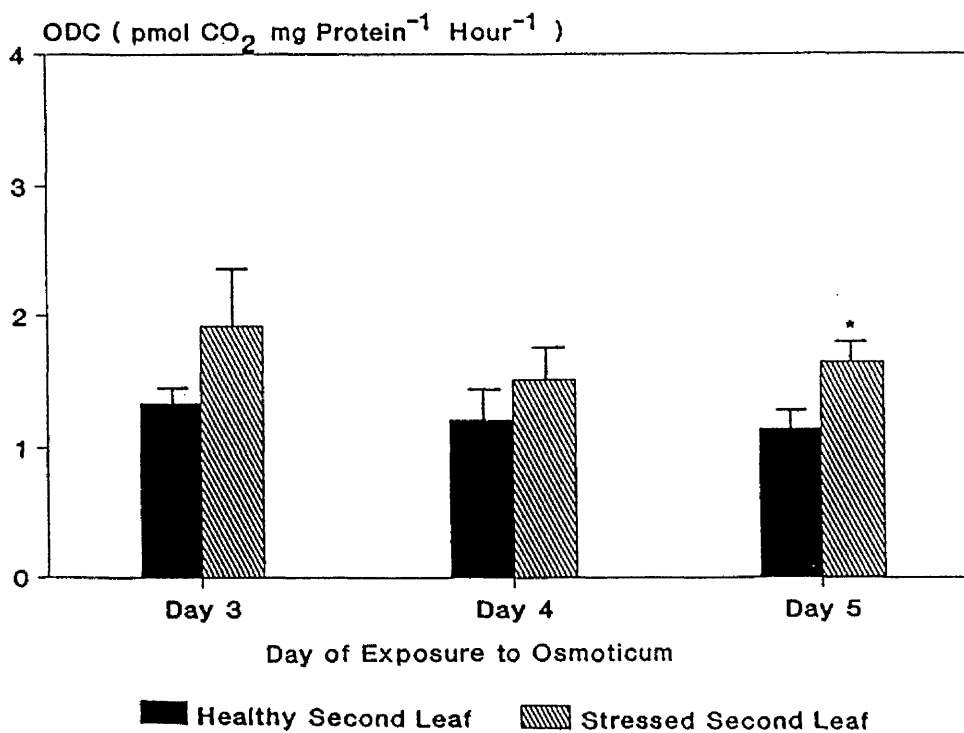
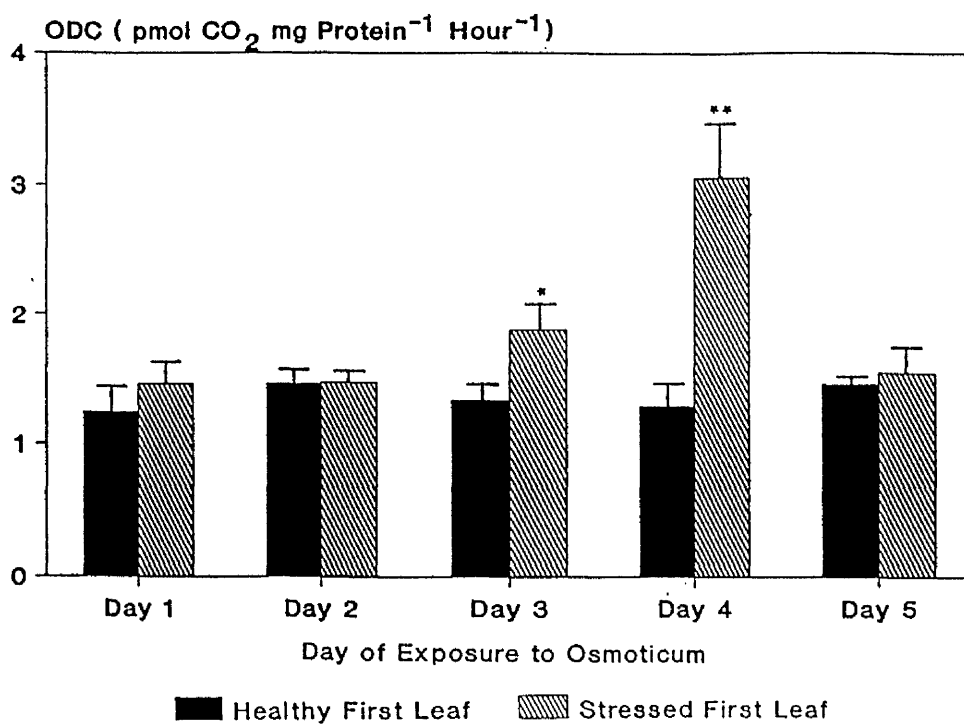


Figure 9: Nuclear ornithine decarboxylase (ODC) activity in first leaves of healthy and mannitol treated wheat plants sampled from the first to the fifth day of exposure to osmoticum. Values are the means of five replicates with standard error bar shown. Significant differences are shown as follows: $P = 0.001$ ***; $P = 0.01$ **.

Figure 10: Nuclear ornithine decarboxylase (ODC) activity in second leaves of healthy and mannitol treated wheat plants sampled from the third to the fifth day of exposure to osmoticum. Values are the means of five replicates with standard error bar shown. Significant differences are shown as $P = 0.01$ **.

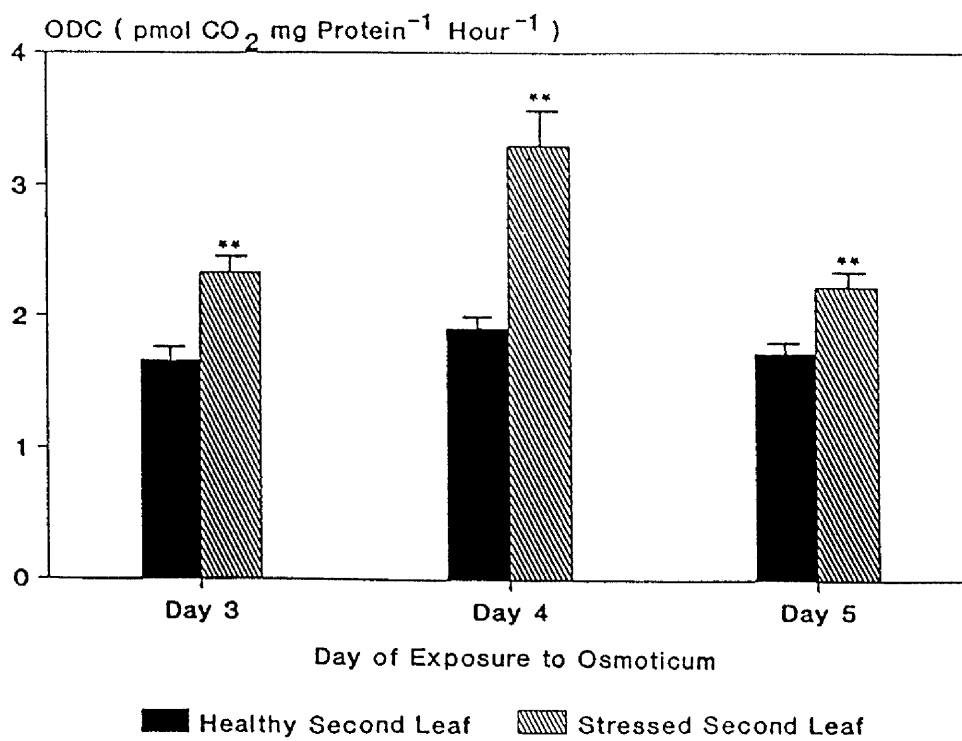
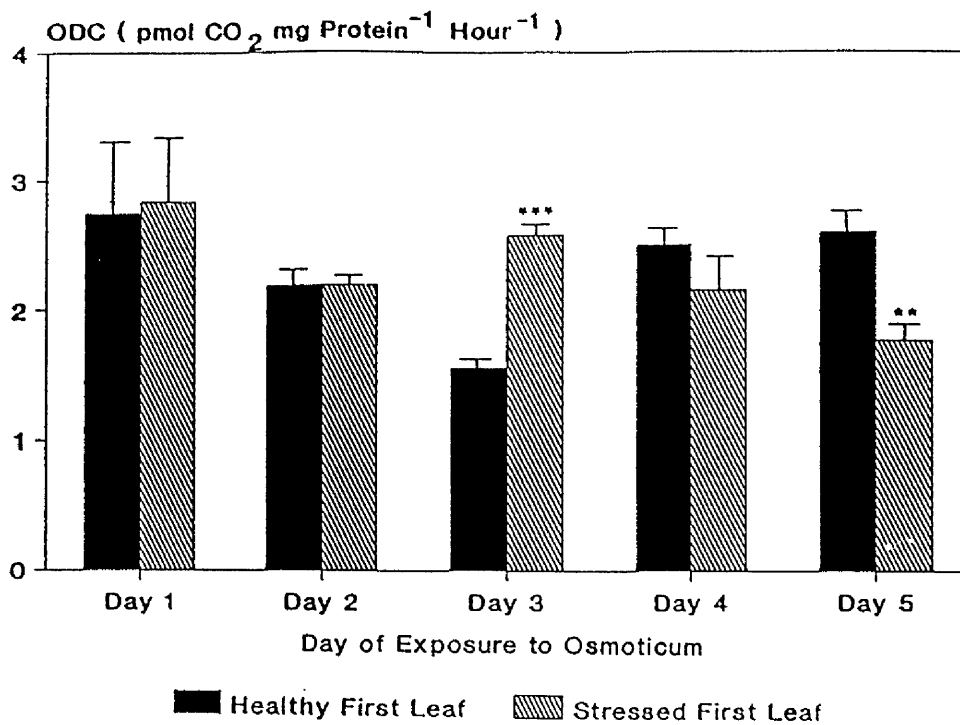
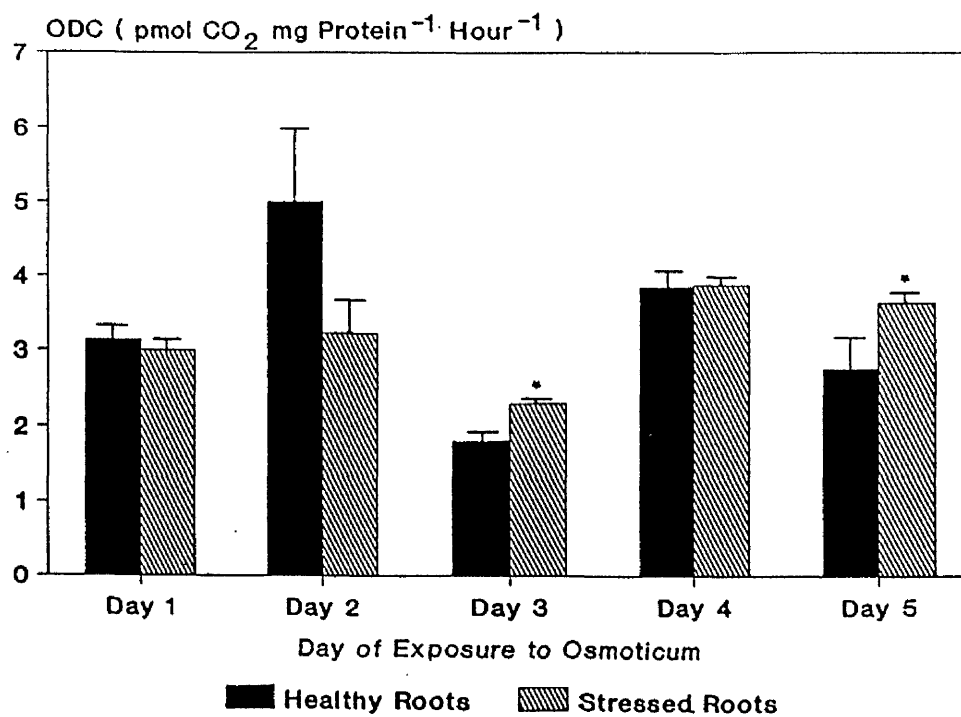
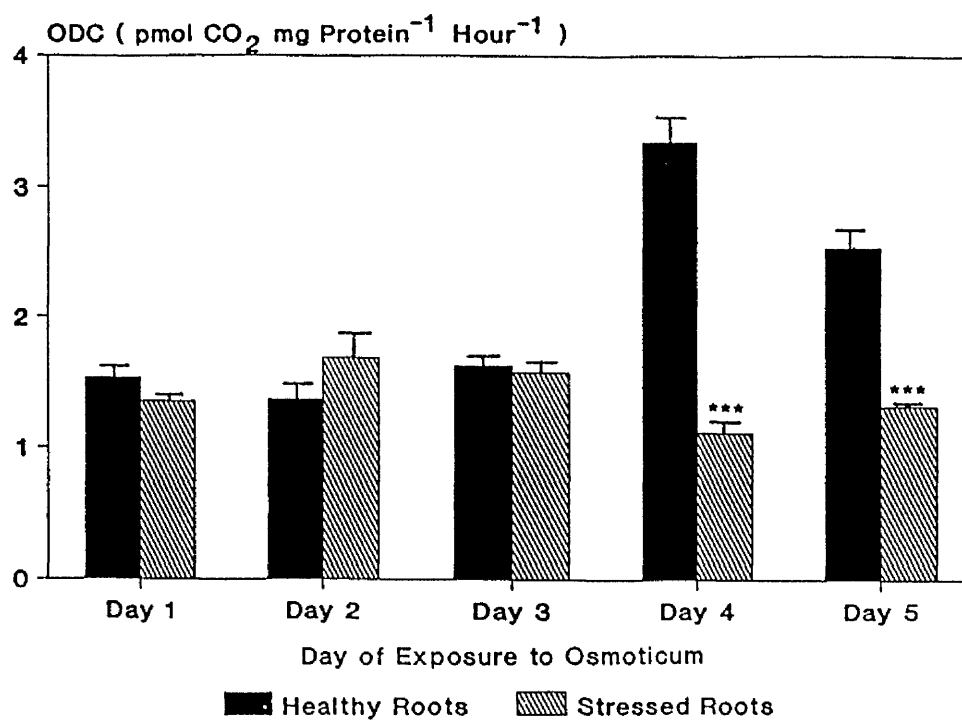


Figure 11: Cytosolic ornithine decarboxylase (ODC) activity in roots of healthy and mannitol treated plants sampled from the first to the fifth day of exposure to osmoticum. Values are the means of five replicates with standard error bar shown. Significant differences are shown as $P = 0.001$ ***.

Figure 12: Nuclear ornithine decarboxylase (ODC) activity in roots of healthy and mannitol treated wheat plants sampled from the first to the fifth day of exposure to osmoticum. Values are the means of five replicates with standard error bar shown. Significant differences are shown as $P = 0.1$ *.



9). ODC activity in treated first leaves on the fifth day of exposure to mannitol was significantly reduced in comparison with control first leaves (Figure 9). The activity of ODC in the second leaves of mannitol treated plants was significantly greater than controls over the experimental period (Figure 10). ODC activity in the roots of mannitol treated plants was significantly greater than controls on the third and fifth days of exposure to mannitol (Figure 12). However, ODC activity in treated roots on the first, second and fourth days of exposure to mannitol was not significantly different from ODC activity in control roots (Figure 12).

3.35 Polyamine concentrations in mannitol treated whole plants

The concentration of putrescine in the first leaves and second leaves of mannitol treated plants on each day of treatment was significantly greater than in control leaves (Table 12). Although putrescine concentration in the roots of mannitol treated plants on the third and fifth day of exposure to osmoticum was significantly greater than in control roots, there was no significant difference on the first and fourth days of treatment (Table 12). A significant reduction in putrescine concentration in treated roots on the second day of exposure to mannitol was observed (Table 12).

Spermidine concentration in the first leaves of mannitol treated plants increased significantly on the

Table 12: Putrescine content in healthy and osmotically-stressed first leaves and roots of wheat from the first to the fifth day after watering with 0.4 M mannitol. Second leaves were analysed from the third to the fifth day. Values are the means of four replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$; (c) $P = 0.1$. ND = Not Determined.

Putrescine (nmol g Fresh Weight ⁻¹)						
	First Leaf		Second Leaf		Roots	
	Healthy	Stressed	Healthy	Stressed	Healthy	Stressed
Day 1	62.1±2.1	110.0±4.0 ^a	ND	ND	80.7 ± 3.1	80.7±2.2
Day 2	80.7±2.2	110.0±2.3 ^a	ND	ND	80.7 ± 2.9	62.1±9.9 ^c
Day 3	42.0±2.6	110.0±3.7 ^a	21.0±0.3	80.7 ± 3.5 ^a	62.1 ± 1.8	80.7±3.3 ^a
Day 4	62.1±1.5	110.0±4.7 ^a	42.0±1.1	124.2 ± 4.1 ^a	62.1 ± 1.6	62.1±1.0
Day 5	80.7±1.5	144.9±3.5 ^a	62.1±1.3	229.8±11.1 ^a	80.7±12.6	124.2±1.9 ^b

first day of exposure to osmoticum in comparison with control first leaves (Table 13). Thereafter, although the concentration of spermidine in treated first leaves was no different to controls on the second, third and fifth days of exposure to mannitol, a significant reduction in spermidine concentration was observed in first leaves exposed to mannitol for four days (Table 13).

With the exception of leaves sampled on the fifth day of treatment, spermidine concentrations in the second leaves of mannitol treated plants were not significantly different from the control second leaves (Table 13). Spermidine concentrations in the roots of mannitol treated plants on the second and fifth days of exposure to mannitol were significantly reduced, in comparison with the controls. However, no significant difference in the concentration of spermidine was observed in treated roots on the first, third and fourth days of exposure to mannitol (Table 13).

With the exception of first leaves and roots on the second day of exposure to osmoticum, the concentration of spermine in all tissues of mannitol treated plants, on each day of treatment, was significantly greater than in control tissue (Table 14).

The concentration of cadaverine in tissue exposed to mannitol treatment, on each day of treatment, was significantly greater than in control tissue with the exception of roots on the fourth day of treatment with mannitol (Table 15).

Table 13: Spermidine content in healthy and osmotically-stressed first leaves and roots of wheat from the first to the fifth day after watering with 0.4 M mannitol. Second leaves were analysed from the third to the fifth day. Values are the means of four replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$. ND = Not Determined.

Spermidine (nmol g Fresh Weight ⁻¹)					
	First Leaf		Second Leaf		Roots
	Healthy	Stressed	Healthy	Stressed	Healthy Stressed
Day 1	42.2 \pm 0.9	51.3 \pm 1.5 ^b	ND	ND	11.5 \pm 2.5 11.5 \pm 0.3
Day 2	17.1 \pm 1.2	17.1 \pm 1.5	ND	ND	11.5 \pm 0.2 5.7 \pm 0.4 ^a
Day 3	22.2 \pm 1.1	22.2 \pm 1.2	34.2 \pm 0.1	29.1 \pm 2.2	11.5 \pm 0.2 11.5 \pm 0.3
Day 4	29.1 \pm 0.6	22.2 \pm 0.9 ^a	34.2 \pm 1.0	34.2 \pm 1.5	11.5 \pm 0.1 11.5 \pm 1.8
Day 5	39.3 \pm 2.1	34.2 \pm 1.8	34.2 \pm 0.5	39.3 \pm 1.2 ^b	22.2 \pm 0.6 17.1 \pm 0.2 ^a

Table 14: Spermine content in healthy and osmotically-stressed first leaves and roots of wheat from the first to the fifth day after watering with 0.4 M mannitol. Second leaves were analysed from the third to the fifth day. Values are the means of four replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$; (c) $P = 0.1$. ND = Not Determined.

Spermine (nmol g Fresh Weight ⁻¹)						
	First Leaf		Second Leaf		Roots	
	Healthy	Stressed	Healthy	Stressed	Healthy	Stressed
Day 1	48.8 \pm 1.2	71.8 \pm 1.8 ^a	ND	ND	19.2 \pm 0.4	28.7 \pm 0.7 ^a
Day 2	48.8 \pm 2.5	48.8 \pm 1.8	ND	ND	19.2 \pm 0.4	28.7 \pm 4.7
Day 3	37.3 \pm 2.6	86.2 \pm 5.3 ^a	66.1 \pm 2.4	123.5 \pm 4.3 ^a	19.2 \pm 0.6	28.7 \pm 0.7 ^a
Day 4	48.8 \pm 0.9	86.2 \pm 4.4 ^a	57.5 \pm 2.1	123.5 \pm 5.4 ^a	19.2 \pm 1.6	28.7 \pm 1.4 ^b
Day 5	123.5 \pm 4.2	146.5 \pm 7.5 ^c	57.5 \pm 1.2	152.2 \pm 10.5 ^a	37.3 \pm 0.6	66.1 \pm 2.8 ^a

Table 15: Cadaverine content in healthy and osmotically-stressed first leaves and roots of wheat from the first to the fifth day after watering with 0.4 M mannitol. Second leaves were analysed from the third to the fifth day. Values are the means of four replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$. ND = Not Determined.

Cadaverine (nmol g Fresh Weight ⁻¹)						
	First Leaf		Second Leaf		Roots	
	Healthy	Stressed	Healthy	Stressed	Healthy	Stressed
Day 1	11.4 \pm 0.7	38.3 \pm 0.9 ^a	ND	ND	9.1 \pm 0.8	18.8 \pm 0.3 ^a
Day 2	11.4 \pm 2.3	38.3 \pm 1.7 ^a	ND	ND	9.1 \pm 0.5	38.3 \pm 1.8 ^a
Day 3	11.4 \pm 1.1	18.8 \pm 2.8 ^a	18.8 \pm 0.3	57.1 \pm 2.1 ^a	9.1 \pm 1.3	18.8 \pm 1.7 ^b
Day 4	18.8 \pm 0.6	38.3 \pm 2.1 ^a	18.8 \pm 0.2	97.1 \pm 3.3 ^a	18.8 \pm 5.2	18.8 \pm 1.2
Day 5	30.3 \pm 1.8	57.1 \pm 1.9 ^a	38.3 \pm 0.6	268.0 \pm 8.2 ^a	18.8 \pm 3.5	38.3 \pm 1.3 ^b

All plants treated with mannitol showed visible signs of wilting by day 5.

