POLYAMINE METABOLISM IN MYCORRHIZAL FUNGI.

JOHN ZARB   C.Biol., M.I.Biol.

Plant Science Department,

Scottish Agricultural College,

Auchincruive,

Ayr, KA6 5HW.

A thesis submitted for the degree of Doctor of Philosophy at the University of Glasgow, September 1994.

ProQuest Number: 10647143

ProQuest.

ProQuest 10647143

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

ACKNOWLEDGEMENTS.

Parts of this work appear in the following papers:

Zarb, J. & Walters, D.R., (1994). The effects of polyamine biosynthesis inhibitors on growth, enzyme activities and polyamine concentrations in the mycorrhizal fungus *Laccaria proxima*. *New Phytologist* **126**, 99-104.

Zarb, J. & Walters, D.R., (1994). The effect of the polyamine biosynthesis inhibitor DFMO on the ectomycorrhizal fungus *Paxillus involutus*. *Letters in Applied Microbiology* **18**, 5-7.

Zarb, J. & Walters, D.R. (1994). The formation of cadaverine, aminopropylcadaverine and N,N' bis (3 aminopropyl)cadaverine in mycorrhizal and phytopathogenic fungi. *Letters in Applied Microbiology* **19** (In press).

3. EFFECTS OF POLYAMINE BIOSYNTHESIS INHIBITORS ON GROWTH, ENZYME ACTIVITY AND POLYAMINE CONCENTRATIONS IN MYCORRHIZAL FUNGI.

4. THE FORMATION OF CADAVERINE, AMINOPROPYLCADAVERINE AND N,N' *bis* (3 AMINOPROPYL)CADAVERINE IN MYCORRHIZAL AND PHYTOPATHOGENIC FUNGI.

5. THE EFFECTS OF TOXIC METALS ON ENZYME ACTIVITITES AND POLYAMINE BIOSYNTHESIS IN THE EM FUNGUS *PAXILLUS INVOLUTUS*.

ABSTRACT.

The polyamine biosynthetic enzymes arginine decarboxylase (ADC), ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (AdoMetDC), and spermidine synthase, are susceptible to inhibition by a variety of compounds originally developed for the inhibition of polyamine biosynthesis in animal cells as an approach to cancer treatment. These compounds have subsequently been used in the study of polyamine metabolism in plants, fungi, bacteria, and protozoa, with important consequences. For example, most fungi are considered to possess only ODC for the initial step in polyamine biosynthesis, whilst plants have both ODC and ADC enzymes. Therefore, specific inhibition of ornithine decarboxylation in a plant-pathogenic fungus should inhibit fungal growth without damaging the host plant. In fact, such an approach to plant disease control has been realised.

Difluoromethylarginine (DFMA) and difluoromethylornithine (DFMO) are inhibitors of ADC and ODC, respectively. Cyclohexylamine (CHA) is a spermidine synthase inhibitor, and methylglyoxal bis (guanylhydrazone) (MGBG), an inihibtor of AdoMetDC. Mycorrhizal fungi, which are intimately associated with most plants, and which are of benefit in terrestrial ecosystems, may be vulnerable to compounds affecting polyamine metabolism. This premise was investigated in the ectomycorrhizal fungi *Laccaria proxima, Paxillus involutus, Hebeloma mesophaeum*, and *Thelephora terrestris*. Furthermore, in view of the pivotal role of polyamines in organisms, the importance of polyamines in stress responses in plants, and the fact that many mycorrhizal fungi are tolerant of industrially-polluted soils, polyamine metabolism was studied in *P. involutus* following exposure of the fungus to toxic metals.

Exposure of *L. proxima* to DFMO led to decreased growth, whilst exposure of the fungus to DFMA enhanced growth. The following data indicate that *L. proxima* possesses both ADC and ODC activities: (1) DFMA depleted putrescine and did not inhibit ODC activity; (2) incubation of mycelium with a [U-^{14}C]arginine substrate led to [^{14}C]putrescine formation; (3) DFMA completely inhibited the formation of polyamines from [U-^{14}C]arginine; (4) DFMO inhibited bound biosynthetic enzyme activity and the formation of putrescine from [U-^{14}C]ornithine.

Although exposure of *P. involutus* to DFMO resulted in reduced growth, and reduced activities of ODC, AdoMetDC and diamine oxidase, this was not accompanied by reductions in

polyamine concentrations. Indeed, spermine concentration was substantially increased. Together, these data suggest the existence of a mechanism in *P. involutus* to maintain intracellular concentrations of free polyamines.

H. mesophaeum exhibited a notable lack of response to inhibitor treatments, the possibilities for which are discussed in Chapter 3. The substantial depletion in spermine in *T. terrestris*, leading to a reduction in growth, following DFMO treatment, suggests the importance of this polyamine for growth in *T. terrestris*, in common with other fungi.

Radiolabelled compounds that co-chromatographed with authentic standards of cadaverine, aminopropylcadaverine (APC) and N,N' *bis* (3 aminopropyl)cadaverine (3 APC), were isolated from reaction mixtures after the decarboxylation of [U-^{14}C]lysine by fungal extracts. The identity of 3 APC was confirmed by nuclear magnetic resonance spectroscopy. In subsequent work, the inhibition of AdoMetDC and spermidine synthase led to significant reductions in the recovery of radiolabelled 3 APC. Results show that a range of ectomycorrhizal and plant-pathogenic fungi can synthesise cadaverine and its higher homologues APC and 3 APC, in reactions catalysed by AdoMetDC and spermidine synthase. Furthermore, these compounds may also be synthesised from the condensation of cadaverine and L-aspartic-β-semialhehyde.

Biosynthetic enzyme activity, polyamine concentrations and the rate of conversion of lysine into cadaverine were measured in *P. involutus* following exposure of the fungus to copper, nickel, lead, or zinc. Metal ions were accumulated in the fungus. Although the concentrations of putrescine, spermidine and spermine were affected to some extent, with copper and nickel treatments associated with reductions in spermidine concentration, these variations in polyamine concentrations did not significantly affect fungal fresh weight. Accumulaton of metals in fungal tissues, and exclusion of ions from the cell by sequestration at extracellular sites, are among the mechanisms that would tend to limit metal toxicity in mycorrhizal plants.