3.36 Distribution of ^{14}C -mannitol in whole plants

The concentration of ^{14}C -mannitol in treated plants was much greater in the roots than in the shoots. The first leaves, however, contained a higher concentration of ^{14}C -mannitol than the second leaves (Table 16).

3.37 Enzyme activities in plants water-stressed by treatment with PEG

The activity of ADC in water-stressed tissue was not significantly different from ADC activity in control tissue (Table 17). Both cytosolic and nuclear ODC activities in water-stressed tissue were not significantly different from ODC activities in control tissues, with the exception of cytosolic ODC activity in water-stressed roots (Table 18).

3.38 Polyamine concentrations in plants water-stressed by treatment with PEG

The concentrations of putrescine and cadaverine in first leaves, second leaves and roots of stressed plants were not significantly different from concentrations in control tissues (Table 19).

The concentration of spermidine in stressed first leaves and second leaves was significantly reduced in

Table 16: Distribution of ^{14}C -mannitol in first leaves, second leaves and roots of wheat. Values are the means of nine replicates \pm standard error.

	^{14}C Mannitol (nmol g Dry Weight $^{-1}$)
First Leaf	0.31 ± 0.04
Second Leaf	0.23 ± 0.03
Roots	2.86 ± 0.32

Table 17: Arginine Decarboxylase activity in healthy and water-stressed first leaves, second leaves and roots of wheat, on the fifth day of exposure to stress. Values are the means of five replicates \pm standard error.

	ADC Activity ($\mu\text{mol CO}_2 \text{ mg Protein}^{-1} \text{ Hour}^{-1}$)
Healthy First Leaf	0.15 ± 0.01
Stressed First Leaf	0.17 ± 0.01
Healthy Second Leaf	0.32 ± 0.08
Stressed Second Leaf	0.28 ± 0.08
Healthy Roots	0.24 ± 0.03
Stressed Roots	0.21 ± 0.01

Table 18: Ornithine Decarboxylase activity in healthy and water-stressed first leaves, second leaves and roots of wheat, on the fifth day of exposure to stress. Values are the means of five replicates \pm standard error. Significant differences are shown as (c) $P = 0.1$.

	ODC Activity ($\mu\text{mol CO}_2 \text{ mg Protein}^{-1} \text{ Hour}^{-1}$)	
	Cytosolic	Nuclear
Healthy First Leaf	3.47 ± 0.39	2.32 ± 0.29
Stressed First Leaf	3.74 ± 0.30	2.07 ± 0.16
Healthy Second Leaf	3.71 ± 0.48	2.39 ± 0.07
Stressed Second Leaf	3.91 ± 0.15	2.68 ± 0.31
Healthy Roots	4.23 ± 0.12	2.35 ± 0.17
Stressed Roots	3.55 ± 0.05^c	2.53 ± 0.35

Table 19: Polyamine content in healthy and water-stressed first leaves, second leaves and roots of wheat, on the fifth day of exposure to stress. Values are the means of four replicates \pm standard error. Significant differences are shown as (b) $P = 0.01$.

	nmol g Fresh Weight ⁻¹			
	Putrescine	Spermidine	Spermine	Cadaverine
Healthy First Leaf	55.2 ± 1.6	45.7 ± 3.6	89.3 ± 5.5	12.7±1.1
Stressed First Leaf	55.2 ± 2.0	30.4 ± 1.1 ^b	63.8 ± 1.4 ^b	25.4±9.8
Healthy Second Leaf	103.7±16.2	85.5 ± 5.6	143.6 ± 4.4	19.0±9.0
Stressed Second Leaf	80.1±18.6	55.2 ± 3.3 ^b	129.7±29.4	22.1±7.8
Healthy Roots	193.0±40.3	30.4 ± 9.3	81.7±12.5	10.2±0.8
Stressed Roots	176.3±47.8	55.2±10.4	89.3±13.8	12.7±4.4

comparison with control tissue. However, no significant difference in the concentration of spermidine in stressed roots was observed (Table 19).

With the exception of stressed first leaves, the concentration of spermine in stressed plants was not significantly different from the spermine concentration in control plants (Table 19).

3.4 Discussion

The observed increases in ADC activity in osmotically-stressed wheat leaf segments are in agreement with results obtained by Flores and Galston for oat (1982). In an attempt to elucidate whether the increase in $^{14}\text{CO}_2$ evolution during enzyme assays was as a result of ADC or ODC activity (since the radiolabelled arginine may have been converted to ornithine via arginase activity) two polyamine biosynthetic inhibitors were used. The inhibitor α -difluoromethylarginine (DFMA) irreversibly inhibits ADC activity whereas α -difluoromethylornithine (DFMO) irreversibly inhibits ODC activity (Metcalf et al, 1978; Kallio, McCann and Bey, 1981). DFMA almost completely inhibited $^{14}\text{CO}_2$ release whereas DFMO enhanced the activity, confirming that the $^{14}\text{CO}_2$ released was a result of ADC and not ODC activity. The enhanced ADC activity in the presence of DFMO has also been described by Flores and Galston (1982), and may signify stimulation of one pathway when the other is blocked. The rise in ADC activity in leaves exposed to osmotic shock reached an

optimum between 1.5 and 2 hours incubation, suggesting a requirement for protein synthesis. Pretreatment of leaves with cycloheximide, a translation inhibitor, almost completely prevented the increase in ADC activity during osmotic stress as did rifamycin, a transcription inhibitor, although to a lesser extent. These results and those obtained by Flores and Galston (1984 a) suggest that during the initial hours of exposure to osmotic shock, an event dependent upon protein synthesis occurs which is subsequently translated into a rise in ADC activity.

Although significant increases in ADC and ODC activities of first leaves and roots of whole plants were found on certain days of exposure to osmoticum, the most consistent increases in enzyme activities were found in stressed second leaves. The increase in ODC activity observed in leaves of osmotically-stressed wheat plants was not found in wheat exposed to chill stress (Nadeau, Delaney and Chouinard, 1987). Although both ADC and ODC are involved in putrescine biosynthesis in plants (Bachrach, 1973; Slocum, Kaur-Sawhney and Galston, 1984; Tabor and Tabor, 1984) it has been suggested that ODC activity is involved in cell division whereas ADC activity is associated with stress responses (Slocum, Kaur-Sawhney and Galston, 1984; Smith, 1985). Indeed, correlations between increased ADC activity and putrescine accumulation have been reported during potassium deficiency (Smith, 1979), acid stress (Young and Galston, 1983) and chill stress (Nadeau, Delaney and Chouinard, 1987), although, this does not rule out the possibility of ODC-mediated

stress-induced changes. In fact, the results presented in this work clearly demonstrate that both ADC and ODC activities can increase during osmotic stress. Similarly, work by Friedman, Altman and Levin (1989) on polyamine biosynthesis during salt stress described an increase in ODC as well as ADC activity suggesting that perhaps ADC and ODC are involved in both growth and stress responses. However, using DFMA and DFMO, the enzyme-activated, irreversible inhibitors of ADC and ODC respectively, Flores and Galston (1984 a) found that DFMA inhibited ADC activity and prevented a rise in putrescine whereas DFMO had no effect on ODC activity and did not prevent the putrescine increase, suggesting that putrescine accumulation during osmotic shock is due entirely to the ADC pathway. These workers cautioned, however, that the use of irreversible inhibitors in plant systems may be variable since in some plant tissues DFMO and DFMA may be subject to interconversion.

Consistent with the increases in ADC and ODC activities in stressed second leaves, in general, increases in putrescine, spermine and cadaverine concentrations were observed. In several studies of water and osmotic stress, the effect on polyamine levels but not biosynthetic enzyme activity was reported (Turner and Stewart, 1986; 1988). An increase, although not significant, in putrescine concentration during osmotic stress of barley leaf sections was reported (Turner and Stewart, 1988). However, these workers also described an increase in putrescine accumulation in the presence of

phosphate buffer, pH 5.8. However, it seems unlikely that putrescine is accumulating as a result of a pH effect since Young and Galston (1983) reported that a pH level below 5.0 was necessary to stimulate putrescine synthesis.

Further, variations in polyamine metabolism between acid and osmotic stress suggest that the osmotic stress-induced increase is not mediated through a reduction in the pH of the external solution (Flores and Galston, 1984 a). In *in vitro* experiments Turner and Stewart (1988) observed that spermidine and spermine levels were not affected in the presence of an osmoticum. The results presented in this thesis are inconsistent with these findings, since spermine concentrations increased in osmotically-stressed wheat plants. However, very little change was observed in spermidine concentrations during osmotic shock. Earlier work by Turner and Stewart (1986) on whole water-stressed plants described an accumulation of putrescine and spermine only when leaves retained turgor. Once leaf turgor was lost they found that putrescine and spermine concentrations decreased. Similarly, Flores and Galston (1984 b) suggested a relationship between putrescine accumulation and leaf turgor since most putrescine accumulated when leaf turgor was only slightly reduced.

The observed putrescine accumulation may be a result of transformation of spermidine back to putrescine by the enzymes spermidine-N¹-acetyltransferase or polyamine oxidase. This would also account for the reduction in

spermidine levels. However, this would appear to be unlikely since Flores and Galston (1984 a) found that decreases in spermidine and spermine during osmotic shock were correlated with increases in diaminopropane, the direct product of polyamine oxidase, rather than putrescine. Flores and Galston (1982; 1984a) found that during osmotic stress of cereal mesophyll protoplasts, conversion of putrescine to spermidine and spermine was inhibited, and therefore the increases observed in spermine concentration, in this work, may be due to the activity of spermine synthase which would also result in depleted pools of spermidine. In addition the increase in putrescine during osmotic shock does not appear to be the result of its release from a bound form (Flores and Galston, 1982).

The greater increase of putrescine, spermine and cadaverine in second leaves of osmotically-stressed plants may reflect a survival mechanism of the plant, as it would be advantageous to show a greater response in younger and more metabolically active organs. Similarly, during osmotic stress of oat leaf segments, Flores and Galston (1984 a) found that the accumulation of putrescine decreased with leaf age.

Since it appears that polyamines are involved in the reduction of RNase activity, protease activity, chlorophyll degradation and membrane leakiness (Slocum, Kaur-Sawhney and Galston, 1984) it might be expected that increased polyamine levels during osmotic shock are

involved in a protective role. The protective effect of polyamines has been attributed to their polycationic nature. Electrostatic binding of the cationic polyamines to negatively charged sites in the membrane exerts a stabilising effect on cell membranes (Naik and Srivastava, 1981). Guye, Vigh and Wilson (1986) demonstrated that chill-tolerant bean genotypes have a greater amount of leaf phospholipid than more sensitive genotypes and thus suggested that the tolerant genotypes may have more negatively charged membrane sites available for polyamine binding. The substantial drop observed in membrane potential of oat slices immersed in mannitol solutions (Rubinstein, Mahar and Tattar, 1977) and the contraction of the membrane due to reduced turgor, would alter the activity of the membrane enzymes (Levitt, 1980). It is possible therefore, that an increase in polyamine concentrations may reflect membrane stabilisation in the work reported here. However, putrescine appears to be the major polyamine present during osmotic shock and in these situations it has been shown to be the least effective polyamine (Slocum, Kaur-Sawhney and Galston, 1984). Moreover, recent work has suggested that high intracellular concentrations of putrescine may actually be involved in membrane damage in certain systems (DiTomaso, Shaff and Kochian, 1989). The increase in spermine however, might suggest involvement in physiological protection during osmotic stress although Turner and Stewart (1986) described a decrease in spermine accumulation when leaf turgor was lost and suggested that

stabilisation of cell membranes and conservation of protein synthesis would have been more crucial during this time. These workers suggested that phosphorus deficiency, as a result of water stress, may be responsible for increased polyamine synthesis since leaf phosphate content has been shown to decrease during water stress, before leaf turgor is affected (Turner, 1985) and putrescine synthesis was found to increase during phosphorus deficiency (Smith, Negrel and Bird, 1983).

An increase of proline concentration during water stress was first observed by Kemble and Macpherson (1954) and is now considered to be a general phenomenon (Hanson and Hitz, 1982). Since proline and putrescine may share common precursors such as glutamate, arginine and ornithine (Stewart and Boggess, 1977) and since both increase during osmotic stress, a possible association between them should be considered. However, the magnitude of increase in concentration of proline and putrescine differed greatly with proline accumulating at a much greater rate (Turner and Stewart, 1986). These workers also found that proline and glycine-betaine levels did not begin to rise until putrescine accumulation and turgor decreased, suggesting that the nature of the response of polyamine metabolism to water stress differed from that of proline and glycine-betaine. Similarly, the increase in putrescine during osmotic stress appears to occur at a similar turgor deficit to that resulting in inhibition of cell wall synthesis, cell growth and abscisic acid (ABA) accumulation (Hsiao, 1973), whereas the accumulation of

proline occurs at an even lower water potential.

The changes in ODC and ADC activities of both healthy and stressed first leaves and roots during the five day sampling period were variable, and may be due to slight differences between each wheat plant sampled. However, the enzyme activities in the second leaves of control plants remained relatively consistent and increased in stressed plants. The increase in ADC activity in the roots of stressed plants was not always accompanied by an increase in putrescine concentration, although an increase in spermine was observed, suggesting translocation of putrescine from roots to shoots. This is in agreement with work by Friedman, Altman and Levin (1989) on polyamine biosynthesis during salt stress and is supported by recent findings of long distance translocation of polyamines within the plant (Friedman, Levin and Altman, 1986). Although the greatest stress-induced increase in ODC and ADC activities and putrescine, spermine and cadaverine concentrations was usually detected in leaf tissue it was decided to determine the distribution of mannitol in plants grown in solutions amended with radiolabelled mannitol, since in nature, the root system of plants is directly exposed to stress conditions such as changes in osmotic concentrations, pH and salinity. The distribution of ^{14}C -mannitol in treated plants was highest in the roots, whereas the most profound effect on polyamine metabolism was observed in the second leaves, suggesting possible regulation of water use and growth by a signal moving from the roots to the shoots. In an

attempt to investigate this possibility, polyamine metabolism was determined in plants water-stressed using polyethylene glycol (PEG), a commonly used water stress agent. Much work has involved the study of plant growth regulators acting as possible 'signals' within the plant during stress conditions. Zhang and Davies (1989) described an increase in ABA content of roots growing in unwatered soil and thus suggested that ABA was transported to the shoots, from the dehydrating roots, providing an indication of the water status of the soil. Similarly, Zhang, Schurr and Davies (1987) described movement of ABA from roots to shoots to regulate stomatal movement during water stress. The results presented in this thesis indicate that the effect of PEG on polyamine metabolism was minimal. This is an agreement with work by Flores and Galston (1984 b). However, the lack of effect does not appear to be as a result of toxicity since proline increased significantly (Flores and Galston, 1984 b) and leaf viability was not affected (Taylor and West, 1980). Thus any possible 'signal' released during osmotic stress which results in changes in polyamine metabolism is not triggered by changes in water stress. Since PEG does not enter the cell wall space (Rubinstein, 1982) and mannitol does, perhaps the observed increase in polyamine metabolism during osmotic stress is dependent upon some interaction at the membrane surface. Clearly, this is an area ripe for future investigation.

S E C T I O N 4

THE EFFECTS OF POLYAMINE BIOSYNTHESIS INHIBITORS
ON INFECTION OF WHEAT BY *PUCCINIA GRAMINIS*
f. sp. *TRITICI* AND MYCELIAL GROWTH, ENZYME ACTIVITY
AND POLYAMINE CONTENT IN THE OAT-INFECTING FUNGUS
PYRENOPHORA AVENAE

4.1 Introduction

The field of polyamine research was enhanced greatly with the development of specific and enzyme-activated irreversible inhibitors of polyamine biosynthetic enzymes. Two such inhibitors are α -difluoromethylornithine (DFMO) and α -difluoromethylarginine (DFMA) which irreversibly inhibit ODC and ADC respectively (Metcalf et al 1978; Kallio, McCann and Bey, 1981). An initial search for polyamine biosynthesis inhibitors to help clarify the role of polyamines in cell growth, led to the synthesis of ornithine analogues such as α -hydrazinoornithine (Harik and Snyder, 1973) and α -methylornithine (Abdel-Monem, Newton and Weeks, 1974). However, although these compounds which are competitive inhibitors of ODC, were effective in reducing the accumulation of polyamines, their effects were readily reversible (Mamont et al, 1976). Thus, the availability of DFMO made it possible to confirm the role of polyamines in cell proliferation in various systems (Mamont et al, 1978).

The development of specific inhibitors of the main enzymes required for polyamine synthesis has not only provided evidence of the need for polyamines for growth, but has also helped in the understanding of polyamine metabolism and in the regulation of enzymes involved in this process. Indeed, DFMO was found to inhibit ODC activity, to decrease the intracellular concentrations of putrescine and spermidine and to inhibit cell growth *in vitro* (Mamont et al, 1978; Sunkara et al, 1980; Luk et

al, 1981) and in animal tumours *in vivo* (Prakash et al 1978; Sunkara, Prakash and Rosenberger, 1982). DFMO was first introduced as an anticancer agent (Metcalf et al, 1978) and demonstrated low toxicity and tolerance in animals and humans (Sunkara and Prakash, 1984; Tabor and Tabor, 1985), although some slightly toxic, but reversible, effects have been described (Sjoerdsma and Schechter, 1984). The dramatic antitumour effects of DFMO on rodent models (Sjoerdsma, 1981; Pegg and McCann, 1982) led to an enthusiastic search of the possibilities of using DFMO on human cancer diseases. The results, however, have proved to be slightly disappointing (Sjoerdsma and Schechter, 1984; Schechter, Barlow and Sjoerdsma, 1987). On comparing the effects of DFMO in animal models and humans, Sjoerdsma and Schechter (1984) suggested that the effects observed were preventative rather than therapeutic, since optimum effects in rodent tumour models were obtained when treatment was started soon after inoculation of malignant cells.

DFMO exhibited contragestational activity in mice, rats and chicks (Fozard et al, 1980) and this compound was also found to be effective at inhibiting growth of three yeast species that are human pathogens (Pfaller, Gerarden and Riley, 1987). The lack of effect of DFMO on growth of bacteria is probably because these organisms have an alternative route for putrescine synthesis via ADC activity. Indeed, ADC activity was induced when ODC activity was inhibited in *Pseudomonas aeruginosa* by DFMO (Kallio and McCann, 1981).

DFMO has been used successfully against some parasite infections. Thus, inhibitory effects of DFMO on coccidiosis in chicks were reported by Hanson *et al* (1982) and *in vitro* replication of the malaria parasite was restricted by the compound (Sjoerdsma and Schechter, 1984). Indeed, the most exciting results have been obtained from research on the role of polyamines in protozoal growth. On finding that DFMO totally cured infections by the African trypanosome *Trypanosoma brucei brucei* in mice (Bacchi *et al*, 1980), the compound was used on potentially fatal cases of late-stage human sleeping sickness (*T.b. gambiense*) in Africa (Sjoerdsma and Schechter, 1984). DFMO is the only effective drug introduced for this disease in the last 40 years (Schechter, Barlow and Sjoerdsma, 1987). Moreover, it demonstrates low toxicity, even when administered in high dosage (Sjoerdsma and Schechter, 1984). The *in vivo* effects of DFMO were reversible by the addition of putrescine, spermidine or spermine (Nathan *et al*, 1981), indicating that the likely mode of action of the compound is via inhibition of the polyamine pathway.

The differences observed between the effects of DFMO on cell proliferation in humans and trypanosomes is surprising since the *T.b. brucei* enzyme and mammalian ODC exhibit similar sensitivities to DFMO and the inhibitor enters both systems by passive diffusion and not by facilitated transport (Bacchi and McCann, 1987). However, a likely target for the efforts of DFMO, unique to trypanosome infections, is the presence of a spermidine-

containing cofactor trypanothione, which is obligatory for glutathione reductase activity. Indeed, some workers have found that spermidine depletion, due to the presence of DFMO, greatly reduces the amount of trypanothione in *T.b. brucei* (Fairlamb et al, 1987). Moreover, trypanosomes may be more dependent on polyamines than mammalian cells because of their rapid doubling time in the blood stream (Bitonti et al, 1985). Giffin et al (1986 b) found that the primary effect of DFMO on cultures of *T.b. brucei* was a depletion of putrescine and a reduction in spermidine, resulting in inhibition of cellular division probably at nucleic acid synthesis level (Bacchi et al, 1983). Giffin and colleagues (1986 b) suggested that DFMO was exerting a cytostatic rather than a cytotoxic effect on *T.b. brucei*. Results obtained by Giffin, McCann and Bacchi (1986 a) indicated that reduced oxygen consumption of trypanosomes cultured in the presence of DFMO was probably due to putrescine depletion thus supporting the idea that the compound's antitrypanosomal action is as a result of inhibition of polyamine biosynthesis. Interestingly, another parasitic protozoan *Pneumocystis carinii*, the most common cause of death in patients with acquired immune deficiency syndrome (AIDS), has shown a favourable clinical response when treated with DFMO (Schechter, Barlow and Sjoerdsma, 1987).

Janne et al (1985) suggested that by using an inhibitor which would block SAMDC activity, in conjunction with ODC inhibitors, complete depletion of putrescine, spermidine and spermine in mammalian cells should be

possible. Indeed, significant studies have been carried out, using DFMO combined with an inhibitor of SAMDC, on patients with primary recurrent malignant brain tumour (Schechter, Barlow and Sjoerdsma, 1987). Two such SAMDC inhibitors are methylglyoxal bis(guanyldrazone) (MGBG) and ethylmethylglyoxal bis(guanyldrazone) (EMGBG). Using MGBG as an anti-tumour agent, Williams-Ashman and Schenone (1972) discovered that the compound was a very competitive inhibitor of SAMDC *in vitro*, blocking the formation of spermidine and spermine.