1. INTRODUCTION.

Mycorrhizal Fungi (Gr. *Myco* = fungus; *rhiza* = root).

Fungi are a very significant group of organisms which vary greatly in morphology, habitat and ecology. They are composed of filamentous, multicellular strands called hyphae which collectively form the mycelium. The hyphae permeate the soil or other substrate from which they obtain nutrition. Mycorrhizal fungi are able to form intimate structural and physiological associations with the roots of most plants *via* hyphae that sheath the plant root and which penetrate into and between root cells. The hyphal sheath and associated root cells together constitute the mycorrhiza. The mycorrhiza is an entity with distinct morphology and physiology, and water, minerals, metabolic products and other similar materials are able to pass across the plant-fungus interface. Mycorrhizas show great diversity of structure and physiology since the plant and fungal partners involved are manifold. Consequently, mycorrhizal relationships can vary from mutualistic, where net movement of carbon is generally from plant to fungus, to agonistic, where the fungus supplies both carbon and mineral nutrients to the plant and in which there is no clear advantage of the relationship to the fungus (Lewis, 1973). However, the fungus confers a consistent benefit to the plant primarily by increasing the capability of the root system to absorb water and minerals *via* the extensive hyphal net. Mycorrhizal fungi are categorised on the basis of their physical and physiological association with the plant.

Ectomycorrhizal (EM) Fungi.

EM fungi are mushrooms and toadstools: Ascomycetes and Basidiomycetes with characteristic macroscopic fruiting bodies of distinctive morphology. They form the so-called sheathing mycorrhizas. The hyphae form a well-developed, compact mantle around the plant root and extend from the mantle to penetrate between root cortex cells of short, lateral roots. Young lateral roots then become infected as they emerge through the cortex of infected roots. Mycorrhizal roots are relatively short-lived and are periodically replaced. Hyphae extend from these mycorrhizal roots into the surrounding soil. EM fungi are associated mostly with trees of temperate to boreal forest. They can be categorised into four ecological groups depending on their ability to live independently of a host and on their degree of host specificity: (1) species which are normally free-living, but which can form mycorrhizas with a suitable host *e.g. Phallus impudicus* (stinkhorn); (2) facultatively mycorrhizal species with a broad host range but which can also be free-living

Xylem

Hartig net

Epidermis

Cortex

Fungal sheath

100 μm
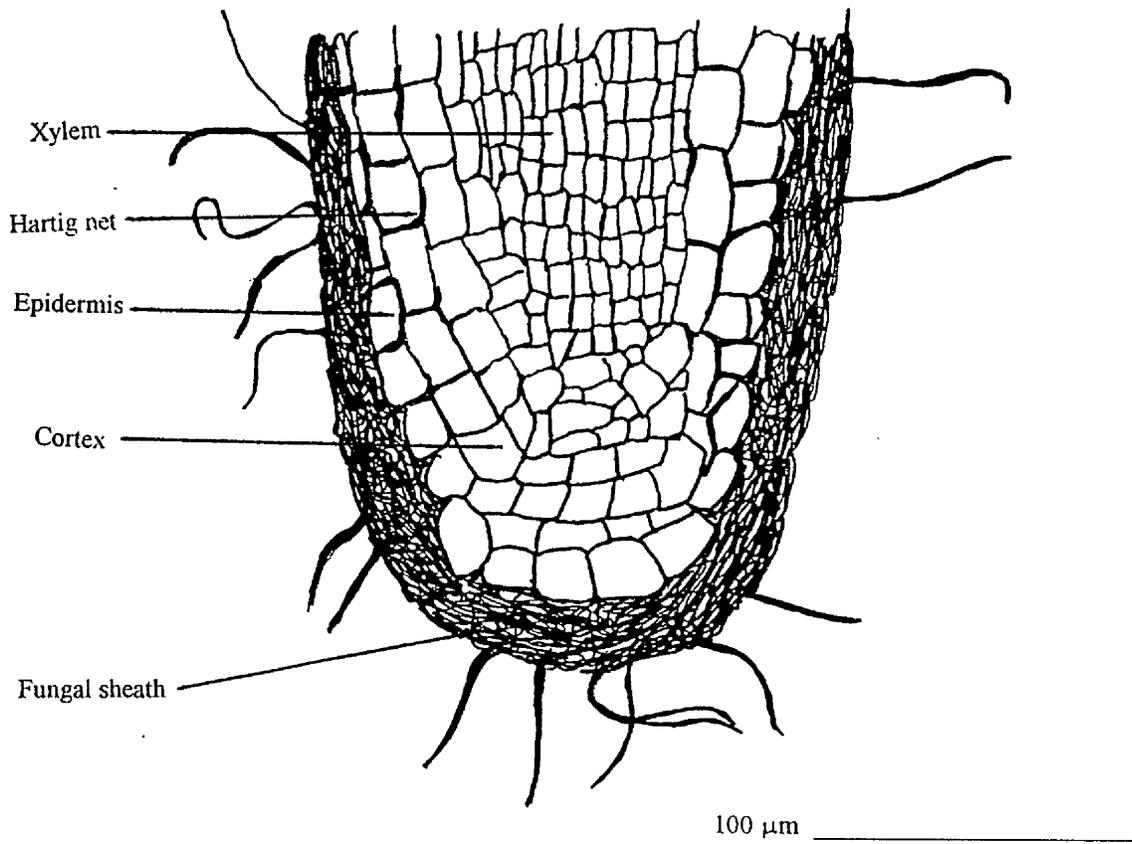
Figure 1. Longitudinal section of root tip infected with EM fungus.
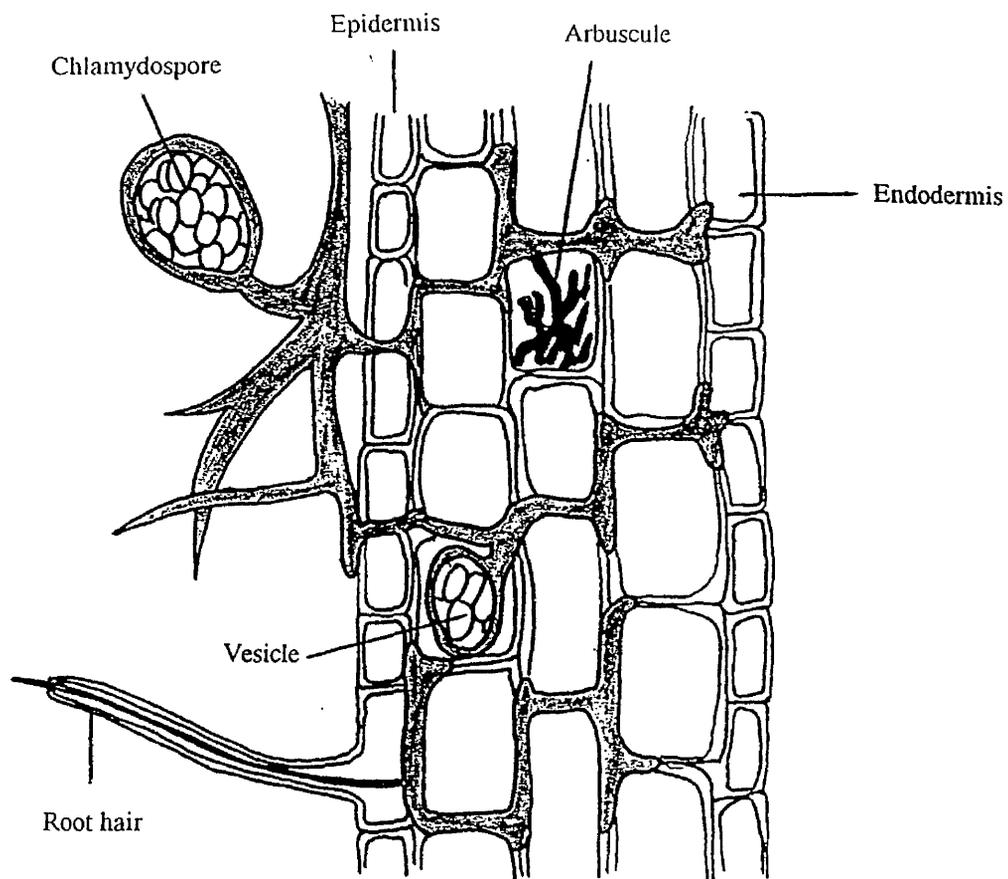
Figure 2. Longitudinal section of root infected with VAM fungus.