Since most fungi appear to possess one route for polyamine biosynthesis, while plants synthesise these amines using two pathways (see Section 1.2), specific inhibitors of polyamine biosynthesis may have potential for use against phytopathogenic fungi. Indeed, work over the past few years has shown that DFMO can give very good control of infection by rust and powdery mildew fungi, which are biotrophic pathogens (eg. Rajam, Weinstein and Galston, 1985; Walters, 1986; West and Walters, 1988). Necrotrophic fungal pathogens, on the other hand, were much less responsive to DFMO and considerable variability between different species was observed (Rajam and Galston, 1985; West and Walters, 1989). However, nothing is known about the effect of other inhibitors of polyamine biosynthesis (eg. MGBG) in rust infection. Thus, the effects of DFMO, MGBG and exogenous polyamines, on infection of wheat by the biotrophic pathogen *Puccinia graminis* were examined. Since rust is notoriously difficult to grow in culture and moreover, these fungi are

very sensitive to polyamine biosynthesis inhibitors, it is very difficult to obtain fungal material for biochemical analysis from inhibitor experiments with rusts. Thus, a fungal pathogen of some importance, which was also cultured with ease and also exhibited some sensitivity to inhibitors of polyamine biosynthesis, was chosen for detailed biochemical examinations. Therefore, a detailed study of the effects of DFMO, MGBG, EMGBG and exogenous polyamines on mycelial growth, polyamine metabolism and respiration rates in the oat pathogen *Pyrenophora avenae* grown *in vitro* was also carried out. Carnitine was used in the presence of MGBG to assess its effectiveness against mitochondrial damage in *P. avenae* as suggested by Nikula *et al* (1984).

4.2 Materials and Methods

4.21 Growth and maintenance of the pathogen *Pyrenophora avenae*

Stock cultures of *Pyrenophora avenae* Ito and Kuribayashi Apud Ito⁺, obtained from the Commonwealth Mycological Institute, Kew, UK were maintained on potato dextrose agar (PDA, Oxoid) in 90 mm sterile plastic Petri dishes. The cultures were maintained at 24°C in the dark. Approximately every 10 days a section of mycelium was removed from the advancing edge of the culture, inverted and placed on fresh PDA plates. For experimental use, mycelial plugs were removed from the advancing edge of 6 day old stock cultures.

⁺Culture No. 296817

4.22 Growth, enzyme and polyamine analysis of *Pyrenophora avenae* grown on inhibitor-amended media

4.221 Growth of *Pyrenophora avenae* on solid media

Ten ml of filter-sterilised solution containing the inhibitor were added to 140 ml of sterile PDA at 45-47°C to obtain final concentrations of 0.1-2.0 mM. Putrescine or spermidine were added to obtain final concentrations of 0.1 mM to determine if they reversed inhibition by DFMO (1.0 mM), MGBG (0.5 mM), EMGBG (0.1 mM) or a mixture containing DFMO (0.5 mM) and MGBG (0.5 mM). Filter sterilisation was carried out using Sartorius Minisart filters (pore size 0.2 µm). Control plates contained culture medium only. Twenty ml of sterile medium containing the inhibitor were added aseptically to each 90 mm single vent sterile plastic Petri dish. To obtain inoculum, a sterile 10 mm diameter cork borer was used to remove plugs of mycelium from the peripheral edge of stock cultures. The mycelial plugs were inverted and placed in the centre of each Petri dish. Inoculated plates were incubated in the dark at 24°C.

Colony diameters were measured in millimetres, excluding the 10 mm plug of inoculum, 1 - 6 days after inoculation. Three measurements were made from each Petri dish, the mean of which represented colony growth for that particular dish. All results are the means of six replicates. Lengths and diameters of cells were measured using a calibrated micrometer 6 days after inoculation. Mycelium was scraped from the Petri dishes and stained

using lactophenol cotton blue. Standard errors were calculated and differences between means were analysed for significance using the Student's *t*-test (see Section 2.25). Standard errors and significance values are given in Appendix IV.

4.222 Growth of *Pyrenophora avenae* in liquid media

Ten ml of filter-sterilised solution containing the inhibitor were added to 140 ml of sterile liquid PDA, in 250 ml flasks, to obtain the following concentrations: 1.0 mM DFMO; 0.1 mM MGBG; 0.1 mM EMGBG; 0.1 mM DFMO/MGBG combined; 0.1 mM putrescine; 0.1 mM spermidine. 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 volume was adjusted to 1 litre (Commonwealth Mycological Institute, 1983).

Each flask was inoculated with a 10 mm disc of mycelium and placed in a Gallenkamp orbital shaker at 140 rpm, 24°C, with a mean irradiance of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After 4 days the fungus was washed with distilled water through a 250 μm mesh sieve (Henry Simon) and centrifuged at 16,000 g for 10 minutes. The pellet obtained was used for enzyme and polyamine analysis.

When measuring respiration rates, carnitine was added to each inhibitor-amended flask (concentrations as described above, except DFMO 0.1 mM) to obtain final

concentrations of 1.0 mM.

4.223 Enzyme assays

Crude enzyme extracts were prepared by grinding 500 mg fungus with 1 ml buffer using a pre-chilled pestle and mortar. The buffer used (as detailed by Stevens, McKinnon and Winther, 1976) 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). The suspensions were sonicated using a Soniprep 150 for 10 cycles of 20 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°C.

For ODC assays, the supernatant (cytosolic fraction) was dialysed against 30 volumes of buffer for 24 hours in the dark at 4°C, using dialysis tubing with a molecular weight cut off of 12,000. The pellet (nuclear fraction) was redissolved in the original volume of buffer and dialysed as described for the cytosolic fraction above.

For SAMDC assays, 430 mg of ammonium sulphate was added per millilitre of supernatant and redissolved pellet (cytosolic and nuclear fractions respectively). The suspensions were centrifuged at 24,000 g for 20 minutes at 0°C. The pellets obtained were redissolved in the original volume of buffer and dialysed as described for ODC assays above.

Enzyme activities were assayed by measuring the $^{14}\text{CO}_2$ released after incubation with $[1-^{14}\text{C}]$ ornithine and S-adenosyl- $[1-^{14}\text{C}]$ methionine for ODC and SAMDC respectively. Radioisotopes were obtained from Amersham International plc. The reaction mixtures used were as described by Stevens, McKinnon and Winther (1976). For ODC assays the reaction mixture consisted of 50 mM Tris/HCl, pH 8.0, 0.05 mM L-ornithine monohydrochloride (58 mCi/mmol) and 0.1 ml of enzyme extract in a total volume of 0.4 ml. For SAMDC assays the reaction mixture contained 0.1 M sodium phosphate, pH 7.4, 0.2 mM S-adenosyl-L-methionine, 1.0 mM putrescine, 0.025 μCi of S-adenosyl-L- $[1-^{14}\text{C}]$ methionine (45 mCi/mmol) and 0.1 ml of enzyme extract in a total volume of 0.4 ml.

Assays were carried out as described in Section 2.231. All results are the means of five replicates. Standard errors were calculated and differences between means were analysed for significance using the Student's *t*-test (see Section 2.25).

4.224 Polyamine analysis

600 mg of *P. avenae* was macerated with 1 ml 4% (v/v) perchloric acid using a chilled pestle and mortar. The samples were sonicated as described in Section 4.223, then centrifuged at 12,000 g for 25 minutes at 0°C. The analysis was then carried out as described in Section 2.24. All results are the means of four replicates. Standard errors were calculated and differences between

means were analysed for significance using the Student's *t*-test (see Section 2.25).

4.225 Respiration rate measurements

500 mg of *P. avenae* in 5 ml of fresh liquid PDA was placed in a Rank oxygen electrode at 24°C. Each sample was aerated to 100% before measuring oxygen uptake. Measurements are expressed as percentage change in oxygen compared to control.

4.23 Growth and maintenance of the pathogen *Puccinia graminis*

The growth and maintenance of *P. graminis* is described in Section 2.21.

4.24 Growth and inoculation of plant material with *Puccinia graminis*

Wheat seedlings (*Triticum aestivum* L.cv.Sappo) were grown and inoculated with spores of *P. graminis* as described in Section 2.22.

4.25 Treatment of *Puccinia graminis*-infected wheat seedlings with inhibitors

Solutions of the inhibitors DFMO, MGBG and DFMO/MGBG combined were made up in 0.01% (v/v) Tween 20 and adjusted to pH 7.0 using sodium hydroxide. The inhibitors were applied at concentrations of 0.05, 0.1 and 1.0 mM.

Putrescine or spermidine were added to obtain final concentrations of 0.1 mM to determine if they reversed inhibition by the inhibitors (0.1 mM). A Preval spray unit was used to apply the inhibitors to the seedlings and the solutions were sprayed to run-off. Control plants were sprayed with 0.01% (v/v) Tween 20 only. For pre-inoculation treatments, the seedlings were sprayed with solutions of the inhibitors, left to dry for approximately 2 hours and then inoculated with *P. graminis* spores as described in Section 2.22. In post-inoculation treatments, plants were inoculated with *P. graminis* spores (see Section 2.22) and left for 4 days before applying the inhibitors using the method described above.

The intensity of infection was assessed 11, 13, 15 and 17 days after inoculation by estimating the per cent of leaf area infected, using a standard area diagram, and by counting the number of pustules per centimetre square of leaf area. The dry weights of the first leaves were determined, for pre- and post-inoculatory treatments 17 days after inoculation, using a Gallenkamp Hotbox oven at 70°C. All values are the means of 20 replicates. Standard errors were calculated and differences between means were analysed for significance using the Student's *t*-test (see Section 2.25). However, when data are not in the form of measurements but take the form of proportions or percentages, the following equation must be used to analyse for significant differences between means (Parker, 1979):

$$u = \frac{\frac{SD_1^2}{N_1}}{\frac{SD_1^2}{N_1} + \frac{SD_2^2}{N_2}}$$

Where SD = standard deviation of the sample.

N = number of replicates in a sample.

$$\frac{1}{f} = \frac{u^2}{(N_1 - 1)} + \frac{(1-u)^2}{(N_2 - 1)}$$

f = degrees of freedom.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{SD_1^2}{N_1} + \frac{SD_2^2}{N_2}}}$$

The probability values were obtained using Student's *t*-tables.

4.3 Results

4.31 Mycelial growth of *Pyrenophora avenae* on solid media

The addition of the inhibitors MGBG, DFMO, EMGBG and DFMO/MGBG combined, reduced mycelial growth of *P. avenae*

(Figures 13-16). Although DFMO was less effective than MGBG, it reduced growth of the fungus by 64% when used at 2.0 mM (Figure 13). In contrast, MGBG almost completely inhibited fungal growth at 2.0 mM (Figure 14). Significant reduction of mycelial growth was obtained using EMGBG, which at 0.1 mM reduced growth by about 50% (Figure 16). DFMO/MGBG combined were very effective in controlling mycelial growth, although no more so than MGBG alone. Nevertheless, a 2.0 mM concentration of this mixture resulted in complete inhibition of growth on the first day of measurement, with only a small tuft of mycelium on the following days (Figure 15).

The addition of putrescine at 0.1 mM had little effect on growth, although a slight reduction was observed on days 4 and 5 (Figure 17). 0.1 mM spermidine increased growth only on day 6 and decreased growth on days 3 and 4 (Figure 18). Inhibitory effects of 0.1 mM EMGBG, 0.5 mM MGBG and 0.5 mM DFMO/MGBG combined were not reversed by the addition of either 0.1 mM putrescine or spermidine. However, both putrescine and spermidine reversed inhibition by 1.0 mM DFMO (Figures 17 and 18).

For all treatments, effects of the inhibitor were more pronounced on the 6th day in comparison with measurements made on previous days.

The addition of DFMO/MGBG combined at 0.5 mM and 1.0 mM significantly decreased the cell lengths of mycelia whereas 0.1 mM DFMO significantly increased cell lengths (Table 20). The cell lengths of mycelia grown on 0.5 mM

Figure 13: The effect of DFMO on the mycelial growth of *Pyrenophora avenae*. Values are the means of six replicates.

Figure 14: The effect of MGBG on the mycelial growth of *Pyrenophora avenae*. Values are the means of six replicates.

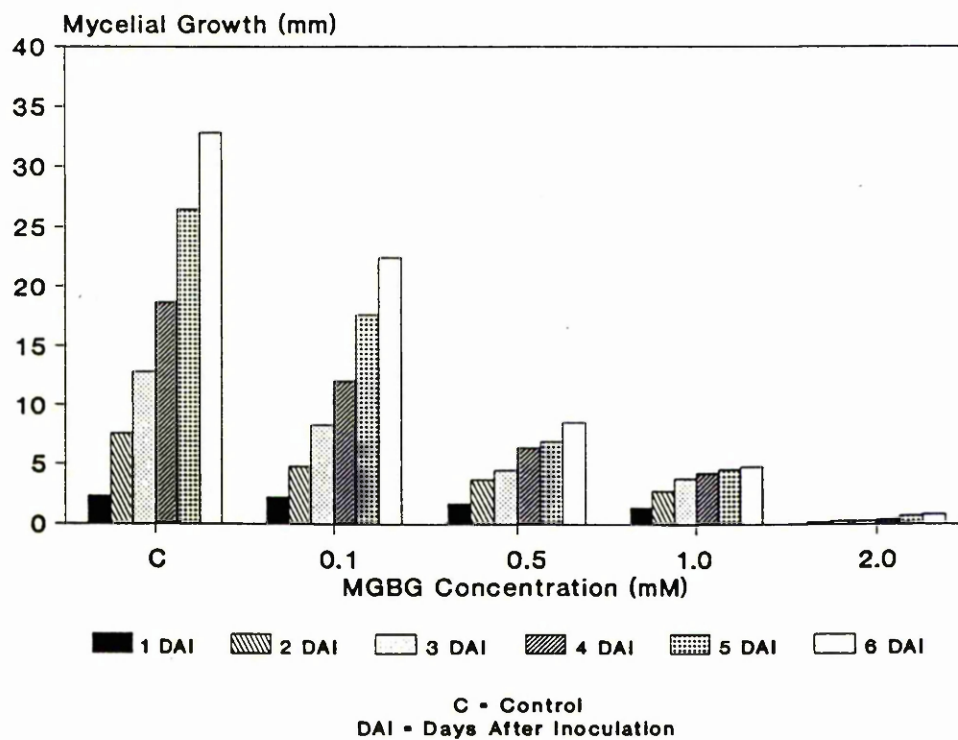
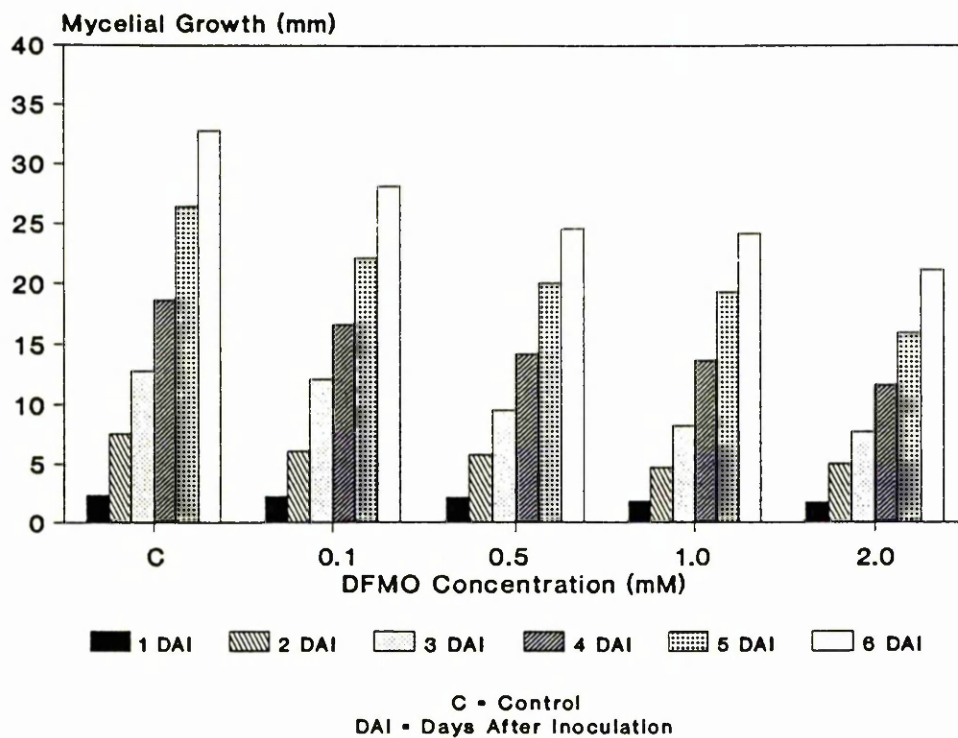


Figure 17: The effect of putrescine, alone or in combination with DFMO, MGBG, DFMO/MGBG and EMGBG, on the mycelial growth of *Pyrenophora avenae*. Values are the means of six replicates.

Figure 18: The effect of spermidine, alone or in combination with DFMO, MGBG, DFMO/MGBG and EMGBG, on the mycelial growth of *Pyrenophora avenae*. Values are the means of six replicates.

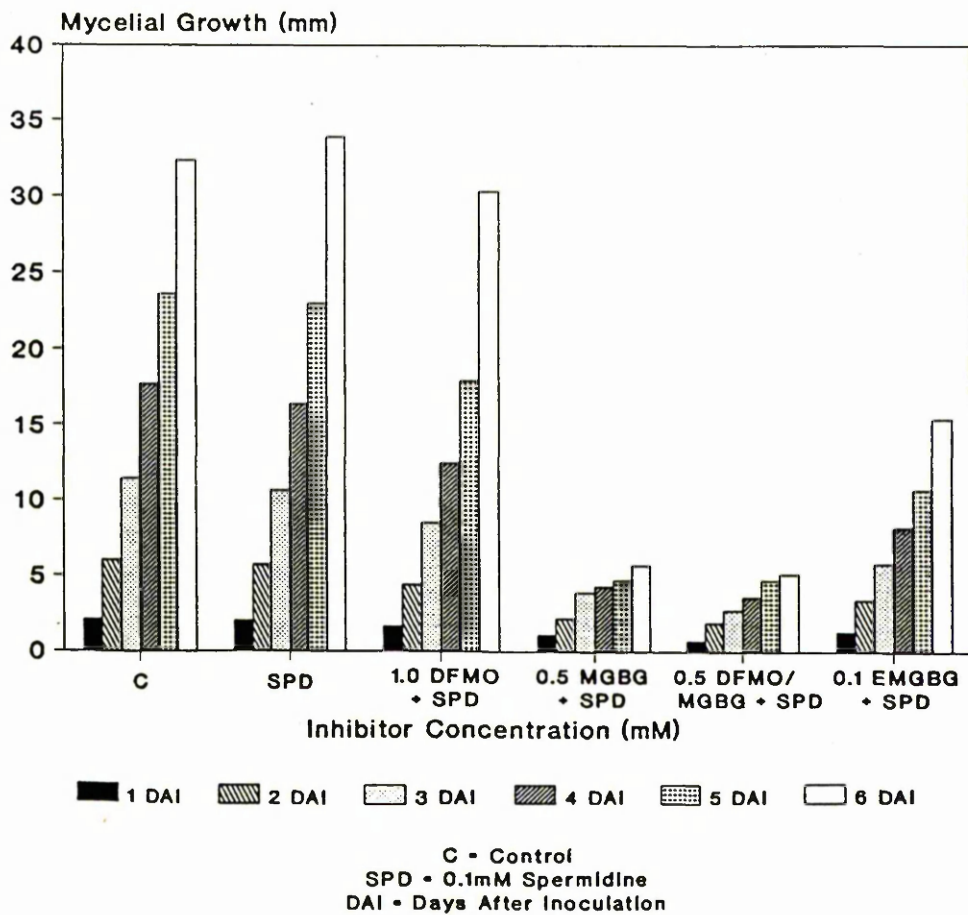
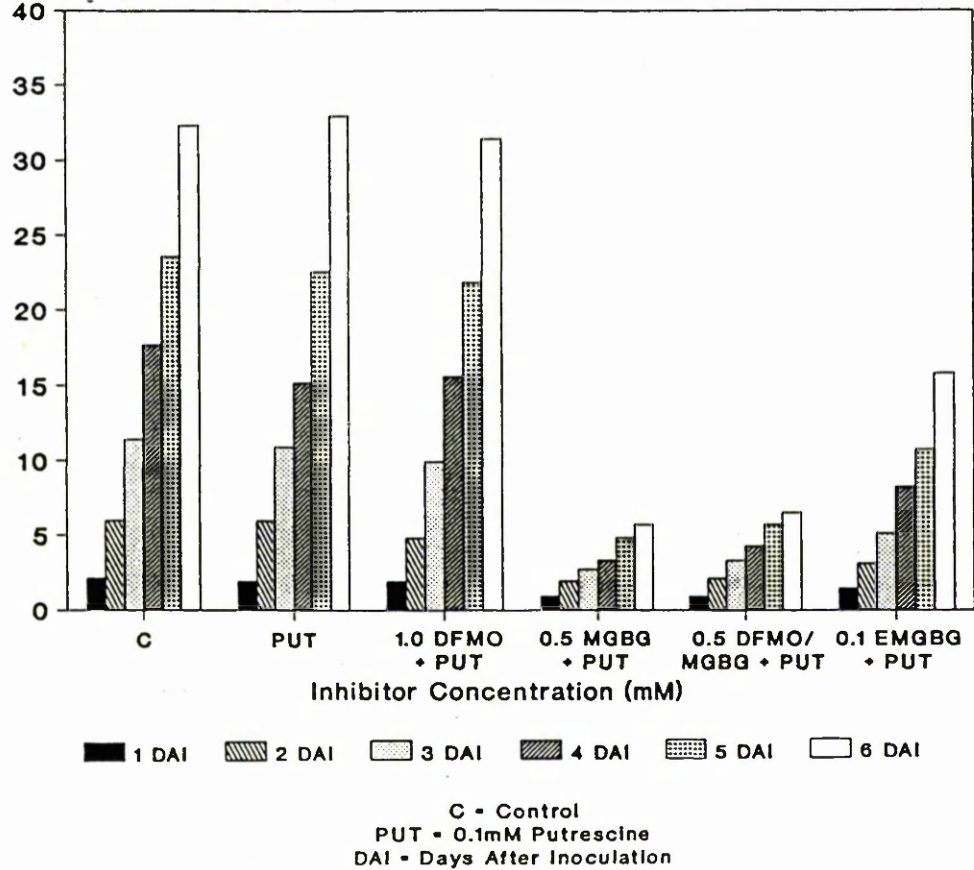


Table 20: Cell length and diameter in μM of *Pyrenophora avenae* following treatment with DFMO, MGBG, EMGBG and DFMO/MGBG combined. Measurements made six days after inoculation. Values represent the means of ten replicates \pm standard error. Significant differences are shown as (c) $P = 0.1$.

ND = Not Determined.

Treatment (mM)	Cell Length (μM)	Cell Diameter (μM)
Control	44.8 ± 3.3	4.2 ± 0.4
DFMO, 0.1	$49.8 \pm 3.3^{\text{c}}$	3.2 ± 0.3
DFMO, 0.5	46.7 ± 6.3	3.2 ± 0.3
DFMO, 1.0	48.3 ± 2.9	2.9 ± 0.3
DFMO, 2.0	36.1 ± 4.5	3.6 ± 0.2
MGBG, 0.1	37.6 ± 3.9	3.6 ± 0.2
MGBG, 0.5	40.3 ± 3.3	3.4 ± 0.3
MGBG, 1.0	36.5 ± 2.3	3.4 ± 0.3
MGBG, 2.0	ND	ND
EMGBG, 0.1	40.7 ± 2.7	3.6 ± 0.2
EMGBG, 0.5	36.1 ± 1.7	2.9 ± 0.3
EMGBG, 1.0	40.7 ± 4.6	3.6 ± 0.2
DFMO/MGBG, 0.1	32.3 ± 3.1	3.2 ± 0.3
DFMO/MGBG, 0.5	$28.5 \pm 3.4^{\text{c}}$	3.6 ± 0.2
DFMO/MGBG, 1.0	$28.8 \pm 3.2^{\text{c}}$	3.4 ± 0.3
DFMO/MGBG, 2.0	ND	ND

DFMO/MGBG combined with 0.1 mM spermidine were also significantly reduced (Table 21). There were no significant differences in cell diameters of mycelia grown on inhibitor and polyamine amended media (Tables 20 and 21).

4.32 Enzyme activities of *Pyrenophora avenae* grown in inhibitor-amended media

The activity of ODC in *P. avenae* grown in media containing MGBG, DFMO and DFMO/MGBG combined, was significantly reduced in both nuclear and cytosolic fractions (Table 22). EMGBG had no effect on ODC activity in the cytosolic fraction although a slight reduction was observed in nuclear ODC activity (Table 22).

SAMDC activity was greatly reduced when *P. avenae* was grown in EMGBG (Table 23). A mixture of DFMO/MGBG had no significant effect on SAMDC activity and MGBG reduced activity only slightly. DFMO significantly increased SAMDC activity (Table 23).

With only a few exceptions, the addition of 0.1 mM putrescine or spermidine did not reverse inhibition of ODC or SAMDC activities and in most cases reduced activity further (Tables 22 and 23).

4.33 Polyamine concentrations in *Pyrenophora avenae* grown in inhibitor-amended media

Putrescine, spermidine, spermine and cadaverine were

Table 21: Cell length and diameter in μM of *Pyrenophora avenae* following treatment with polyamines alone and in combination with inhibitors. PUT represents 0.1 mM putrescine and SPD represents 0.1 mM spermidine. Values are the means of ten replicates \pm standard error. Significant differences are shown as (c) $P = 0.1$.

Treatment (mM)	Cell Length (μM)	Cell Diameter (μM)
Control	44.8 \pm 3.3	4.2 \pm 0.4
PUT	41.4 \pm 3.8	3.9 \pm 0.2
SPD	51.3 \pm 5.8	4.2 \pm 0.3
DFMO, 1.0 + PUT	39.1 \pm 3.9	4.2 \pm 0.3
DFMO, 1.0 + SPD	41.8 \pm 4.8	4.2 \pm 0.3
MGBG, 0.5 + PUT	51.7 \pm 5.8	4.8 \pm 0.8
MGBG, 0.5 + SPD	37.6 \pm 3.1	4.9 \pm 0.5
DFMO/MGBG, 0.5 + PUT	40.7 \pm 5.3	4.2 \pm 0.3
DFMO/MGBG, 0.5 + SPD	34.9 \pm 2.7 ^c	4.2 \pm 0.3

Table 22: Effects of inhibitors on the activity of ornithine decarboxylase in *Pyrenophora avenae*. Concentrations of inhibitors 0.1 mM, except DFMO 1.0 mM. PUT represents 0.1 mM putrescine and SPD represents 0.1 mM spermidine. Values are the means of five replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$; (c) $P = 0.1$.

Treatment	ODC Activity (pmol CO ₂ mg Protein ⁻¹ Hour ⁻¹)	
	Cytosolic	Nuclear
Control	6.14 \pm 1.45	9.53 \pm 1.67
MGBG	2.72 \pm 0.17 ^c	4.54 \pm 0.26 ^b
MGBG + PUT	1.98 \pm 0.08 ^c	3.43 \pm 0.52 ^c
MGBG + SPD	1.93 \pm 0.10 ^c	2.99 \pm 0.55 ^b
DFMO/MGBG	2.82 \pm 0.75 ^c	5.98 \pm 0.54 ^c
DFMO/MGBG + PUT	1.58 \pm 0.09 ^c	3.53 \pm 0.29 ^b
DFMO/MGBG + SPD	1.26 \pm 0.17 ^c	1.53 \pm 0.08 ^a
DFMO	0.93 \pm 0.09 ^b	1.7 \pm 0.20 ^a
DFMO + PUT	0.93 \pm 0.07 ^b	3.6 \pm 0.22 ^b
DFMO + SPD	1.24 \pm 0.10 ^c	2.3 \pm 0.21 ^b
EMGBG	6.0 \pm 0.41	4.41 \pm 0.40 ^b
EMGBG + PUT	3.9 \pm 0.91	3.1 \pm 0.22 ^b
EMGBG + SPD	1.64 \pm 0.20 ^c	1.8 \pm 0.05 ^a

Table 23: Effects of inhibitors on the activity of S-adenosylmethionine decarboxylase in *Pyrenophora avenae*. Concentrations of inhibitors 0.1 mM, except DFMO 1.0 mM. PUT represents 0.1 mM putrescine and SPD represents 0.1 mM spermidine. Values are the means of five replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$; (c) $P = 0.1$.

Treatment	SAMDC Activity (pmol CO ₂ mg Protein ⁻¹ Hour ⁻¹)	
	Cytosolic	Nuclear
Control	8.68 \pm 1.04	8.56 \pm 2.38
MGBG	6.93 \pm 0.58	6.9 \pm 1.17
MGBG + PUT	4.57 \pm 0.20 ^b	4.07 \pm 0.34 ^c
MGBG + SPD	1.08 \pm 0.08 ^a	3.74 \pm 0.43 ^c
DFMO/MGBG	11.8 \pm 1.45	10.62 \pm 1.74
DFMO/MGBG + PUT	4.69 \pm 2.17	5.36 \pm 0.54
DFMO/MGBG + SPD	9.43 \pm 1.56	7.54 \pm 0.46
DFMO	19.35 \pm 1.92 ^a	31.28 \pm 7.18 ^c
DFMO + PUT	15.02 \pm 2.8 ^c	38.84 \pm 1.96 ^a
DFMO + SPD	5.53 \pm 0.22 ^c	15.89 \pm 0.84 ^b
EMGBG	3.65 \pm 0.21 ^a	0.30 \pm 0.07 ^b
EMGBG + PUT	3.09 \pm 0.24 ^a	1.07 \pm 0.15 ^b
EMGBG + SPD	0.48 \pm 0.07 ^a	2.76 \pm 1.57 ^c

detected in *P. avenae* (Table 24). Putrescine, spermidine and cadaverine concentrations were significantly reduced when *P. avenae* was grown in media amended with DFMO and DFMO/MGBG combined (Table 24). EMGBG and MGBG reduced the concentrations of spermidine and cadaverine. Spermine levels decreased in the presence of MGBG.

4.34 Respiration rate of *Pyrenophora avenae* grown in inhibitor-amended media

The respiration rate of *P. avenae* was reduced when grown in media containing MGBG, a mixture of DFMO/MGBG and a mixture of MGBG/carnitine. An increase in respiration was obtained in the presence of DFMO and EMGBG (Table 25).

4.35 Effect of pre- and post-inoculation application of inhibitors on the growth of *Puccinia graminis* on wheat seedlings

Pre-inoculation treatment with DFMO and MGBG, singly and in combination, significantly reduced the percentage of leaf area infected with *P. graminis* and the number of pustules per centimetre square of leaf (Figures 19-24). 1.0 mM DFMO, 0.1 mM MGBG and 1.0 mM DFMO/MGBG almost completely eliminated *P. graminis* infection. However, the 1.0 mM MGBG treatment was less effective than the 0.1 mM MGBG treatment (Figures 21 and 22). Similarly, the 0.1 mM DFMO/MGBG treatment was less effective than the 0.05 mM DFMO/MGBG spray (Figures 23 and 24).

With the exception of 0.1 mM MGBG, 11 days after

Table 24: Effect of inhibitors on the polyamine concentrations in *Pyrenophora avenae*. Concentrations of inhibitors 0.1 mM, except DFMO 1.0 mM. Values are the means of four replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$; (c) $P = 0.1$.

Treatment	Polyamine Concentration (nmol g Fresh Weight ⁻¹)			
	Putrescine	Spermidine	Spermine	Cadaverine
Control	72.4 \pm 1.5	182.5 \pm 7.4	105.3 \pm 7.8	76.2 \pm 2.0
MGBG	72.4 \pm 2.1	125.5 \pm 6.3 ^a	47.8 \pm 3.3 ^a	38.1 \pm 1.3 ^a
DFMO/MGBG	31.0 \pm 4.1 ^a	45.6 \pm 2.2 ^a	95.7 \pm 13.4	38.1 \pm 3.3 ^a
DFMO	51.7 \pm 3.1 ^a	56.9 \pm 1.9 ^a	105.3 \pm 4.5	38.1 \pm 5.4 ^a
EMGBG	91.0 \pm 6.8 ^c	57.0 \pm 1.7 ^a	86.2 \pm 10.1	45.7 \pm 9.2 ^b

Table 25: Effect of inhibitors and carnitine on the respiration rate in *Pyrenophora avenae*. Concentrations of inhibitors and carnitine 0.1 mM and 1.0 mM respectively. Values are expressed as a percentage of the control (100 %).

Treatment	Respiration Rate
	(mg oxygen l ⁻¹ min ⁻¹)
Control	100
Carnitine	117
DFMO	107
DFMO + Carnitine	115
MGBG	92
MGBG + Carnitine	73
DFMO/MGBG	92
DFMO/MGBG + Carnitine	97
EMGBG	132
EMGBG + Carnitine	137

Figure 19: The effect of pre-inoculation treatment with DFMO on the per cent of leaf area infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as $P = 0.001$ ***.

Figure 20: The effect of pre-inoculation treatment with DFMO on the number of pustules per centimetre square of leaf infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as follows: $P = 0.01$ **; $P = 0.001$ ***.

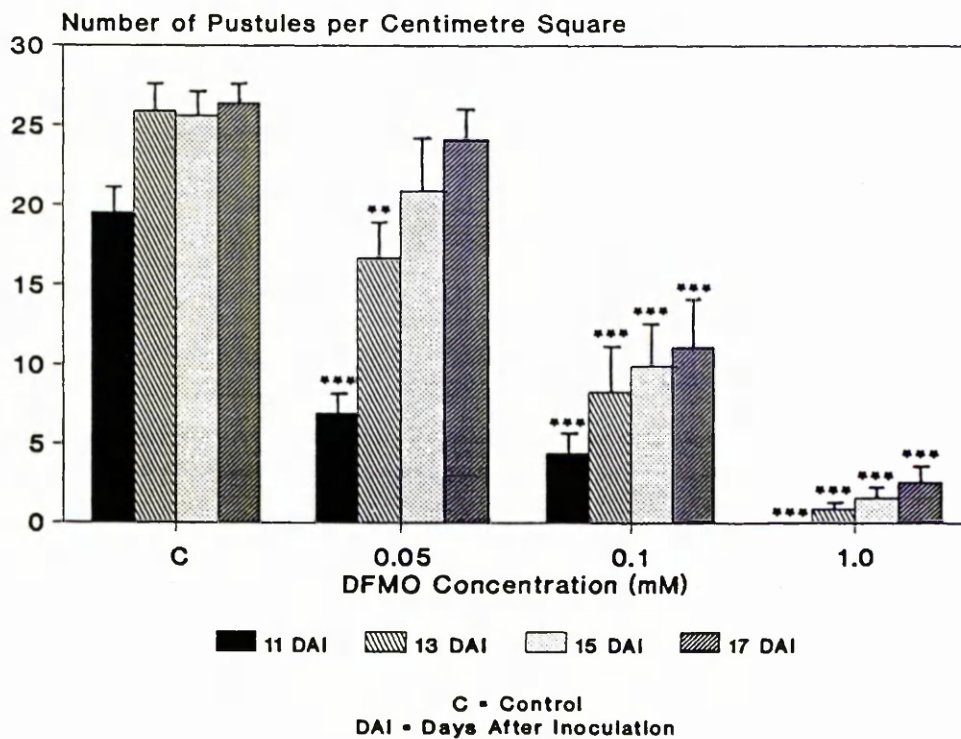
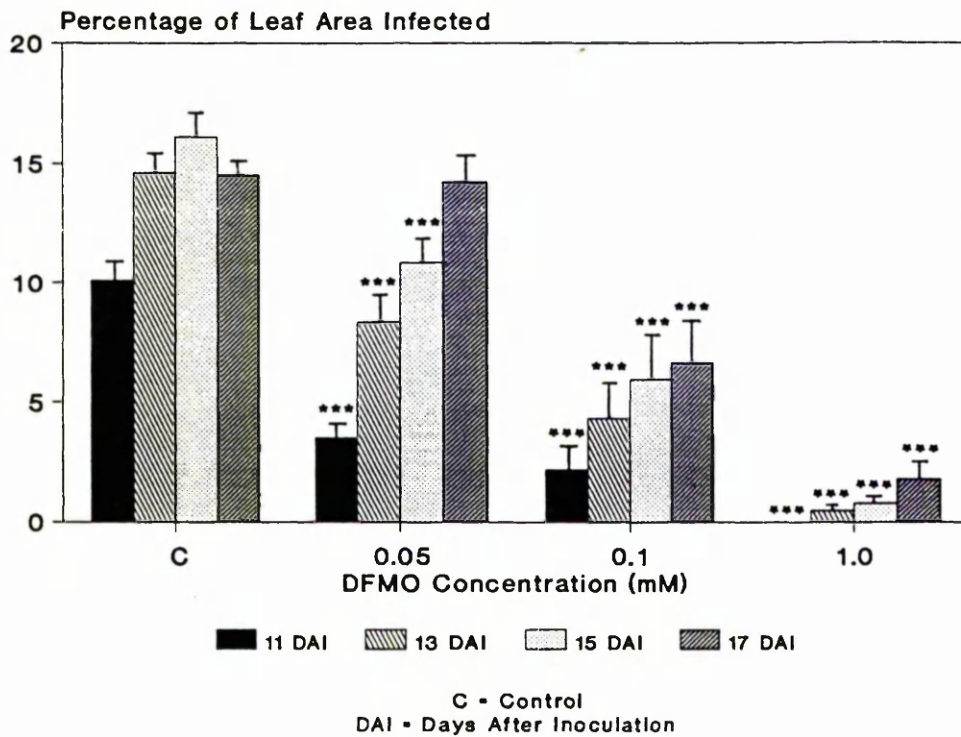


Figure 21: The effect of pre-inoculation treatment with MGBG on the per cent of leaf area infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as $P = 0.001$ ***.

Figure 22: The effect of pre-inoculation treatment with MGBG on the number of pustules per centimetre square of leaf infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as $P = 0.001$ ***.

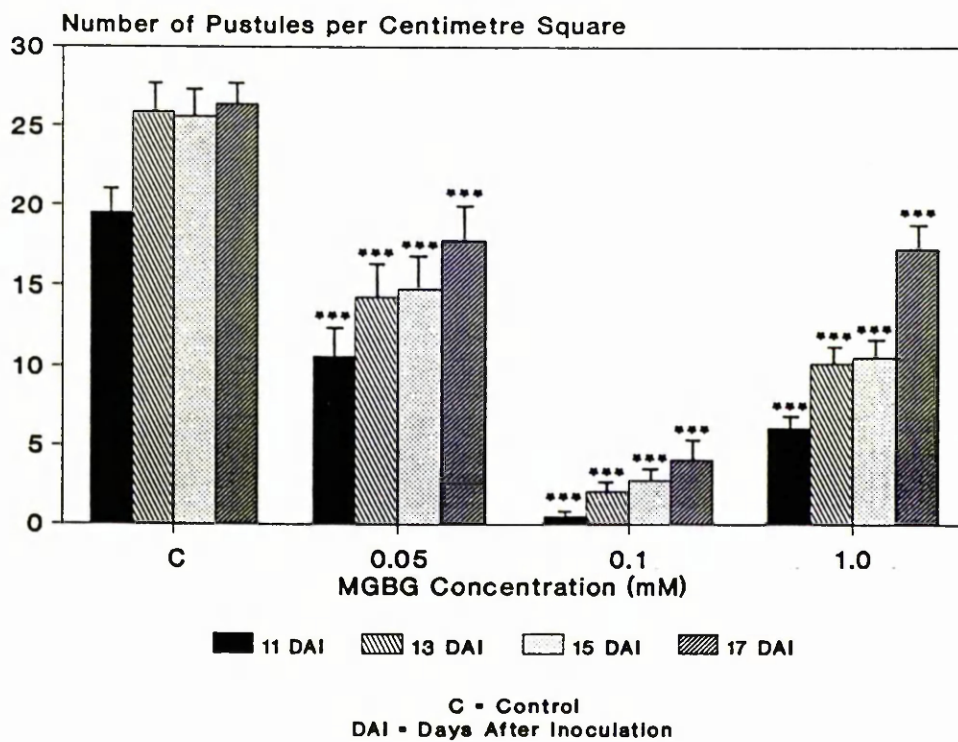
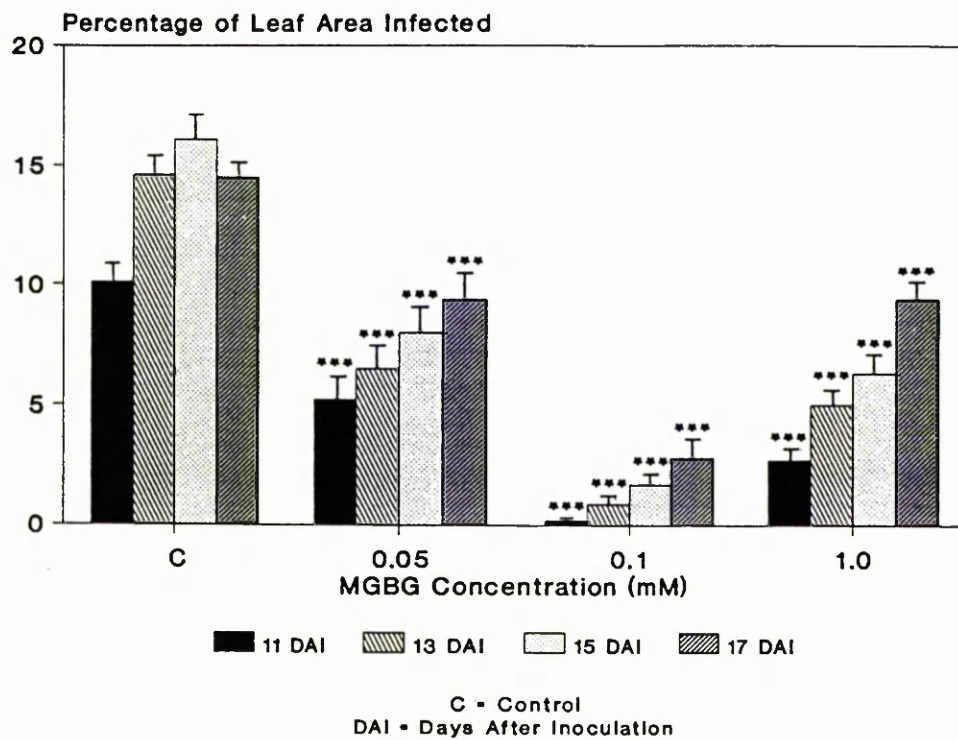
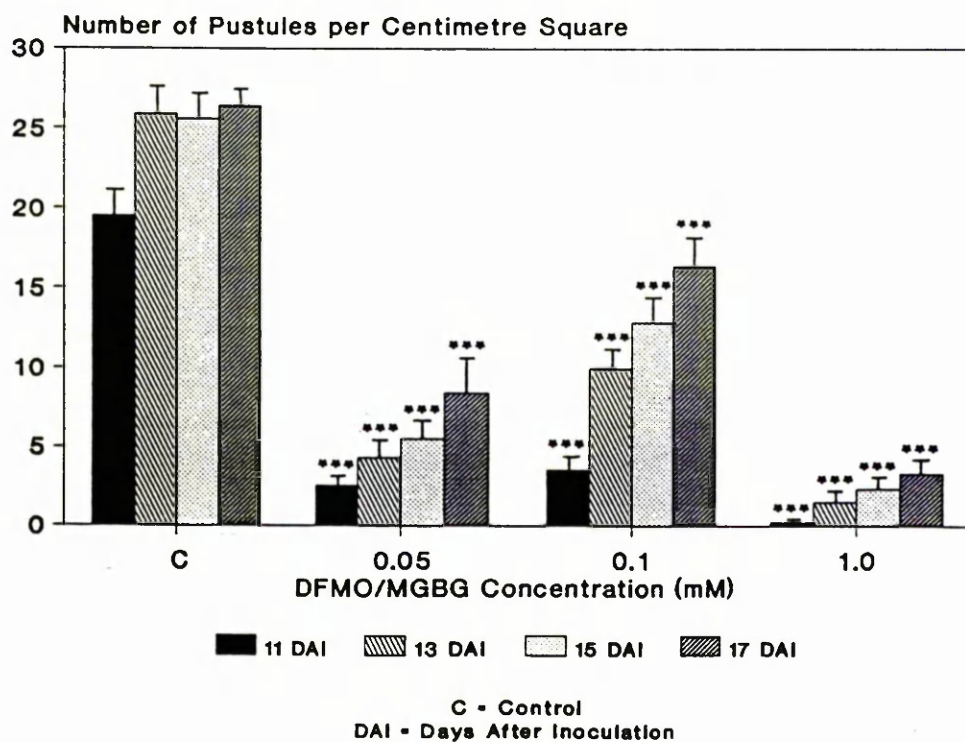
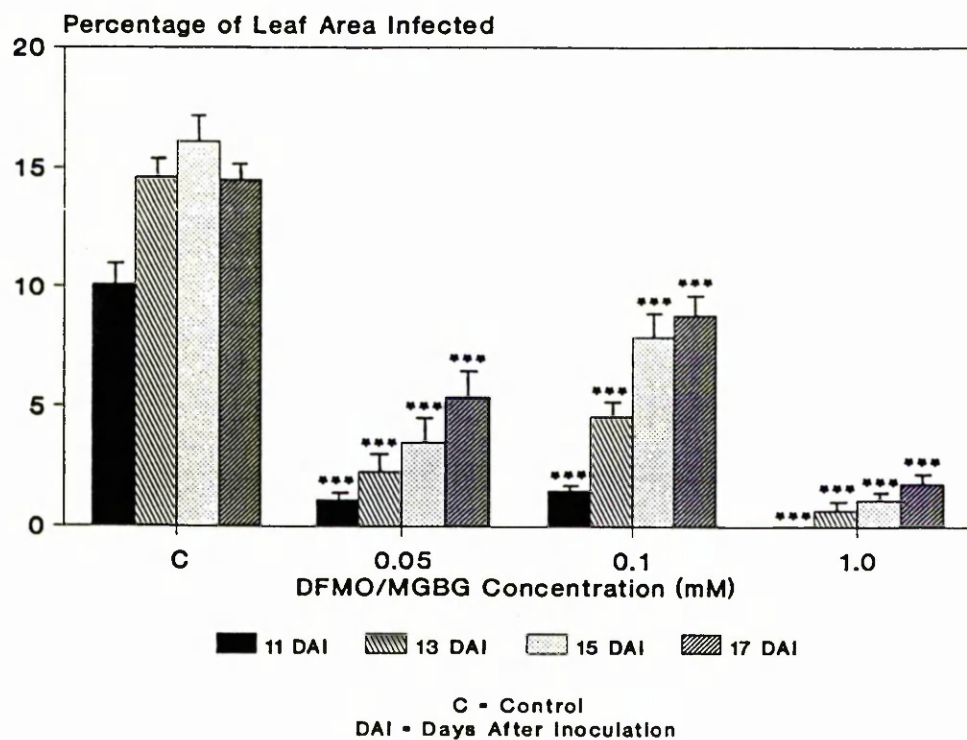