e.g. Scleroderma aurantium (Scleroderma citrinum , common earth ball); (3) ecologically obligate mycorrhiza-formers with a broad host range that are not normally found free-living. These comprise the majority of EM fungi; (4) ecologically obligate mycorrhiza-formers with a narrow host range that are not normally free-living *e.g. Boletus elegans (Suillus grevillei* , Larch bolete) on larch. Free-living types have a greater ability to decompose soil organic matter than obligately mycorrhizal types. The latter therefore depend largely on the plant for carbon requirements.

Root infection takes place in living primary root cortical cells from a variety of infective propagules including viable mycelium, hyphal strands or spores. A number of complex biotic and abiotic factors which are poorly understood influence the initiation of infection and subsequent infection events.

Vesicular Arbuscular Mycorrhizal (VAM) Fungi.

VAM fungi are associated with the vast majority of vascular plants, including those of tropical forests. The hyphae outside the root are diffuse and contiguous with those that penetrate the plant cells. Within root cells, hyphae give rise to short-lived, branched structures known as arbuscules which are involved in nutrient ion exchange between the symbionts, and enlarged vesicles which are thought to be fungal storage organs. Infective propagules of VAM fungi include chlamydospores (vegetative spores arising from hyphae), vesicles in residues of previously-infected roots, or possibly viable hyphal segments remaining in the soil. Infection is initiated when a hypa extending from any of these structures contacts a suitable plant root. Root cell penetration may be direct or there may be the formation of an appressorium prior to penetration. Subsequent root colonisation occurs in the cortical zone of differentiated, elongating roots. Arbuscules are formed within the cortical cells surrounding the vascular cylinder. They are in direct contact with a large volume of cytoplasm. Arbuscules begin to break down as soon as they are fully developed. It is not known whether this process is controlled by the fungus or the plant. The host cell concomitantly resumes the cytological characteristics of uninfected cortical cells.

There are about 30 species of VAM fungi, about half of which are contained in the genus *Glomus*. VAM fungi are microscopic and are obligate parasites not amenable to axenic culture. They have an extremely broad host range. Thus the distribution of VAM fungi in the British flora is extensive. However, mycorrhizas are absent from, or rare, in the Brassicaceae, Caryophylleceae and Cyperaceae and in the genera *Oenanthe* (Apiaceae) and *Astralagus* (Fabaceae). Non-mycorrhizal plants occur in habitats unsuitable for mycorrhizal fungi such as disturbed, soil-less, aquatic or wetland habitats, and in soils

4

lacking a suitable fungal inoculum. Some plants have acquired resistance to infection (Peat & Fitter, 1993). Resistance to infection by VAM fungi may be a response to selection pressure from phytopathogenic fungi and may be based on several mechanisms. Antifungal compounds such as glucosinolates exuded from the plant root are important in suppressing VAM infection *in vitro* in some *Betula* species (Schreiner & Koide, 1993). Some plants may lack the chemical signals required to initiate infection or there may be physical barriers to infection that are pre-formed, or produced in response to infection such as structural adaptations of the root cell wall to prevent fungal invasion. The benefits of generalised resistance to infection must outweigh the disadvantages of the loss of mycorrhiza formation. Interestingly, mycorrhizal and non-mycorrhizal plants co-exist in most habitats.

Physiological Aspects of the Association Between Plants and Mycorrhizal Fungi.