Figure 23: The effect of pre-inoculation treatment with DFMO/MGBG combined on the per cent of leaf area infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as $P = 0.001$ ***.

Figure 24: The effect of pre-inoculation treatment with DFMO/MGBG combined on the number of pustules per centimetre square of leaf infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as $P = 0.001$ ***.



inoculation, post-inoculation treatment with DFMO, MGBG and DFMO/MGBG combined significantly reduced the percentage of leaf area infected with *P. graminis* (Figures 25, 27 and 29). The number of pustules per centimetre square of leaf area was significantly reduced in the presence of DFMO and DFMO/MGBG combined, with the exception of 0.05 mM DFMO, 17 days after inoculation (Figures 26 and 30). Although the effects of post-inoculation treatment with MGBG on the number of pustules per centimetre square of leaf area varied, consistently significant reductions were observed at concentrations of 1.0 mM (Figure 28). Post-inoculation applications of DFMO were more effective at controlling infection by *P. graminis* than MGBG, whereas DFMO/MGBG combined almost completely inhibited fungal infection.

The pre-inoculation treatments with 1.0 mM DFMO, 0.1 mM MGBG and 0.05 mM and 0.1 mM DFMO/MGBG were more effective at reducing the percentage of leaf area infected with *P. graminis* and the number of pustules per centimetre square of leaf area than post-inoculation treatments. However, in general, post-inoculation treatments with 0.05 mM and 0.1 mM DFMO, 0.05 mM and 1.0 mM MGBG and 1.0 mM DFMO/MGBG were more effective, than pre-inoculatory sprays.

4.36 Sprays of inhibitors plus polyamines

The addition of putrescine at 0.1 mM to pre-inoculation applications of 0.1 mM DFMO and 0.1 mM DFMO/MGBG treatments substantially reduced the effects of

Figure 25: The effect of post-inoculation treatment with DFMO on the per cent of leaf area infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as follows: $P = 0.01$ **; $P = 0.001$ ***.

Figure 26: The effect of post-inoculation treatment with DFMO on the number of pustules per centimetre square of leaf infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as: $P = 0.1$ *; $P = 0.01$ **; $P = 0.001$ ***.

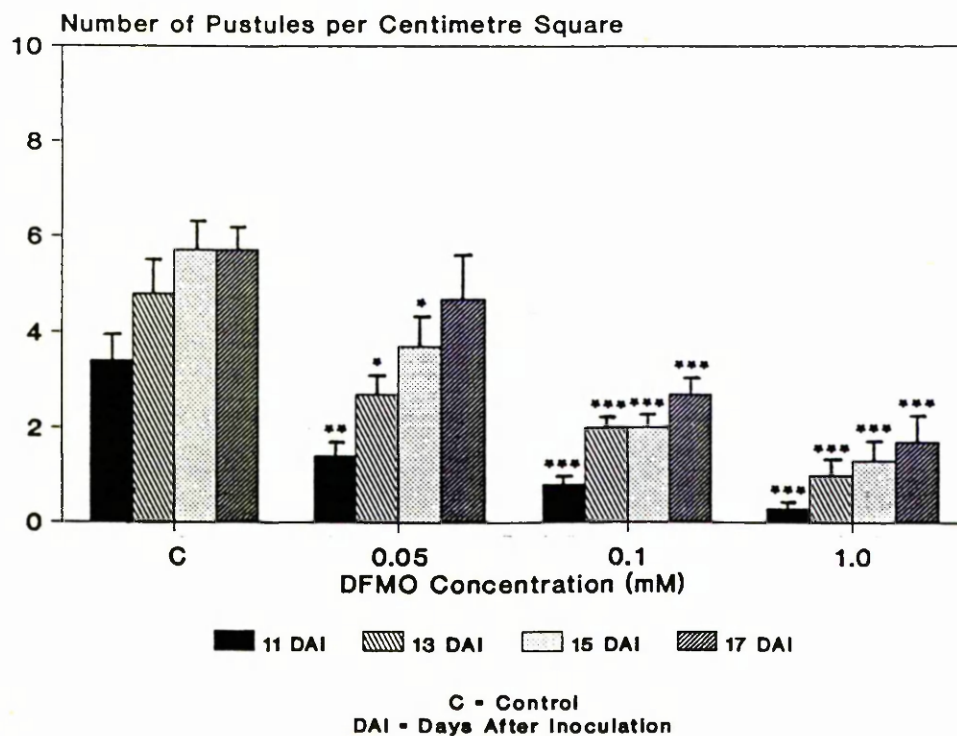
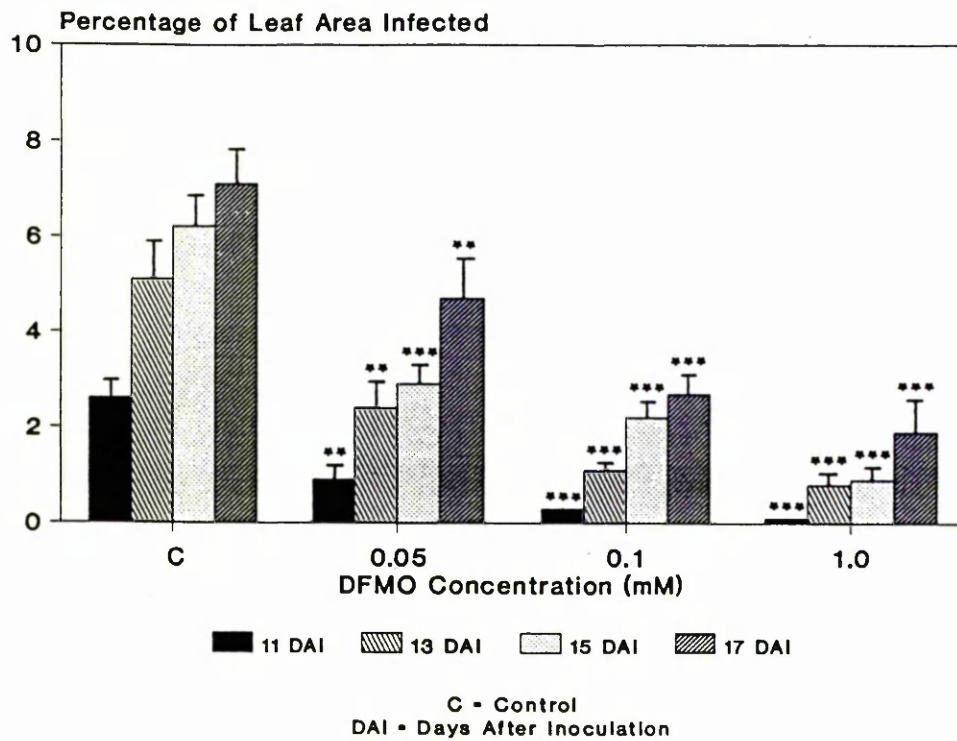


Figure 27: The effect of post-inoculation treatment with MGBG on the per cent of leaf area infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as follows: P = 0.1 *; P = 0.01 **; P = 0.001 ***.

Figure 28: The effect of post-inoculation treatment with MGBG on the number of pustules per centimetre square of leaf infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as follows: P = 0.1 *; P = 0.01 **; P = 0.001 ***.

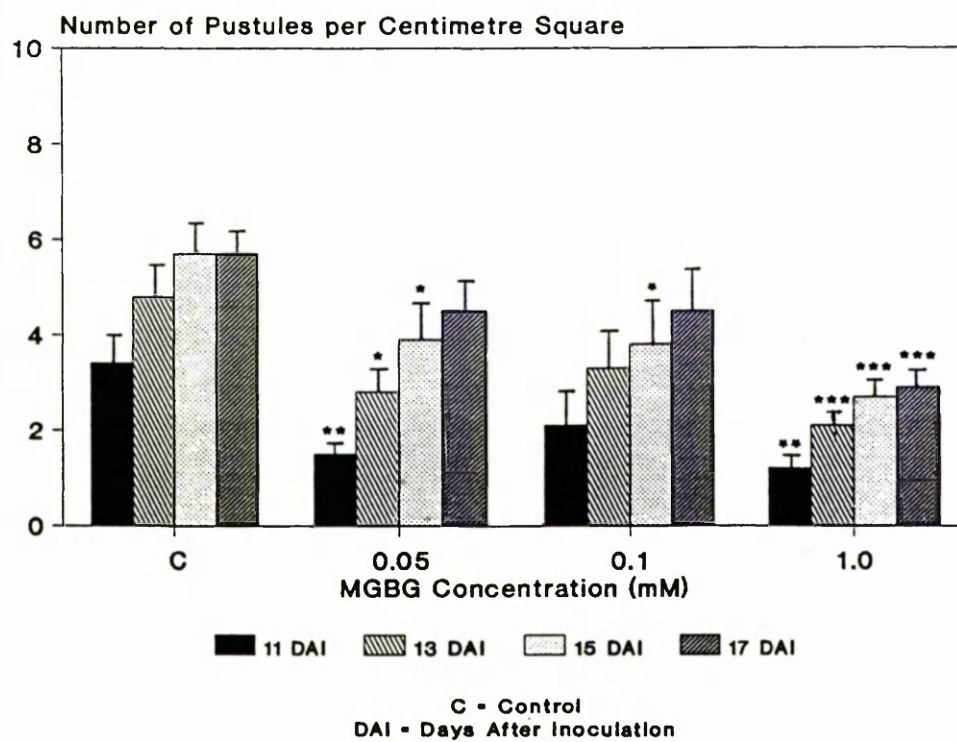
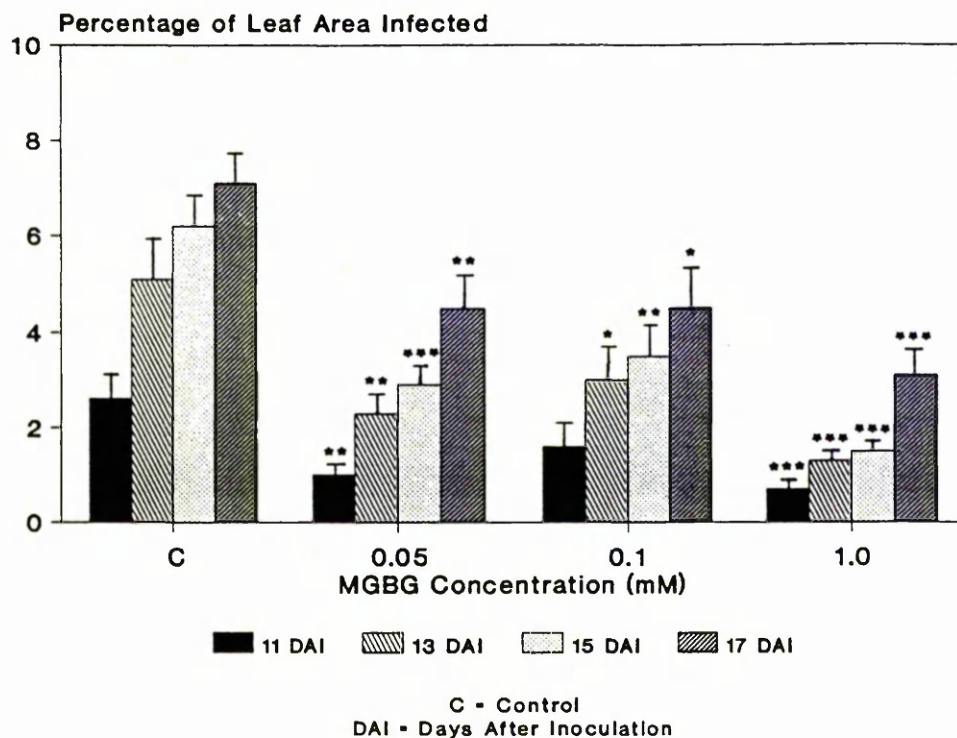
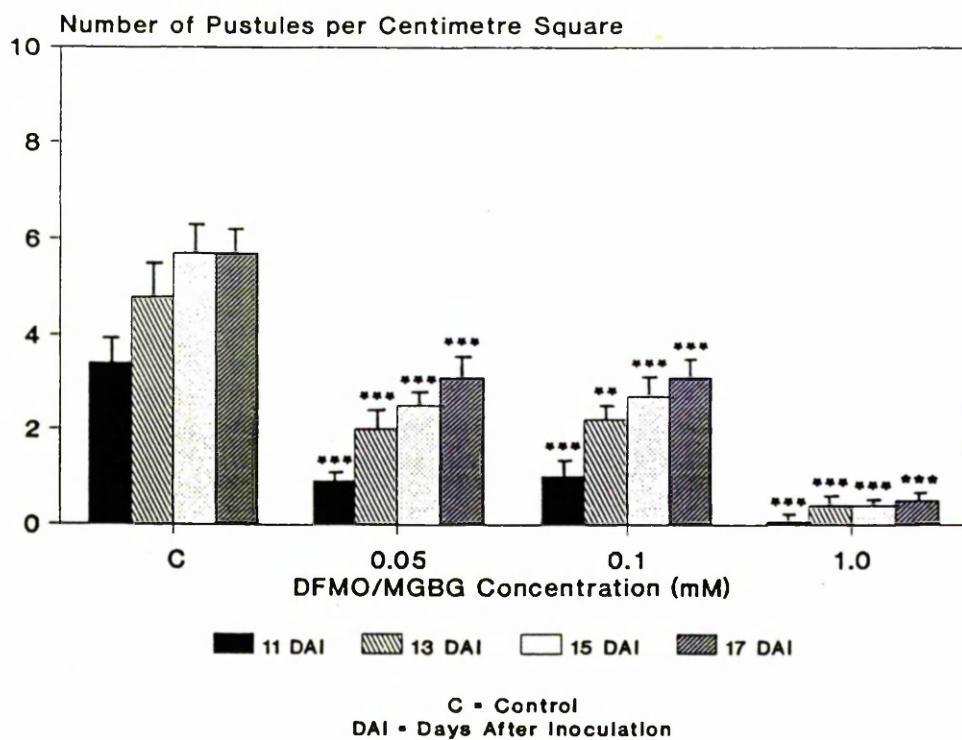
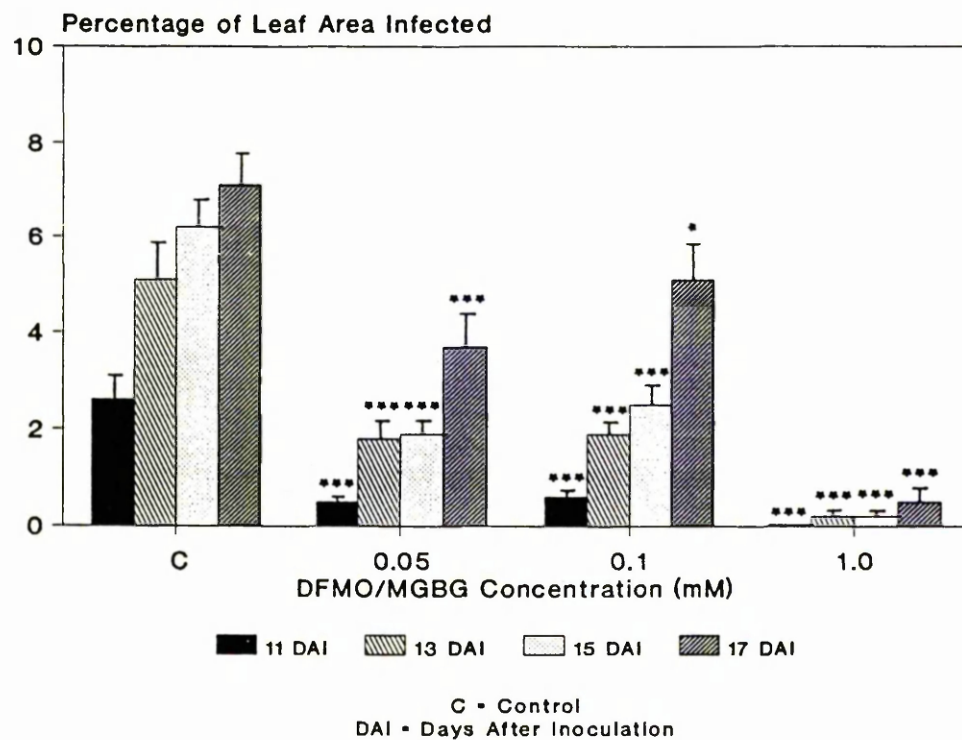


Figure 29: The effect of post-inoculation treatment with DFMO/MGBG combined on the per cent of leaf area infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as follows: P = 0.1 *; P = 0.001 ***.

Figure 30: The effect of post-inoculation treatment with DFMO/MGBG combined on the number of pustules per centimetre square of leaf infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as follows: P = 0.01 **; P = 0.001 ***.



the inhibitors. However, complete reversal of inhibition by pre-inoculation application of 0.1 mM MGBG was observed in the presence of 0.1 mM putrescine (Figures 31 and 32). Similarly, 0.1 mM spermidine partially reversed inhibition by pre-inoculation applications of 0.1 mM DFMO and 0.1 mM DFMO/MGBG and completely reversed inhibitory effects of 0.1 mM MGBG (Figures 33 and 34).

Although the addition of putrescine at 0.1 mM reduced the effects of post-inoculation application of the inhibitors (Figures 35 and 36), 0.1 mM spermidine almost completely reversed inhibitor effects and even increased infection by the fungus in some instances (Figures 37 and 38).

4.37 The effects of pre- and post-inoculation treatment with inhibitors on leaf dry weight

In general, leaf dry weight decreased in plants exposed to both pre- and post-inoculation application of inhibitors, with the largest reduction usually occurring at the higher concentrations of inhibitors (Tables 26 and 27). The addition of polyamines to the inhibitors reversed this effect with the exception of pre-inoculation application of 0.1 mM DFMO/MGBG with 0.1 mM spermidine (Table 26) and post-inoculation application of 0.1 mM MGBG with 0.1 mM putrescine (Table 27).

4.4 Discussion

The application of inhibitors substantially reduced

Figure 31: The effect of pre-inoculation treatment with putrescine in combination with DFMO, MGBG and DFMO/MGBG on the per cent of leaf area infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as follows: P = 0.1 *; P = 0.01 **; P = 0.001 ***.

Figure 32: The effect of pre-inoculation treatment with putrescine in combination with DFMO, MGBG and DFMO/MGBG on the number of pustules per centimetre square of leaf infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as follows: P = 0.1 *; P = 0.01 **; P = 0.001 ***.