Mycorrhizal plants show increased growth (Thompson *et al.*, 1994; Bloss & Pfeiffer, 1984) and are generally more tolerant of adverse conditions such as drought (Parke *et al.*, 1983), poor soil nutrient status (Smith, 1988), soil pathogens (Cooper & Grandison, 1986; Dehne, 1982), transplantation (Haas *et al.*, 1986), and soil pollution (*e.g.* Gadd, 1993), compared to non-mycorrhizal controls. Improved plant growth and increased tolerance of adverse conditions can often be attributed to enhanced water and nutrient acquisition made possible by an extensive hyphal network which effectively increases the absorptive area of the root. The effectiveness of mycorrhizal fungi in increasing plant growth is not always directly related to the extent of root colonisation or hyphal growth. In *Eucalyptus globus*, plant dry weights were positively correlated with the length of colonised root for some EM species, but for others, plant growth resposes to innoculation could not be related to hyphal development (Thompson *et al.*, 1994). In field experiments on mycorrhizal guayle (*Parthenium argentatum*), latex content and biomass of mycorrhizal plants were increased but plant dry weight was unaffected by mycorrhizal infection. Increased survival rate of transplanted mycorrhizal seedlings was attributed to enhanced nutrient acquisition, but causal relationships for latex and biomass increases could not be established.

A primary effect of mycorrhizal symbiosis is improved P nutriton. The effects of mycorrhizal infection on plant survival can be substituted by P in non-mycorrhizal controls in many cases. However, studies of the interaction between mycorrhizal fungi and sedentary parasitic nematodes have provided evidence that resistance to soil pathogens can be related to factors other than improved plant nutrition. Investigations of the interaction between root knot nematode (*Meloidogyne hapla*) and VAM fungi on

5

susceptible cultivars of tomato and white clover revealed that phosphate concentration influenced nematode numbers (Cooper & Grandison, 1986). In mycorrhizal root systems, nematode numbers increased in low phosphate soils but were unaffected or reduced in high phosphate. Number of nematodes per gram of root was always less in mycorrhizal soils and plants pre-infected with mycorrhizal fungi showed improved growth compared to uninnoculated controls. The increased resistance to the pathogen in mycorrhizal plants was possibly due to alterations in root physiology, in addition to better plant nutrition. Indeed, mycorrhizal infection can stimulate induced resistance which is limited to the site of infection and which is effective against soil-borne pathogens (Dehne, 1982). Furthermore, mycorrhizal fungi are known to be able to inhibit organisms in the rhizosphere by the excretion of organic acids (Rasanayagam & Jeffries, 1992). There are thus several interacting factors in the relationship between mycorrhizas, plants and soil pathogens.

The undoubted advantage of mycorrhizal infection to drought-stressed plants is not based solely on increased water uptake by mycorrhizal roots. Drought-stressed mycorrhizal Douglas fir seedlings fixed CO_2 at a rate ten times that of non-mycorrhizal plants (Parke, Linderman & Black, 1983). Other workers have found relationships between photosynthesis and drought stress, in which improved nutrition of mycorrhizal plants in nutrient-poor soils is important. In Sitka spruce infected with *Paxillus involutus* in conditions of nutrient defficiency, stomatal conductance, net photosynthetic rate and water potential were higher than in non-mycorrhizal controls (Lehto, 1992a). During drought however, although mycorrhizal plants were in greater water deficit, these parameters were found to be similar in both treatments. A correlation was expressed between shoot P concentration and photosynthetic rate and the improved performance of mycorrhizal plants was considered to be due to improved nutrition and water uptake. With adequate nutrition, mycorrhizal plants had a lower water potential but no effect of mycorrhizal infection on stomatal conductance or net photosynthetic rate was observed (Lehto, 1992b). Again, it is clear that several physiological and environmental phenomena affect the plant-fungus relationship in the field.

Toxic metal pollution, arising from various industrial activities including mining and ancillary industries, is of serious local concern in many areas. In view of the beneficial effects of mycorrhizal infection on plant tolerance of toxic metals, there is much interest in the manipulation of mycorrhizal fungi for the amelioration of industrially polluted sites. Many reports describe the amelioration of zinc toxicity by mycorrhizal fungi. Zinc concentration around mycorrhizal root tips of birch (*Betula sp.*) was found to be significantly lower than that around the peripheral mycelium (Denny & Wilkins, 1987). It was postulated that as the mycelium radiates outwards, zinc is adsorbed by hyphae, and since it is relatively immobile, it is

replaced around the hyphal tips by diffusion at a slower rate than that by which it is taken up. This effectively reduces the concentration of zinc in the soil solution to a greater extent in mycorrhizal than non-mycorrhizal root tips. The fungal mycelium provides a large number of binding sites at which to sequester metal ions, thereby preventing their translocation to the plant.

The ecological importance of mycorrhizal fungi warrants their further study and their conservation. One aspect of concern is their potential susceptibility to fungicides. Because of increasing problems with fungicide resistance, new targets for fungicide action are being continually sought. New fungicide targets should be (1) specific to pathogenic fungi and (2) non-damaging to the host plant and to non-target organisms, including other fungi. A novel fungicide target which appears to meet these criteria in many respects is polyamine metabolism.

Polyamines.

The term *polyamine* collectively describes a large number of naturally-occuring aliphatic diamines, triamines and tetra-amines. They are relatively simple cationic organic compounds of low molecular weight. They exist as free bases or as N-acetyl derivatives strongly bound to cell components. A vital role of polyamines was first observed in 1948 when putrescine was found to be essential for the growth of the bacterium *Hemophilus parainfluenzae* (Herbst & Snell, 1948). In fact, the common polyamines, putrescine, spermidine and spermine are ubiquitous and are now known to be essential for the normal growth and development of plants, animals and micro-organisms (Tabor & Tabor, 1985; Porter & Sufrin, 1986; Galston & Kaur-Sawhney, 1990; Alhonen-Hongisto *et al.*, 1982). Although the precise function of polyamines remains uncertain, their vital role has been assumed for over 40 years on the basis of their ubiquity in nature, the correlation between polyamine biosynthesis and a variety of physiological events in plants, animals and micro-organisms, and the fact that specific inhibition of polyamine biosynthesis in these organisms leads to a cessation in growth and depletion of cellular polyamines.

Polyamine Biosynthesis.

Putrescine can be synthesised directly from ornithine by ornithine decarboxylase (ODC, EC 4.1.1.17) or indirectly from arginine by arginine decarboxylase (ADC, EC 4.1.1.19) *via* agmatine and N-carbamoylputrescine (Fig. 3a). Higher plants, bacteria and some fungi utilise both of these pathways for putrescine synthesis (Smith, 1985; Tabor & Tabor, 1985; Biondi, Polgrossi & Bagni, 1993). In contrast,

Figure 3 a. Polyamine biosynthetic pathways.

AMINO ACIDS
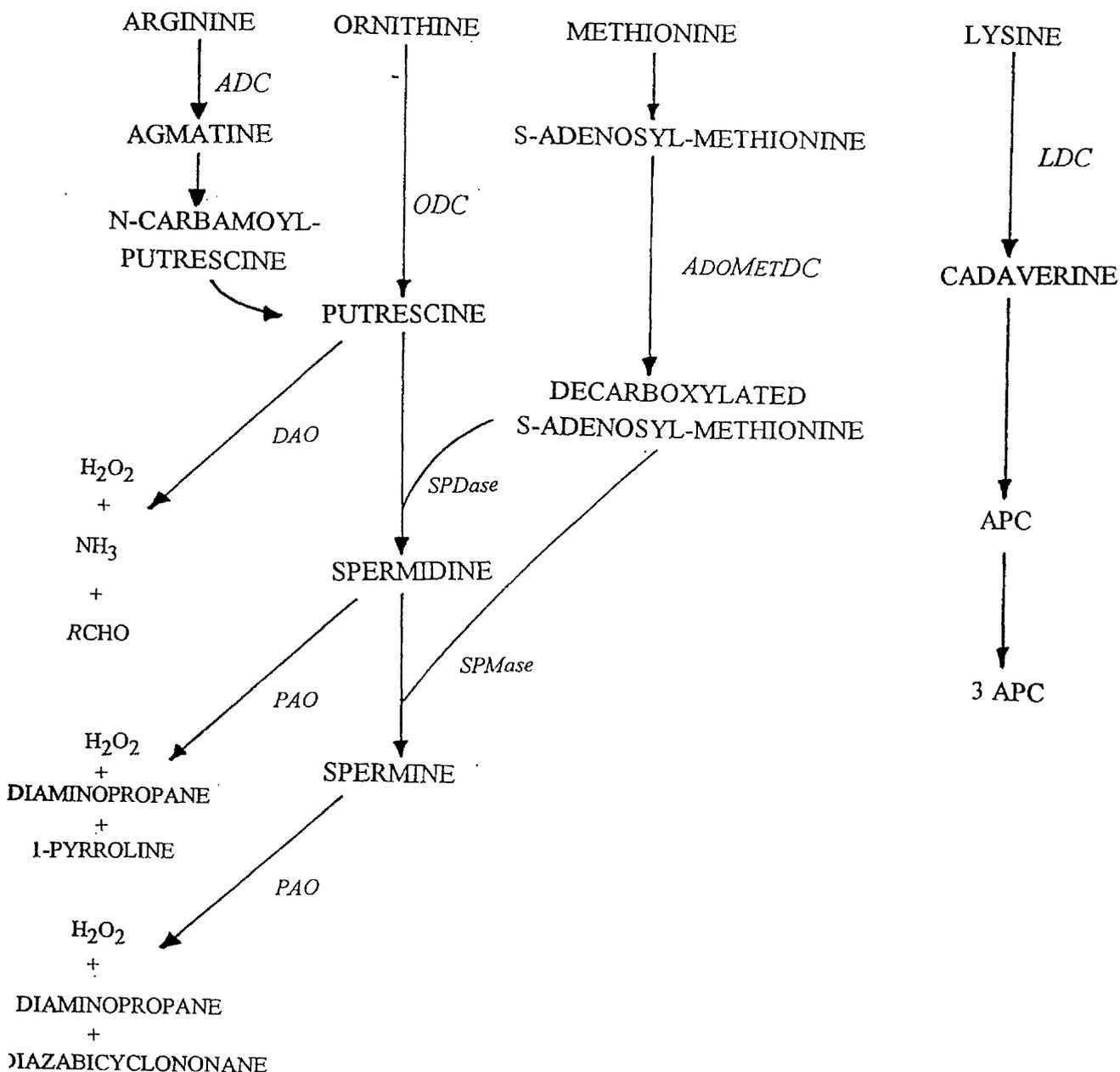
ARGININE   ORNITHINE   METHIONINE   LYSINE

ADC

AGMATINE   S-ADENOSYL-METHIONINE   *LDC*

N-CARBAMOYL-
PUTRESCINE   *ODC*   *ADOMETDC*   CADAVERINE

PUTRESCINE

DECARBOXYLATED
S-ADENOSYL-METHIONINE

DAO

H_2O_2
+
NH_3
+
RCHO   *SPDase*   APC

SPERMIDINE

SPMase

PAO   3 APC

H_2O_2
+
DIAMINOPROPANE   SPERMINE
+
1-PYRROLINE

PAO

H_2O_2
+
DIAMINOPROPANE
+
)IAZABICYCLONONANE

Abbreviations: **DFMO**, difluoromethylornithine; **DFMA**, difluoromethylarginine; **ADC**, arginine decarboxylase; **AdoMetDC**, S-adenosylmethionine decarboxylase; **PAO**, polyamine oxidase; **DAO**, diamine oxidase; **ODC**, ornithine decarboxylase; **APC**, aminopropylcadaverine; **3 APC**, N,N' bis (3 aminopropyl)cadaverine; **MGBG**, methylglyoxal bis(guanylhydrazone); **CHA**, cyclohexylamine; **SPDase**, spermidine synthase; **SPMase**, spermine synthase; **LDC**, lysine decarboxylase.

8

mammals and most fungi utilise only the ODC pathway (Porter & Sufrin, 1986). Specific inhibition of ornithine decarboxylation in a plant-pathogenic fungus should therefore inhibit fungal growth without damaging the host plant. The possibility of such an approach to disease control has been supported by several studies using difluoromethylornithine, an ODC inhibitor, and polyamine analogues, against powdery mildew and brown rust in cereals, and against bean rust and chocolate spot in broad bean (Rajam, Weinstein & Galston, 1985; Walters *et al.*, 1992). However, nothing is known about the effects of polyamine biosynthesis inhibitors on mycorrhizal fungi.

Spermidine and spermine are formed from putrescine by subsequent additions of aminopropyl groups [$NH_2(CH_2)_3$] from decarboxylated S-adenosylmethionine (SAM). The formation of these aminopropyl groups from SAM is catalysed by SAM decarboxylase (AdoMetDC, EC 4.1.1.50) and the aminopropyl group additions to putrescine catalysed successively by spermidine synthase (EC 2.5.1.16) and spermine synthase (EC 2.5.1.22) (Fig. 3a).