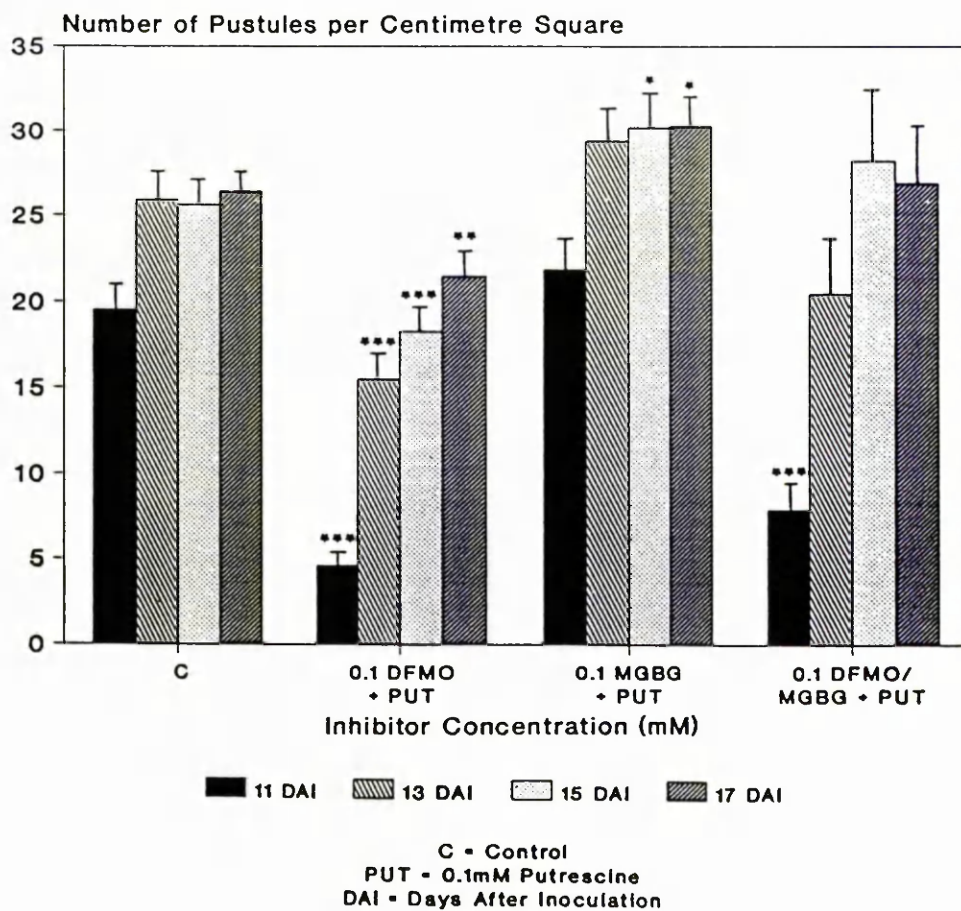
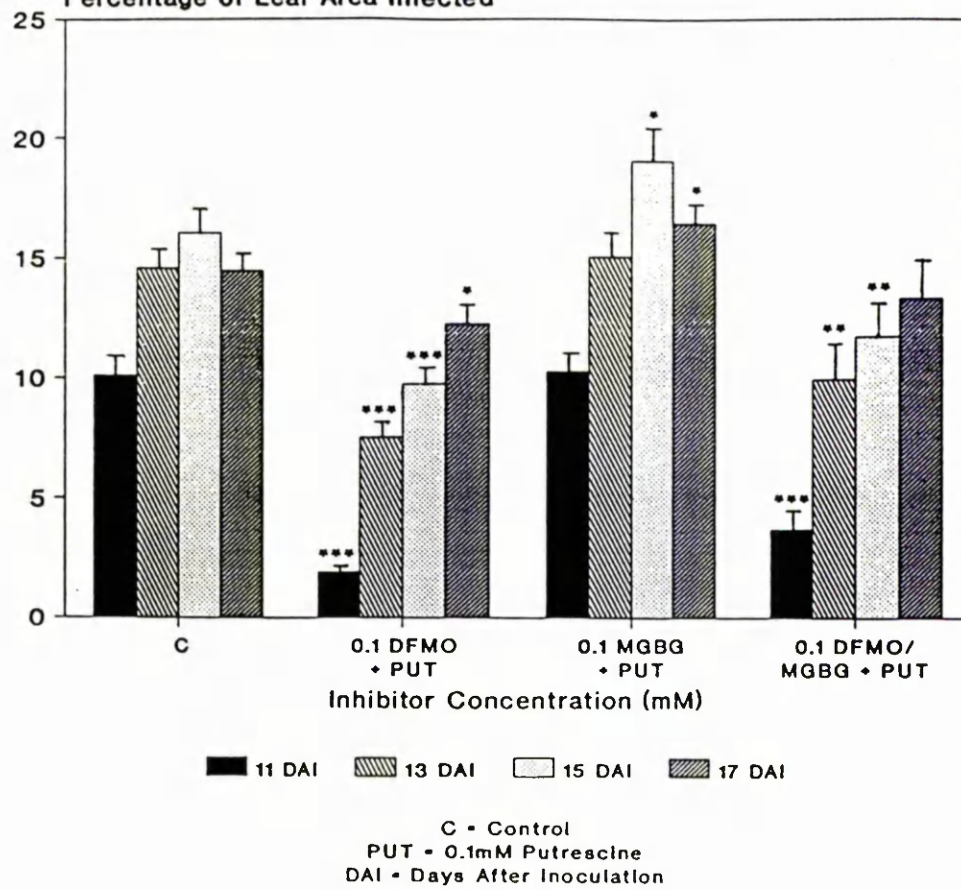


Figure 33: The effect of pre-inoculation treatment with spermidine in combination with DFMO, MGBG and DFMO/MGBG on the per cent of leaf area infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as follows: P = 0.1 *; P = 0.01 **; P = 0.001 ***.

Figure 34: The effect of pre-inoculation treatment with spermidine in combination with DFMO, MGBG and DFMO/MGBG on the number of pustules per centimetre square of leaf infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as follows: P = 0.1 *; P = 0.01 **; P = 0.001 ***.

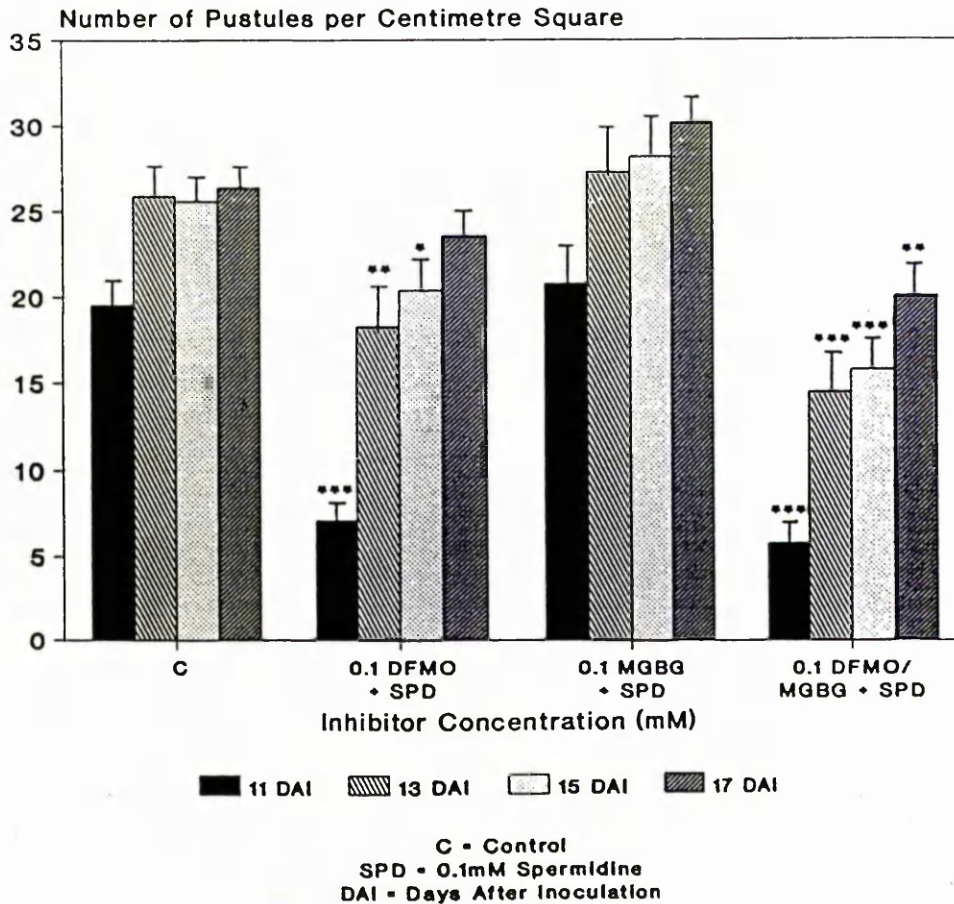
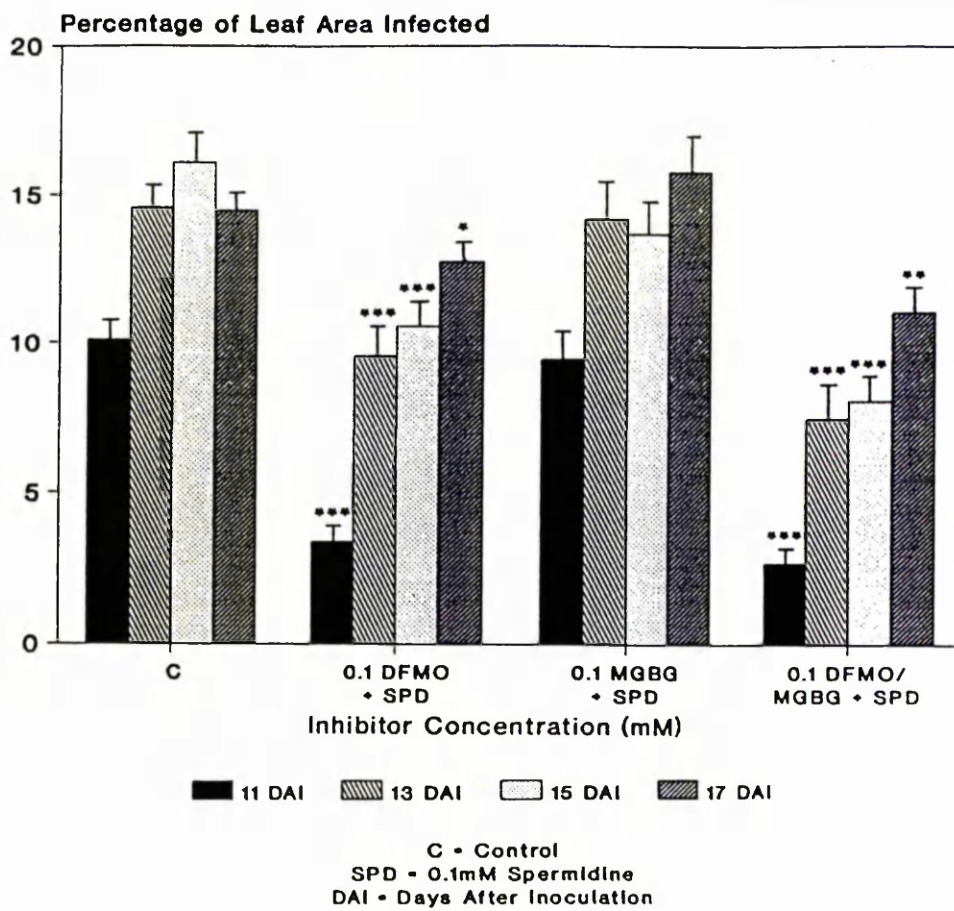


Figure 35: The effect of post-inoculation treatment with putrescine in combination with DFMO, MGBG and DFMO/MGBG on the per cent of leaf area infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant differences are shown as follows: P = 0.1 *; P = 0.01 **; P = 0.001 ***.

Figure 36: The effect of post-inoculation treatment with putrescine in combination with DFMO, MGBG and DFMO/MGBG on the number of pustules per centimetre square of leaf infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown.

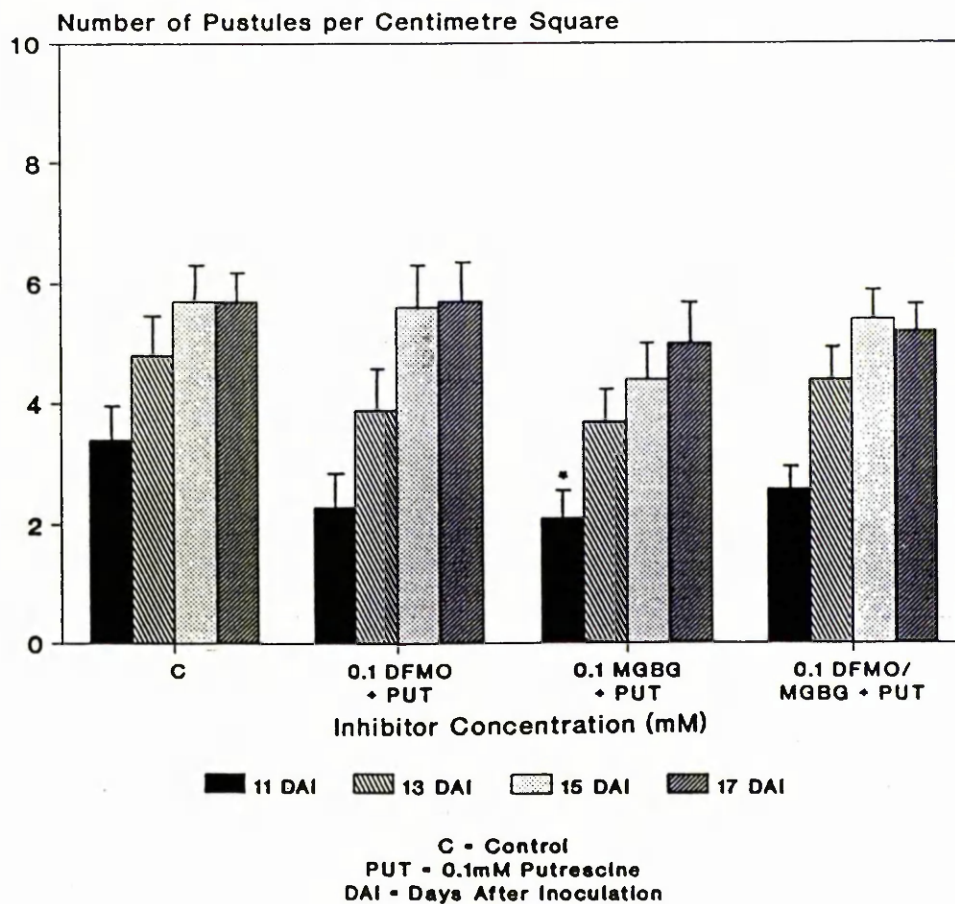
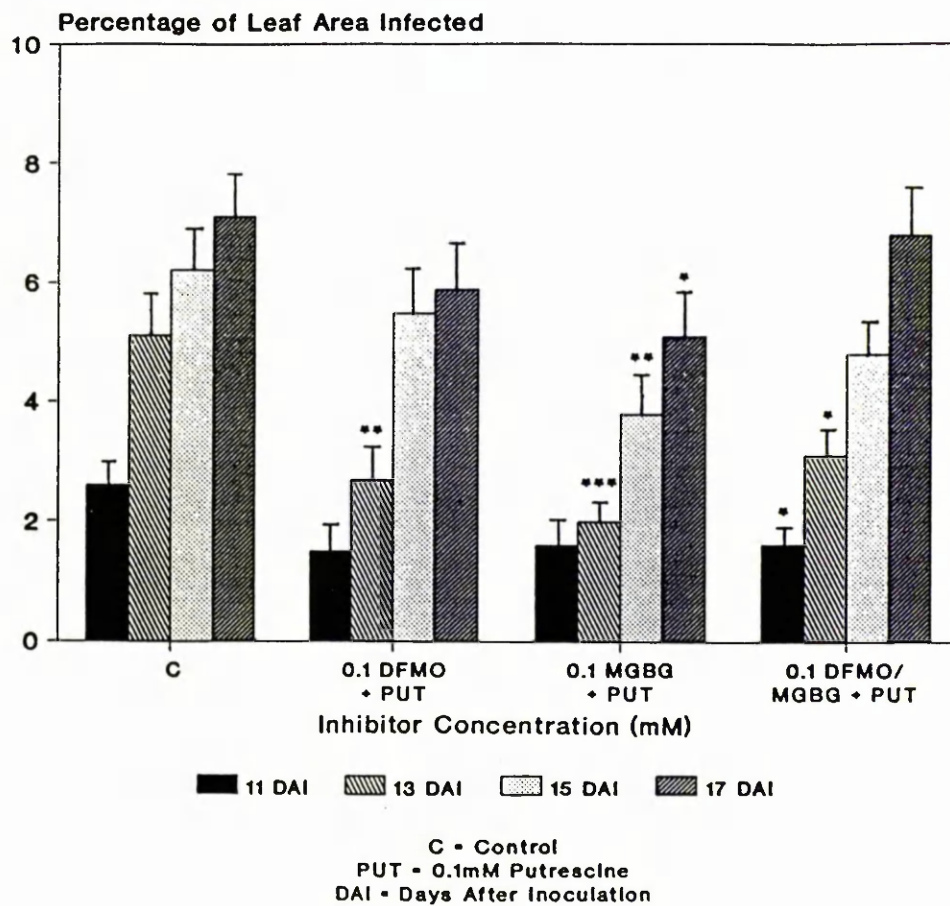


Figure 37: The effect of post-inoculation treatment with spermidine in combination with DFMO, MGBG and DFMO/MGBG on the per cent of leaf area infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown. Significant difference is shown as $P = 0.1 *$.

Figure 38: The effect of post-inoculation treatment with spermidine in combination with DFMO, MGBG and DFMO/MGBG on the number of pustules per centimetre square of leaf infected with *Puccinia graminis*. Values are the means of twenty replicates with standard error bar shown.

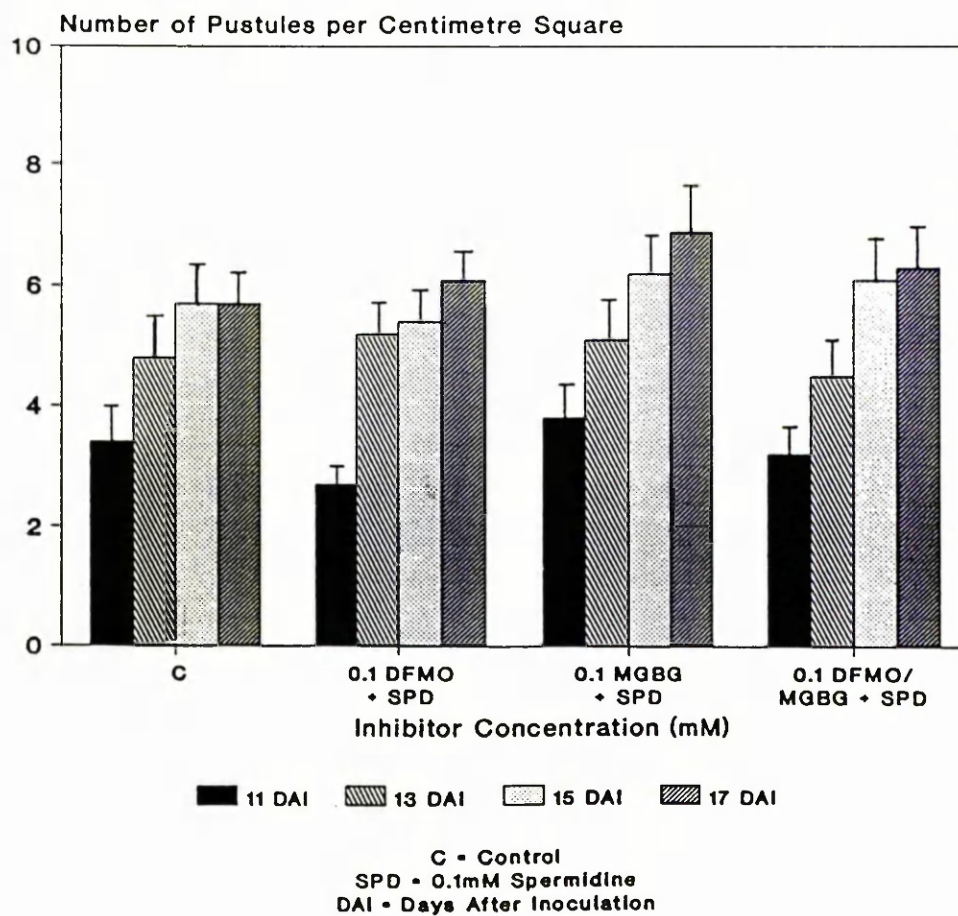
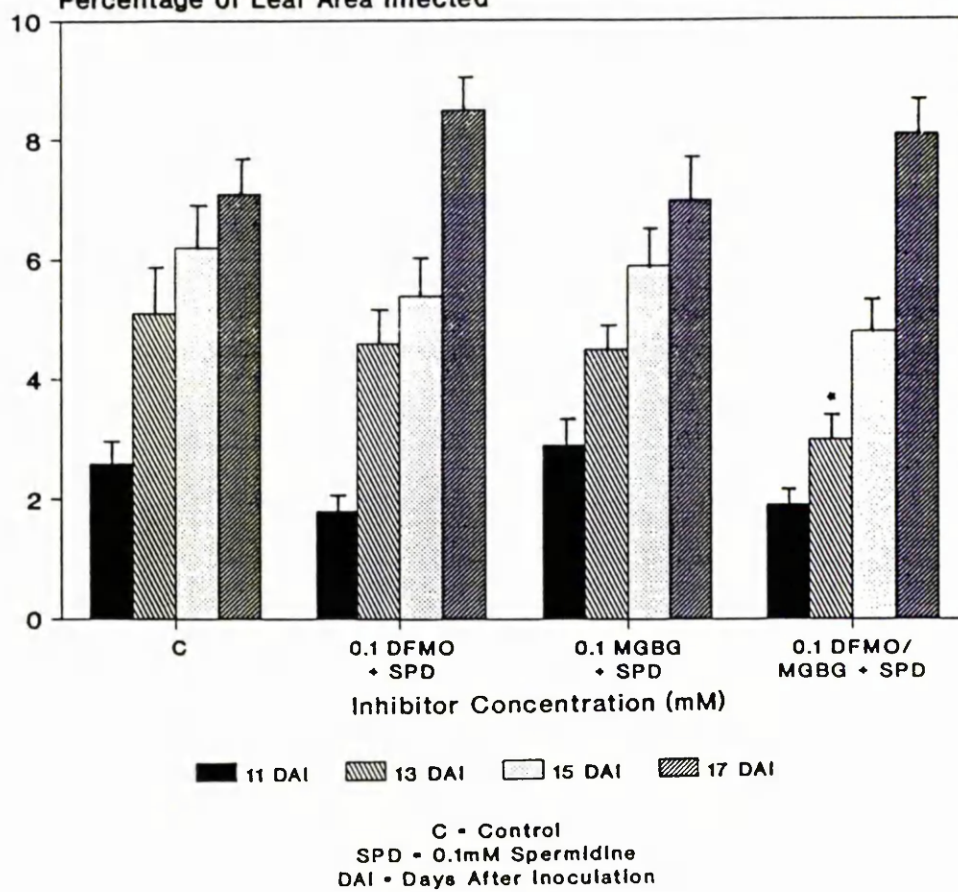


Table 26: Dry weight of wheat leaves treated with inhibitors and polyamines prior to inoculation with *Puccinia graminis*. Tissue harvested seventeen days after inoculation. SPD represents 0.1 mM spermidine and PUT represents 0.1 mM putrescine. Values are the means of twenty replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (c) $P = 0.1$.