Cadaverine is a diamine formed from the decarboxylation of lysine (Fig. 3a). The occurence of cadaverine among organisms is sporadic. The aminopropylation of cadaverine leads to the formation of the higher homolgues aminopropylcadaverine (APC) and N,N' *bis* (3 aminopropyl)cadaverine (3 APC). These reactions are thought to be catalysed by AdoMetDC (for the formation of decarboxylated AdoMet) and the aminopropyltransferases, spermidine and spermine synthase. These cadaverine homologues have also been reported only infrequently in several bacteria (Hamana *et al.*, 1988, 1990), in human tumor cells (Alhonen-Hongisto & Janne, 1980), and in *Neurospora crassa* (Paulus *et al.*, 1982). The formation, distribution and significance of cadaverine will be discussed in more detail in Chapter 4.

Table 1. Chemical Structures of Polyamines.

POLYAMINE	STRUCTURE
Putrescine	$NH_2(CH_2)_3NH_2$
Spermidine	$NH_2(CH_2)_3NH_2(CH_2)_4NH_2$
Spermine	$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$
Cadaverine	$NH_2(CH_2)_5NH_2$
APC	$NH_2(CH_2)_3NH(CH_2)_5NH_2$
3 APC	$NH_2(CH_2)_3NH(CH_2)_5NH(CH_2)_3NH_2$

Polyamine Catabolism.

Experiments in which a polyamine, or its degradation product, is supplied to micro-organisms as the sole nitrogen, or carbon and nitrogen, source have suggested that there are separate routes for the degredation of free polyamines and acetylated forms. The oxidation of putrescine is catalysed by diamine oxidase (DAO, EC 1.4.3.6) and yields H_2O_2, NH_3 and RCHO compounds. Polyamine oxidase (PAO, EC 1.5.5.3) oxidises amines at the secondary amino group to give H_2O_2 and 1-pyrrolone and diaminopropane from spermidine, or diazabicyclononane and diaminopropane from spermine. These oxidation products may be cytotoxic (Large, 1992) and the generation of hydrogen peroxide and free radicals has been reported to account for putrescine-induced damage in maize seedlings (DiTomaso, Hart & Kochian, 1992).

Regulation of Polyamine Biosynthesis.

The polyamine biosynthetic pathway is tightly controlled so that changes in one part of the pathway invariably lead to changes in another, usually by some compensatory mechanism that attempts to sustain cellular polyamine levels. In a study of the effects of DFMO on ODC and ADC activities in *E. coli* and *Pseudomonas aeruginosa*, for example, Kallio & McCann (1981) observed that any decrease in intracellular ODC activity in the two organisms was invariably accompanied by a concomitant increase in ADC activity. Another aspect of this phenomenon is the capability of various diamines to fulfill the role of natural polyamines as substrates for spermidine and spermine synthesis, at least for short periods (Alhonen-Hongisto *et al.*, 1982). These workers, studying the effects of polyamine depletion in acsites tumour cells, suggested that the formation of cadaverine was induced by spermidine depletion in their experimental system. Cadaverine syntheis was repressed by increased levels of either spermidine or spermine. Thus increased AdoMetDC activity was associated with enhanced cadaverine production. Under these conditions, cadaverine provided a feeble substrate for spermidine synthase and was converted to the aminopropyl derivative, itself a close analogue of spermidine. Significantly, these authors point out that the existence of inducible, alternative pathways of polyamine biosynthesis make it difficult to acheive total polyamine depletion by inhibition of a single reaction of the pathway.

Functions of Polyamines.

Specific functions of polyamines have not been rigorously established. One role of polyamines in cells is linked to their positive charge which enables them to bind ionically with vital cell components such as nucleic acids, cell membranes and proteins. Because of their basic nature, the polyamines spermidine and spermine bind strongly with the acidic phosphate groups of nucleic acids. By binding to these structures, polyamines are important in confering stability to DNA and RNA. Indeed, polyamines, especially spermidine and spermine, are essential for cell division. Mutants of *Saccaromyces cerevisiae* deficient in ODC, AdoMetDC or putrescine aminopropyltransferase showed an absolute requirement for putrescine, spermidine and spermine for normal growth. Spermidine and/ or spermine were shown to be essential for sporulation in the organism and spermine was found to play a vital role in the control of ODC activity (Tabor, 1981). Other essential roles of these amines have been highlighted in other organisms. The filamentous fungus, *Neurospora crassa*, when deprived of polyamines, showed significantly elevated ODC activity. Normal enzyme activity was restored in the presence of either putrescine or spermidine, indicating the importance of these amines in the control of the degradation and synthesis of ODC in the fungus (Barnett, Sayfzadeh & Davis, 1988). This corroborated earlier work showing that putrescine functions in ODC inactivation, whilst spermidine is concerned with the formation of the active enzyme in *N. crassa*, although it is probably only a small percentage of total celluar spermidine that is active in this process (Davis *et al.*, 1985). A requirement for putrescine for conidial germination in *Aspergillus nidulans*, and other fungi, has been linked to the increase in ODC activity which accompanies germination (Stevens & Stevens, 1981). In fact, relatively little is known of polyamine function and metabolism in fungi.

Binding of polyamines to anions, especially in experimental acellular systems, may be due to non-specific ionic interactions rather than defined physiological processes, but much evidence points to their active role in cell biology. Polyamines are able to alter the stability and permeability of cell membranes *in vivo* by binding to phospholipid groups. It has been shown that in this way polyamines can stabilise chloroplast thylakoid membranes and reduce chlorophyll loss in senescing barley leaf tissue (Cohen, Popovik & Zalik, 1979). They also promote steps in the transcription-translation sequence during protein biosynthesis (Smith, 1985).

Several micro-organisms provide useful systems for studying the physiological role of polyamines because mutants lacking various components of the polyamine biosynthetic apparatus are available. In

11

functions related to growth, a definite molecular stereospecificity for spermidine in *E. coli* has been reported (Jorstad, Harada & Morris, 1980). Together with magnesium, spermidine may also be important in maintaining the tertiary structure of tRNA (Porter & Sufrin, 1986). Links between polyamines and DNA activity have been established. Thus, Geiger & Morris (1980) showed that spermidine and spermidine analogues affected the movement of the DNA replication fork in polyamine-deficient *E. coli* mutants. There also appears to be a relationship between Mg^{2+} and polyamines in nucleic acid metabolism, since polyamines can decrease, but not abolish, the requirement for Mg^{2+} in many in vitro microbial systems (Tabor & Tabor, 1985).

The universal occurrence of putrescine, spermidine and spermine in plants suggests their importance in these organisms. There are electrostatic interactions between polyamines and nucleic acids in plants. Polyamines are known to be involved with plant growth substances, responses to light and stress, and in growth regulation and senescence. Indole-3-butyric acid promoted putrescine synthesis from both ornithine and arginine in mung bean hypocotyl cuttings, indicating that auxin-induced root formation may depend on increased polyamine biosynthesis (Friedman, Altman & Bachrach, 1985). The identification of polyamines from vascular exudates of several plant species in concentrations directly related to plant age suggests that they undergo long-distance transport and supports the contention that they have a regulatory role in plant growth (Friedman, Levin & Altman, 1986). More putrescine was found in the exudate of salt-stressed plants than in unstressed controls. Indeed, putrescine accumulation, especially in cereals, is known to occur in response to a range of stresses including drought, high pH, deficiency or excess of some monovalent cations, atmospheric pollutants such as cadmium ion and anaerobiosis (Galston & Kaur-Sawhney, 1990). ADC activation appears to be the mediator of increased putrescine concentration under these circumstances.

Inhibitors of Polyamine Biosynthesis.

Interest in polyamine metabolism as a chemotherapeutic target was originally in the field of cancer treatment and is based on the following observations: (1) polyamines are essential in neoplastic cell proliferation and in normal cells stimulated to proliferate; (2) there is enhanced polyamine uptake and biosynthesis in neoplastic tissues; (3) inhibitors of polyamine biosynthesis can selectively slow neoplastic cell growth without apparent damage to the host cell. Several inhibitors of polyamine biosynthesis were

manufactured by Marion Merrell-Dow Pharmaceuticals in the 1970s, specifically for cancer treatment (Table 2).

Table 2. Inhibitors of Polyamine Biosynthesis.

INHIBITOR	SITE OF INHIBITION
DFMO α -difluoromethylornithine	ODC
MFMO (monofluoromethyl) dehydro-ornithine	ODC
MFMO.CH$_3$ (monofluoromethyl) dehydro-ornithine methyl ester	ODC
RRMAP (2R, 5R)-6-heptyne-2,5-diamine	ODC
DFMA difluoromethylarginine	ADC
CHA cyclohexylamine	spermidine synthase
MGBG methylglyoxal bis (guanylhydrazone)	AdoMetDC

Inhibitors of ODC.

Inhibitors of ODC can be categorised into three main groups: (1) product and substrate analogues, *e.g.* α -methylornithine; (2) molecules affecting the enzyme co-factor, pyridoxal phosphate, *e.g.* α-hydrazino-ornithine; (3) enzyme-activated inhibitors e.g. difluoromethylornithine. Molecules in groups (1) and (2) are reversible, competitive inhibitors of ODC. Despite the effectiveness of α-methylornithine and α -hydrazino-ornithine at inhibiting polyamine biosynthesis *in vitro*, their efficacy in *vivo* is limited by their tendancy to cause ODC accumulation, leading to increased putrescine production following metabolism of the inhibitors.

Enzyme-activated inhibitors are chemically inert molecules able to bind at the enzyme active site. The inhibitor molecule contains a moiety which is transformed by catalytic turnover of the enzyme, into a species capable of inactivating the enzyme. A number of substrate and product analogues of ODC are effective enzyme-activated inhibitors. The ornithine analogue, α-difluoromethylornithine (DFMO), (Table 2 &Fig. 3b) has made a significant contribution to the study of polyamine metabolism. Monofluoromethyl-dehydro-ornithine (MFMO), and its methyl ester, have been used in micro-organisms for the study of polyamine synthesis. They possess similar properties but the methyl ester moiety enhaces uptake of the

13

inhibitor into animal cells. Application of the polyamine analogue, (2R, 5R)-6-heptyne-2,5-diamine (RRMAP) in animal sytems leads to reduction in polyamine pools. The molecule is able to penetrate cells rapidly (Porter & Sufrin, 1986).

Inhibitors of AdoMetDC.

The regulation of AdoMetDC activity in cells is of fundamental importance in the control of polyamine biosynthesis, and accompanying processes, since the enzyme occupies a pivotal point in cell metabolism. Mammalian AdoMet decarboxylases are putrescine-activated. Methylglyoxal-bis(guanylhydrazone) (MGBG) is a potent competitive inhibitor of the mammalian enzyme (Fig. 3b). It is less active against some bacterial, plant and microbial AdoMet decarboxylases, possibly because these are either Mg^{2+}-activated, or require no putrescine or metal ion activation. The effect of MGBG on polyamine biosynthesis appears to be through competition with spermidine transport into the cell (Pegg & Williams-Ashman, 1987). However, MGBG has effects on other aspects of polyamine metabolism that limit its practical use as a specific inhibitor of AdoMetDC, including the inhibition of diamine oxidase, interference with polyamine transport, and the induction of polyamine acetyltransferase enzymes.

Inhibitors of Spermidine Synthase.

Experiments in which spermidine synthesis has been abolished in micro-organisms have shown the importance of spermidine synthase in providing polyamines for growth. Cyclohexylamine (CHA) inhibits spermidine synthesis by competing with the usual substrate, putrescine, for the active site (Bitonti & McCann, 1987, Fig. 3b). Spermidine synthases from a variety of plants, animals and micro-organisms, including trypanosomes, are sensitive to CHA.