Treatment	Dry Weight (mg)
Control	19.4 \pm 0.7
0.05 mM DFMO	18.6 \pm 1.1
0.1 mM DFMO	13.5 \pm 1.0 ^a
1.0 mM DFMO	11.5 \pm 0.8 ^a
0.05 mM MGBG	16.6 \pm 0.9 ^c
0.1 mM MGBG	11.0 \pm 0.7 ^a
1.0 mM MGBG	16.9 \pm 1.0 ^a
0.05 mM DFMO/MGBG	13.6 \pm 0.8 ^a
0.1 mM DFMO/MGBG	15.9 \pm 0.7 ^a
1.0 mM DFMO/MGBG	10.9 \pm 0.6 ^a
0.1 mM DFMO + SPD	18.6 \pm 0.9
0.1 mM DFMO + PUT	18.3 \pm 0.9
0.1 mM MGBG + SPD	19.7 \pm 0.9
0.1 mM MGBG + PUT	20.6 \pm 0.7
0.1 mM DFMO/MGBG + SPD	17.0 \pm 0.8 ^c
0.1 mM DFMO/MGBG + PUT	18.1 \pm 1.2

Table 27: Dry weight of wheat leaves treated with inhibitors and polyamines after inoculation with *Puccinia graminis*. Tissue harvested seventeen days after inoculation. SPD represents 0.1 mM spermidine and PUT represents 0.1 mM putrescine. Values are the means of twenty replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$; (c) $P = 0.1$.

Treatment	Dry Weight (mg)
Control	16.2 \pm 0.9
0.05 mM DFMO	12.4 \pm 0.9 ^b
0.1 mM DFMO	11.3 \pm 0.5 ^a
1.0 mM DFMO	11.6 \pm 0.5 ^a
0.05 mM MGBG	13.0 \pm 0.6 ^b
0.1 mM MGBG	14.5 \pm 0.9
1.0 mM MGBG	11.7 \pm 0.4 ^a
0.05 mM DFMO/MGBG	11.8 \pm 0.5 ^a
0.1 mM DFMO/MGBG	13.8 \pm 0.6 ^c
1.0 mM DFMO/MGBG	9.7 \pm 0.5 ^a
0.1 mM DFMO + SPD	18.3 \pm 0.8 ^c
0.1 mM DFMO + PUT	14.8 \pm 1.3
0.1 mM MGBG + SPD	16.1 \pm 1.1
0.1 mM MGBG + PUT	12.9 \pm 0.8 ^b
0.1 mM DFMO/MGBG + SPD	14.6 \pm 1.0
0.1 mM DFMO/MGBG + PUT	15.3 \pm 1.1

P. graminis infection and mycelial growth of the fungus *P. avenae*. This agrees with work using DFMO to inhibit fungal growth *in vitro* (Rajam and Galston, 1985; Birecka *et al*, 1986) and *in vivo* (Rajam, Weinstein and Galston, 1985; Walters, 1986).

The effects of pre- and post-inoculation sprays of inhibitors on *P. graminis* infection varied and neither appeared to be consistently better than the other. However, results obtained by other workers indicate that post-inoculation sprays of inhibitors are usually more effective (Walters, 1986; Weinstein, *et al*, 1987; West and Walters, 1988). Weinstein *et al* (1987) suggested that pre-inoculation sprays with DFMO were less effective since the compound would enter the leaf and react with the plant cell ODC and thus would be unavailable when the leaf was later inoculated with fungal spores. Similarly MGBG may be bound to SAMDC within the cell. Although DFMO was generally more effective, MGBG was also very efficient at reducing infection by *P. graminis*. Applications of MGBG were also effective against powdery mildew infection of barley (West and Walters, 1988). Pre- and post-inoculation sprays of DFMO and MGBG combined were effective at controlling *P. graminis* infection and, with the exception of 0.1 mM DFMO/MGBG combined, the mixtures of the inhibitors were more effective than either DFMO or MGBG applied separately. The compounds together probably exert a synergistic effect, since several studies on a number of mammalian cell systems in culture have indicated the ability of DFMO to increase the cytotoxicity of other

anti-tumour agents (Sunkara et al, 1980; Sunkara et al, 1983). Indeed, the uptake and cytotoxicity of MGBG in Ehrlich ascites cells increased on pre-treatment with DFMO (Seppanen, Alhonen-Hongisto and Janne, 1981). This probably occurred because DFMO depleted the cellular level of polyamines and in an attempt to restore polyamine levels, cellular uptake of MGBG, a structural analogue of spermidine (Janne et al, 1981), was increased.

It is possible that the inhibitory effects of DFMO and MGBG on infection by *P. graminis* observed here were due to reductions in spore germination on the leaf surface. Indeed, recent work by Rajam, Weinstein and Galston (1989) indicates that DFMO reduces germination of bean rust uredospores both *in vitro* and *in vivo*. The control of rust infection observed here could also be the result of reduced mycelial growth following the uptake of inhibitors from within the leaf. It is interesting to note that DFMO has been shown to be readily taken up by the plant and to be mobile in both xylem and phloem (Walters and Kingham, 1990). Moreover, these authors have shown that significant reductions in mildew growth could be achieved if DFMO was only available via uptake from within the plant.

The addition of putrescine or spermidine to the inhibitor sprays reversed inhibition and in some treatments restored growth of *P. graminis* to levels higher than the controls, indicating that DFMO and MGBG inhibition is reversible. Similarly, West and Walters

(1988) described a reversal of DFMO inhibition of powdery mildew infection of barley in the presence of exogenous polyamines.

The observed reductions in leaf dry weights treated with inhibitors are inconsistent with the findings of Walters (1986) who demonstrated that DFMO sprays did not affect growth of the broad bean plant. However, the reduced dry weights of leaves sprayed with inhibitors do not necessarily infer a phytotoxic effect and may merely reflect a reduction in weight due to fungal infection.

Treatment with DFMO strongly reduced ODC activities in *P. avenae*. Furthermore, the intracellular concentrations of putrescine, spermidine and cadaverine were substantially reduced but spermine levels remained unchanged in *P. avenae* grown in the presence of DFMO. Since DFMO did not reduce the respiration rate in *P. avenae*, this suggests that the DFMO-induced growth reduction in this fungus was due to a reduction in polyamine concentration. Indeed, inhibition by DFMO of mycelial growth and, to a lesser extent, ODC activity was almost completely reversed by the addition of exogenous putrescine and spermidine, thus supporting suggestions that polyamines are essential for fungal growth (Stevens and Winther, 1979). The large increase in SAMDC in *P. avenae* which occurred in the presence of DFMO may be due to stabilisation of the active protein as found in animal systems by Shirahata and Pegg (1985). Another possible explanation is that the accumulating and highly basic

decarboxylated SAM could substitute for a polyamine (though it is not a polyamine analogue) ensuring that the small levels of putrescine, made in the presence of DFMO, are converted into spermine (Pegg, 1984). This would explain why DFMO does not reduce spermine content in most cells (Pegg and McCann, 1982; Table 24). However, workers using DFMO in the treatment of cancer have outlined some limitations of the compound. Uptake of DFMO into the cells is slow and probably enters by diffusion rather than active transport (Erwin and Pegg, 1982), the compound is also rapidly excreted by animals (Grove, Fozard and Mamont, 1981) and the rapid turnover of the target enzyme, ODC, allows the cells to recover quickly from inhibition. Thus the effects of DFMO are cytostatic rather than cytotoxic (Pegg and McCann, 1982) although some workers have described cytotoxic effects of the compound (Luk et al, 1981). It has been suggested that the cytostatic response of DFMO may be related to the general inability of the compound to totally deplete cellular polyamine levels (Mamont et al, 1978). Various compensatory mechanisms have also been reported to occur in cells treated with DFMO, in an attempt to prevent total loss of polyamines, such as reduced excretion of polyamines, increased uptake and accumulation of extracellular polyamines and an increase in secondary enzyme production (Janne et al, 1981). However, despite the increase in SAMDC activity in *P. avenae*, only spermine levels were maintained at control values.

This is the first report of cadaverine from a plant

pathogenic fungus, although this diamine has been reported from *Aspergillus oryzae* and *Coprinus atramentarius* (Stevens and Winther, 1979) and *Neurospora crassa* (Paulus, Kiyono and Davis, 1982). The substantial reduction in cadaverine concentrations in *P. avenae* grown in the presence of DFMO, MGBG and EMGBG is difficult to explain since there are no data on the activity of its biosynthetic enzyme, lysine decarboxylase, or of diamine oxidase, which could breakdown cadaverine. Moreover, other research has shown that cadaverine concentration increased considerably in *Neurospora* and in mycoplasma-infected tumour cells depleted in intracellular polyamines (Alhonen-Hongisto et al, 1982; Paulus, Kiyono and Davis, 1982).

MGBG, singly and in combination with DFMO, was more effective at reducing mycelial growth of *P. avenae* than DFMO alone. This is the opposite of what was found for *P. graminis*-infected wheat treated with inhibitors. These differences may be due to the different nutritional requirements of the fungi, since *P. avenae* is a necrotroph and *P. graminis* is a biotroph. Another possibility is that *P. graminis* which was studied *in vivo* and *P. avenae* which was studied *in vitro*, are exhibiting genus-dependent differences in sensitivity to the inhibitor (Birecka et al, 1986). Indeed, West and Walters (1989) described species-dependent responses of some fungi to polyamine biosynthesis inhibitors. Thus, differences observed between *P. teres* (West and Walters, 1989) and *P. avenae* in their response to inhibitors may reflect variations

between different species of the same genus to take up inhibitors or differences in sensitivity of the enzymes to the inhibitors.

Since DFMO inhibits putrescine and spermidine synthesis, but has no effect on spermine levels, the addition of MGBG, thus inhibiting SAMDC, should prevent spermine accumulation. Thus, DFMO and MGBG are probably demonstrating synergistic effects as described above. The results presented in this thesis indicate that MGBG was not very efficient at inhibiting SAMDC activity, although a decrease in spermine concentration was observed. Other workers have found large increases in SAMDC activity when looking at animal cells treated with MGBG and suggest that the increase is due to enzyme stabilisation against proteolytic degradation, by the bound inhibitor (Pegg, Corti and Williams-Ashman, 1973). MGBG did, however, cause a significant reduction in ODC activity in *P. avenae* and although putrescine levels remained unchanged, this may have been the result of a reduced diamine oxidase activity, as has been reported by Kallio and Janne (1983). This MGBG-induced (and EMGBG-induced) reduction in ODC activity is surprising since other workers have reported substantial increases in ODC activity in cultured tumour cells, induced by treatment with MGBG and its analogue (Janne et al, 1985; see Porter and Sufrin, 1986). Janne et al (1985) suggested that this may be due to stabilisation of ODC protein in MGBG treated cells.

It has been reported that as well as inhibiting

SAMDC, MGBG also inhibits cellular respiration resulting in severe mitochondrial damage (see Janne *et al*, 1985). Nikula *et al* (1984; 1985) found that MGBG inhibited mitochondrial fatty acid oxidation and that the addition of carnitine prevented this. They suggest that damaged fatty acid oxidation due to MGBG may be one of the "mechanisms" which leads to mitochondrial damage. The results presented in this thesis show that the use of MGBG and DFMO/MGBG combined resulted in decreased respiration rates. This was reversed, in most cases, in the presence of carnitine. However, the addition of carnitine to MGBG treated *P. avenae* resulted in a further reduction in respiration rate. The cause of this effect is not known and merits further study. It would appear, therefore, that the effect on mycelial growth of *P. avenae*, using MGBG, may not be due solely to inhibition of polyamine biosynthesis. This effect of MGBG may account for the lack of response when exogenous putrescine and spermidine were added to the media. However, since MGBG and polyamines have been reported to share a common cellular transport system (Dave and Caballes, 1973) the prevention of effects when exogenous putrescine or spermidine were added may be due to competition for this transport with MGBG.

EMGBG, an analogue of MGBG, is a very potent inhibitor of SAMDC (Elo *et al*, 1986; Table 23). However, unlike MGBG, it showed no antiproliferative activity against mouse leukaemia cells *in vitro* (Elo *et al*, 1986). It was surprising, therefore, to find that EMGBG was a

very effective inhibitor of mycelial growth in *P. avenae*. The reason for this difference between animal and fungal cells in their response to EMGBG is not known. It would appear from the data presented here that, unlike MGBG, EMGBG does not have a toxic effect on the mitochondria. However, the addition of putrescine or spermidine did not reverse the inhibitory effects of EMGBG on mycelial growth of *P. avenae*, as might have been expected.

It is interesting to note that in all cases where spermidine was added to the incubation media, in an attempt to reverse the effects of the inhibitor, SAMDC activity of *P. avenae* was reduced substantially below that found in cells treated with inhibitor only. Similarly, ODC activity was reduced well below that found in inhibitor treated cells, when spermidine or putrescine were added to the media, suggesting a negative regulation of polyamine biosynthesis by intracellular polyamine levels. This apparent regulation of ODC and SAMDC activities by polyamines has been found previously in animal cells (Pegg and McCann, 1982; Pegg, 1984), and appears to be an attempt by the cells to maintain a balanced and constant supply of putrescine, spermidine and spermine for cellular functions related to growth. Indeed, results obtained using animal cells treated with DFMO and spermidine described an effect on the half-life of SAMDC (Shirahata and Pegg, 1985) and the content of its mRNA (Shirahata and Pegg, 1986).

Cell lengths and diameters of *P. avenae* remained

largely unaffected by the addition of inhibitors and/or polyamines. This does not agree with work by Rajam and Galston (1985) who showed reduced cell lengths and increased cell diameters when various fungi were exposed to DFMO. Increases in cell length and diameter were also reported in the presence of exogenous polyamines. As suggested by Birecka et al (1986) these differences may be accounted for by differing sensitivities between genera and perhaps also in the uptake and distribution of the inhibitor within the cell. The results presented here indicate that polyamine biosynthesis inhibitors have much potential as fungicides. However, a precise understanding is required of the mechanisms underlying the differing fungal responses to polyamine biosynthesis inhibitors and the effects these compounds have on the fungus - plant relationship.

S E C T I O N 5

CONSIDERATIONS FOR FUTURE RESEARCH

The research presented and discussed in the previous chapters highlight the importance of polyamines in plant growth and development. However, there is still much controversy regarding the possible roles of polyamines in plants. The results presented here on polyamine metabolism in diseased plants (Section 2), osmotically-stressed plants (Section 3) and control of phytopathogenic fungi by use of polyamine biosynthesis inhibitors (Section 4), indicate an association of these compounds with a wide variety of both biotic and abiotic plant and fungal responses. Even so, there are still many questions which remain unanswered.

The subcellular localisation of polyamines in the chloroplasts and mitochondria of some plant cells has been reported (Torrighiani *et al*, 1986). The significance of polyamines in these organelles is not known and is an area worthy of further research. Indeed, results obtained from work with animal systems have described an enhancement, by spermine, of respiration in rat liver mitochondria (Chaffee *et al*, 1977; Chaffee, Arine and Rochelle, 1979). This suggests a correlation between the localisation of polyamines and associated physiological changes within the cell. Similar information on the subcellular localisation of polyamines in plants would be useful.

Another neglected aspect of polyamine metabolism in plants concerns conjugated forms of polyamines and their release to the free state. There have been suggestions that there may be movement of polyamines between

conjugated and free pools, which might serve to regulate the level of free polyamine pools in cells (Slocum and Galston, 1985). Indeed, recent work on mildewed barley (Coghlan and Walters, unpublished) has shown that there is movement from conjugated to free polyamines in mildewed barley leaves. This area is worthy of further investigation in *P. graminis*-infected wheat (Section 2) since increases in free polyamine concentrations could not be accounted for in terms of biosynthesis or catabolism. Moreover, research on conjugated polyamines will be important in our understanding, not only of the regulation of polyamine levels in cells, but also the role of polyamines in the multifarious plant responses with which they have been associated.

The results presented on polyamine metabolism in wheat during osmotic stress (Section 3) takes the study of polyamines in such responses one step further, since the whole plant was examined in detail. However, again, information on conjugated forms of polyamines in such responses would be useful. Moreover, it would be interesting to examine the translocation and subcellular localisation of polyamines in stressed plants and to correlate changes in polyamines closely with other physiological changes occurring in the plant.

Control of infection by *P. graminis* using inhibitors of polyamine biosynthesis confirmed previous work on biotrophs (Rajam, Weinstein and Galston, 1986; Walters, 1986), which are highly sensitive to this form of

inhibition. Recent results presented by Khan and Minocha (1989a,b) on the presence of a biosynthetic ADC in some phytopathogenic fungi, indicate that perhaps evidence of fungal polyamine biosynthetic enzymes should be obtained prior to use of inhibitors such as DFMO or DFMA. Indeed using *P. avenae*, a fungus easily culturable, but sensitive to inhibitors, the biochemical effects of polyamine biosynthesis inhibitors were examined. The results obtained indicate that studies on feedback inhibition of enzymes could be very important in future work in this area. Moreover, the use of polyamine analogues rather than substrate analogues may prove useful in the inhibition of polyamine biosynthesis. Indeed, Masse, Laberche and Jeanty (1988) described the inhibitory effects of norspermidine, a natural polyamine, on the *in vitro* growth of potato and suggested that the effect was due to inhibition of spermidine biosynthesis. Further work on the development of novel product analogues could lead to the development of potentially useful fungicidal compounds.

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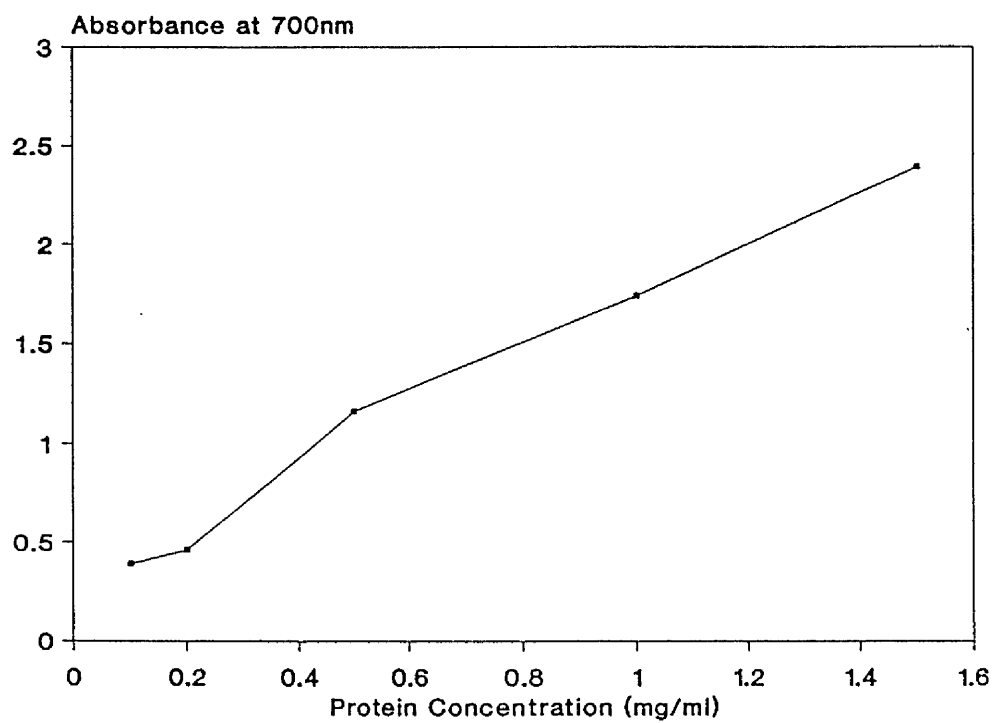
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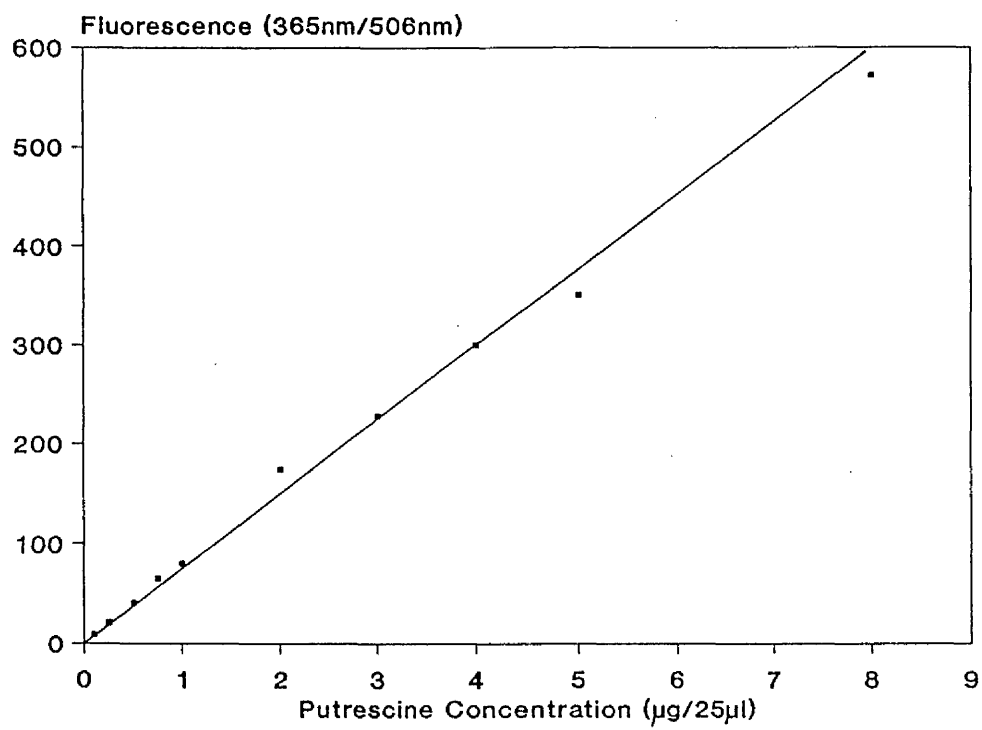
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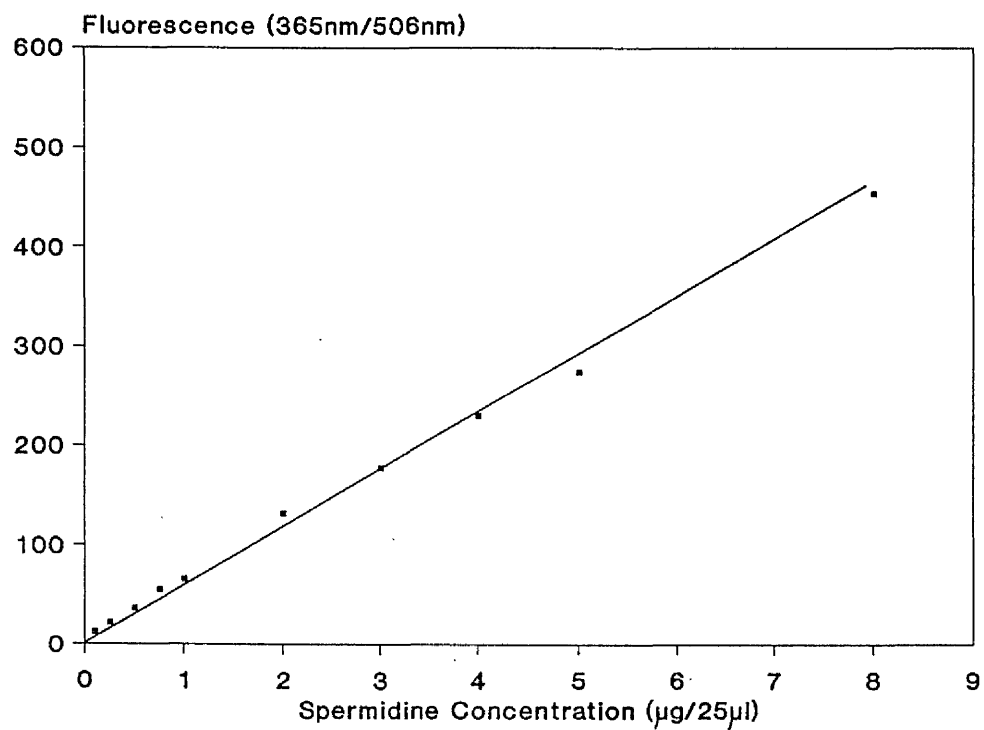
CALIBRATION CURVES



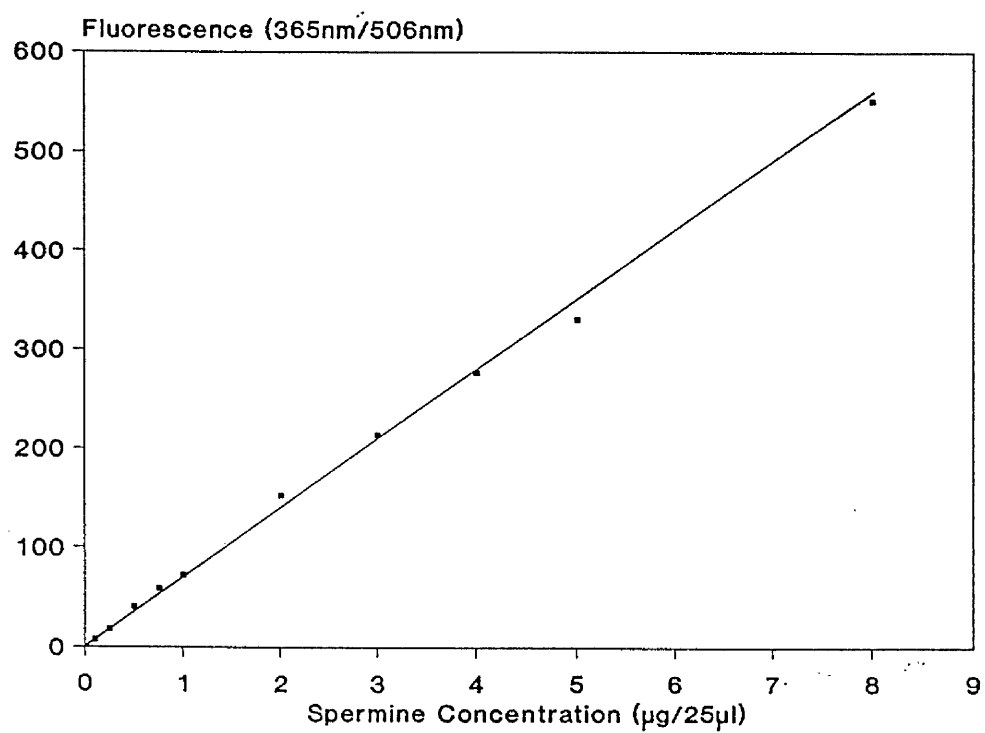
Protein Calibration Curve



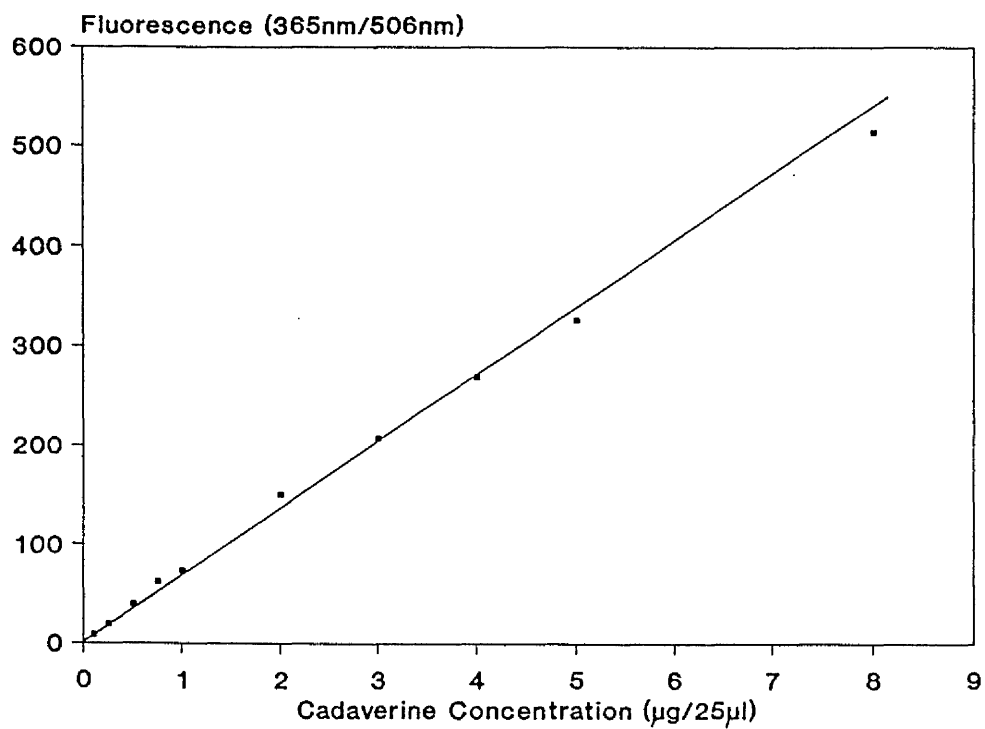
Putrescine Standard Curve



Spermidine Standard Curve



Spermine Standard Curve



Cadaverine Standard Curve

A P P E N D I X I I

STANDARD ERRORS AND SIGNIFICANCE VALUES FOR
ADC ACTIVITY IN OSMOTICALLY-STRESSED WHEAT
LEAF SEGMENTS

Table 1: The effect of osmotic stress alone and in the presence of the inhibitors DFMO or DFMA on arginine decarboxylase activity in segments of first leaves of wheat. Values are the means of five replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$; (c) $P = 0.1$.

Treatment/Incubation Time (hours)		ADC (pmol CO ₂ mg protein ⁻¹ hour ⁻¹)
Control	0.25	0.29 \pm 0.09
	0.50	0.06 \pm 0.02
	0.75	0.12 \pm 0.04
	1.00	0.29 \pm 0.11
	1.50	0.14 \pm 0.04
	2.00	0.05 \pm 0.01
	2.50	0.11 \pm 0.06
	3.00	0.05 \pm 0.005
	4.00	0.09 \pm 0.014
	5.00	0.05 \pm 0.007
Stressed	0.25	0.21 \pm 0.02
	0.50	0.33 \pm 0.02 ^a
	0.75	0.39 \pm 0.03 ^a
	1.00	0.59 \pm 0.03 ^c
	1.50	0.81 \pm 0.03 ^a
	2.00	0.47 \pm 0.02 ^a
	2.50	0.36 \pm 0.03 ^b
	3.00	0.22 \pm 0.02 ^a
	4.00	0.28 \pm 0.02 ^a
	5.00	0.36 \pm 0.03 ^a
Stressed + DFMO	1.50	0.53 \pm 0.07 ^a
	2.00	1.25 \pm 0.11 ^a
	2.50	1.43 \pm 0.19 ^a
	3.00	0.91 \pm 0.10 ^a
Stressed + DFMA	1.50	0.04 \pm 0.004 ^c
	2.00	0.04 \pm 0.002
	2.50	0.03 \pm 0.005
	3.00	0.03 \pm 0.008 ^c

Table 2: The effect of treatment with rifamycin or cycloheximide prior to osmotic stress on arginine decarboxylase (ADC) activity in segments of first leaves of wheat. Values are the means of five replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$; (c) $P = 0.1$.

Treatment/Incubation Time (hours)		ADC (pmol CO ₂ mg protein ⁻¹ hour ⁻¹)
Control	1.00	0.48 \pm 0.05
	1.50	0.56 \pm 0.06
	2.00	0.82 \pm 0.01
Stressed	1.00	0.77 \pm 0.07 ^b
	1.50	0.61 \pm 0.07
	2.00	1.08 \pm 0.04 ^a
Rifamycin + stressed	1.00	0.46 \pm 0.01
	1.50	0.78 \pm 0.05 ^c
	2.00	0.79 \pm 0.10
Cycloheximide + stressed	1.00	0.37 \pm 0.05
	1.50	0.59 \pm 0.06
	2.00	0.88 \pm 0.21

A P P E N D I X I I I

LETCOMBE LABORATORY NUTRIENT SOLUTION

Table 1: Letcombe Laboratory Nutrient Solution.

Nutrient	Concentration	
	mM/litre	µM/litre
$\text{Ca}(\text{NO}_3)_4\text{H}_2\text{O}$	1.5	-
KNO_3	5.0	-
KH_2PO_4	1.0	-
$\text{MgSO}_4\cdot 7\text{H}_2\text{O}$	1.5	-
NaNO_3	2.0	-
Fe EDTA	-	9.22
H_3BO_3	-	9.22
$\text{CuSO}_4\cdot 5\text{H}_2\text{O}$	-	0.16
KCl	-	14.10
$\text{MnSO}_4\cdot 4\text{H}_2\text{O}$	-	3.6
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$	-	0.016
$\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$	-	0.77

A P P E N D I X I V

STANDARD ERRORS AND SIGNIFICANCE VALUES FOR
MYCELIAL GROWTH OF *PYRENOPHORA AVENAE*
GROWING ON MEDIA AMENDED WITH POLYAMINE
BIOSYNTHESIS INHIBITORS

Table 1: The effect of DFMO on the mycelial growth of *Pyrenophora avenae* from the first to the sixth day after inoculation. Values represent the mean of six replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$; (c) $P = 0.1$.

DAI represents days after inoculation.

Mycelial Growth (mm)					
DAI	Control	DFMO Concentration (mM)			
		0.1	0.5	1.0	2.0
1	2.3 \pm 0.1	2.2 \pm 0.2	2.1 \pm 0.1 ^c	1.8 \pm 0.05 ^a	1.7 \pm 0.1 ^b
2	7.6 \pm 0.3	6.1 \pm 0.2 ^b	5.8 \pm 0.4 ^b	4.7 \pm 0.1 ^a	5.0 \pm 0.1 ^a
3	12.8 \pm 0.2	12.1 \pm 0.1 ^b	9.5 \pm 0.4 ^a	8.2 \pm 0.5 ^a	7.7 \pm 0.5 ^a
4	18.7 \pm 0.3	16.6 \pm 0.4 ^a	14.2 \pm 0.3 ^a	13.7 \pm 0.5 ^a	11.6 \pm 0.5 ^a
5	26.5 \pm 0.7	22.1 \pm 0.4 ^a	20.0 \pm 0.5 ^a	19.3 \pm 0.4 ^a	15.9 \pm 0.6 ^a
6	32.8 \pm 0.6	28.1 \pm 0.4 ^a	24.5 \pm 0.3 ^a	24.1 \pm 0.3 ^a	21.1 \pm 0.6 ^a

Table 2: The effect of MGBG on the mycelial growth of *Pyrenophora avenae* from the first to the sixth day after inoculation. Values represent the mean of six replicates \pm standard error. Significant differences are shown as (a) $P = 0.001$.

DAI represents days after inoculation.

Mycelial Growth (mm)					
		MGBG Concentration (mM)			
DAI	Control	0.1	0.5	1.0	2.0
1	2.3 \pm 0.1	2.2 \pm 0.04	1.7 \pm 0.06 ^a	1.4 \pm 0.06 ^a	0.2 \pm 0.1 ^a
2	7.6 \pm 0.3	4.8 \pm 0.2 ^a	3.7 \pm 0.1 ^a	2.8 \pm 0.1 ^a	0.3 \pm 0.1 ^a
3	12.8 \pm 0.2	8.3 \pm 0.5 ^a	4.5 \pm 0.06 ^a	3.8 \pm 0.2 ^a	0.3 \pm 0.1 ^a
4	18.7 \pm 0.3	12.0 \pm 0.4 ^a	6.4 \pm 0.1 ^a	4.3 \pm 0.2 ^a	0.4 \pm 0.1 ^a
5	26.5 \pm 0.7	17.6 \pm 0.4 ^a	6.9 \pm 0.2 ^a	4.6 \pm 0.2 ^a	0.7 \pm 0.1 ^a
6	32.8 \pm 0.6	22.4 \pm 0.3 ^a	8.5 \pm 0.2 ^a	4.8 \pm 0.2 ^a	0.8 \pm 0.2 ^a

Table 3: The effect of DFMO/MGBG combined on the mycelial growth of *Pyrenophora avenae* from the first to the sixth day after inoculation. Values represent the mean of six replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (c) $P = 0.1$. ND = no detectable growth.

DAI represents days after inoculation.

Mycelial Growth (mm)					
DFMO/MGBG Concentration (mM)					
DAI	Control	0.1	0.5	1.0	2.0
1	2.3 \pm 0.1	1.9 \pm 0.2 ^c	1.6 \pm 0.06 ^a	1.3 \pm 0.2 ^a	ND
2	7.6 \pm 0.3	4.8 \pm 0.1 ^a	3.8 \pm 0.1 ^a	2.2 \pm 0.3 ^a	ND
3	12.8 \pm 0.2	9.1 \pm 0.4 ^a	4.6 \pm 0.1 ^a	2.7 \pm 0.5 ^a	0.6 \pm 0.1 ^a
4	18.7 \pm 0.3	14.3 \pm 0.7 ^a	6.4 \pm 0.1 ^a	3.0 \pm 0.5 ^a	0.7 \pm 0.1 ^a
5	26.5 \pm 0.7	19.9 \pm 0.5 ^a	7.7 \pm 0.2 ^a	3.8 \pm 0.5 ^a	0.7 \pm 0.2 ^a
6	32.8 \pm 0.6	26.0 \pm 0.4 ^a	8.5 \pm 0.2 ^a	4.4 \pm 0.4 ^a	0.9 \pm 0.2 ^a

Table 4: The effect of EMGBG on the mycelial growth of *Pyrenophora avenae* from the first to the sixth day after inoculation. Values represent the mean of six replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (c) $P = 0.1$.

DAI represents days after inoculation.

Mycelial Growth (mm)				
		EMGBG Concentration (mM)		
DAI	Control	0.1	0.5	1.0
1	2.3 \pm 0.1	1.7 \pm 0.2 ^c	0.9 \pm 0.2 ^a	1.5 \pm 0.1 ^a
2	7.6 \pm 0.3	3.8 \pm 0.2 ^a	2.1 \pm 0.5 ^a	3.1 \pm 0.2 ^a
3	12.8 \pm 0.2	5.2 \pm 0.3 ^a	2.8 \pm 0.6 ^a	3.1 \pm 0.05 ^a
4	18.7 \pm 0.3	6.8 \pm 0.7 ^a	3.2 \pm 0.6 ^a	3.7 \pm 0.14 ^a
5	26.5 \pm 0.7	9.6 \pm 0.4 ^a	4.2 \pm 0.8 ^a	4.6 \pm 0.3 ^a
6	32.8 \pm 0.6	12.7 \pm 0.2 ^a	4.3 \pm 0.8 ^a	5.1 \pm 0.2 ^a

Table 5: The effect of putrescine, alone or in combination with DFMO, MGBG, DFMO/MGBG and EMGBG, on the mycelial growth of *Pyrenophora avenae* from the first to the sixth day after inoculation. Values represent the mean of six replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$. PUT represents 0.1 mM putrescine and DAI represents days after inoculation.

		Mycelial Growth (mm)				
		Inhibitor Concentration (mM)				
DAI	Control	PUT	1.0 DFMO + PUT	0.5 MGBG + PUT	0.5 DFMO/ MGBG + PUT	0.1 EMGBG + PUT
1	2.1 \pm 0.1	1.9 \pm 0.1	1.9 \pm 0.2	0.9 \pm 0.2 ^a	0.9 \pm 0.1 ^a	1.4 \pm 0.1
2	6.0 \pm 0.2	6.1 \pm 0.3	4.8 \pm 0.2 ^a	1.9 \pm 0.1 ^a	2.1 \pm 0.2 ^a	3.1 \pm 0.1 ^a
3	11.4 \pm 0.3	10.9 \pm 0.2	9.9 \pm 0.2 ^a	2.7 \pm 0.2 ^a	3.3 \pm 0.2 ^a	5.1 \pm 0.2 ^a
4	17.7 \pm 0.2	15.2 \pm 0.4 ^a	15.6 \pm 0.3 ^a	3.3 \pm 0.2 ^a	4.3 \pm 0.1 ^a	8.2 \pm 0.2 ^a
5	23.6 \pm 0.3	22.6 \pm 0.3 ^b	21.8 \pm 0.5 ^b	4.8 \pm 0.2 ^a	5.7 \pm 0.1 ^a	10.7 \pm 0.3 ^a
6	32.3 \pm 0.4	32.9 \pm 0.1	31.4 \pm 0.3	5.7 \pm 0.4 ^a	6.5 \pm 0.2 ^a	15.8 \pm 0.3 ^a



Table 6: The effect of spermidine, alone or in combination with DFMO, MGBG, DFMO/MGBG and EMGBG, on the mycelial growth of *Pyrenophora avenae* from the first to the sixth day after inoculation. Values represent the mean of six replicates \pm standard error. Significant differences are shown as follows: (a) $P = 0.001$; (b) $P = 0.01$. SPD represents 0.1 mM spermidine and DAI represents days after inoculation.

Mycelial Growth (mm)						
Inhibitor Concentration (mM)						
DAI	Control	SPD	1.0 DFMO + SPD	0.5 MGBG + SPD	0.5 DFMO/ MGBG + SPD	0.1 EMGBG + SPD
1	2.1±0.1	2.0±0.2	1.6±0.1 ^b	1.1±0.3 ^b	0.7±0.1 ^a	1.3±0.1
2	6.0±0.2	5.8±0.4	4.4±0.2 ^a	2.2±0.2 ^a	1.9±0.1 ^a	3.4±0.2 ^a
3	11.4±0.3	10.6±0.2 ^b	8.5±0.2 ^a	3.9±0.1 ^a	2.7±0.2 ^a	5.8±0.3 ^a
4	17.7±0.2	16.4±0.2 ^a	12.5±0.2 ^a	4.3±0.1 ^a	3.6±0.3 ^a	8.2±0.3 ^a
5	23.6±0.3	23.0±0.3	18.0±0.5 ^a	4.7±0.1 ^a	4.7±0.4 ^a	10.7±0.2 ^a
6	32.3±0.4	33.9±0.2 ^b	30.3±0.3 ^b	5.7±0.3 ^a	5.1±0.3 ^a	15.4±0.3 ^a