The application of combinations of inhibitors may arrest the synthesis of compensatory polyamines and lead to more substantial growth inhibition. Both DFMO and MFMO.CH_3 have been applied to the treatment of cancer and trypanomiasis with success. Due to its minimal host toxicity, a major application of DFMO as an anticancer agent was in modulating the activities of existing drugs. As such, it has been used with MGBG and interferon. With the former, prior polyamine depletion by DFMO increases cellular uptake of MGBG by stimulating polyamine uptake mechanisms but the basis for synergism with interferon is unknown. Although MGBG has been used clinically since the late 1960s, its use is now restricted following patient toxicity recorded in 1979. The most significant practical success in the clinical

14

Figure 3 b. Polyamine biosynthetic pathways showing sites of inhibitor action.

AMINO ACIDS
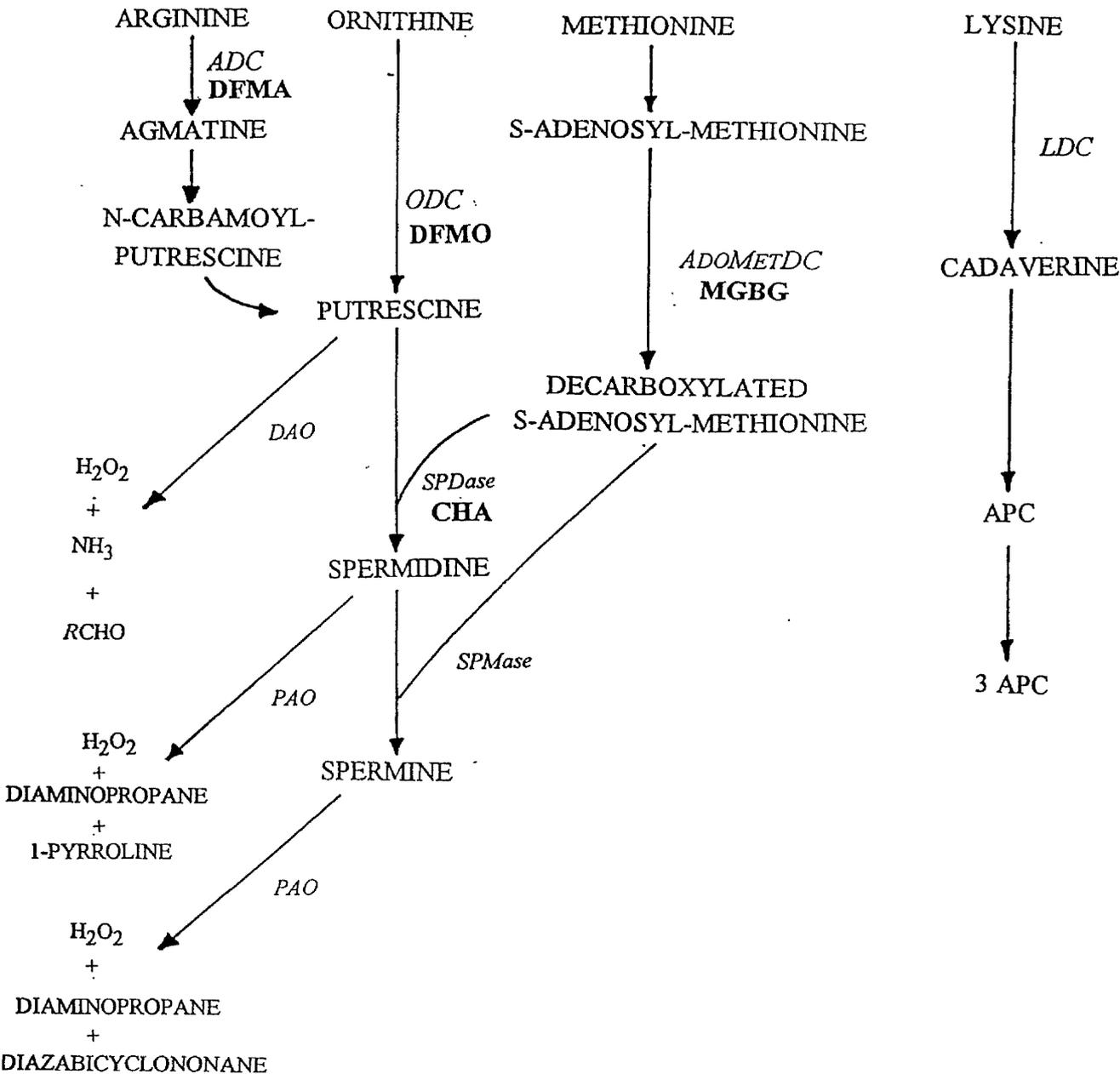
Abbreviations: **DFMO**, difluoromethylornithine; **DFMA**, difluoromethylarginine; **ADC**, arginine decarboxylase; **AdoMetDC**, S-adenosylmethionine decarboxylase; **PAO**, polyamine oxidase; **DAO**, diamine oxidase; **ODC**, ornithine decarboxylase; **APC**, aminopropylcadaverine; **3 APC**, N,N' bis (3 aminopropyl)cadaverine; **MGBG**, methylglyoxal bis(guanylhydrazone); **CHA**, cyclohexylamine; **SPDase**, spermidine synthase; **SPMase**, spermine synthase; **LDC**, lysine decarboxylase.

application of DFMO is in the treatment of sleeping sickness caused, by the protozoan *Trypanosoma brucei*, where the drug is marketed commercially for this purpose. These inhibitors provide useful tools for the study of polyamine metabolism in animals, plants and micro-organisms.

Control of Fungal Plant Diseases Using Inhibitors of Polyamine Biosynthesis.

The efficacy of polyamine biosynthesis inhibitors in the experimental control of fungal pathogens of plants is now well-established, but it was not until the 1980s that this potential was realised. Rajam & Galston (1985) originally demonstrated the inhibitory effects of DFMO and DFMA on fungal growth. DFMO and DFMA, added to solid growth media at concentrations of up to 1 mM, were able to inhibit the mycelial growth of four phytopathogenic fungi. This inhibition could be reversed by the addition of putrescine and spermidine to the culture medium. Rajam, Weinstein & Galston (1985) proposed the use of DFMO as a fungicide based on work showing that DFMO applied as a spray, at a concentration of 0.5 mM or greater, could protect bean (*Phaseolus vulgaris*) from infection by uredospores of the bean rust fungus *Uromyces phaseoli*.

A range of phytopathogenic fungi are susceptible to treatment with DFMO and DFMA including *Tilletia caries* and *T. controversa*, (Trione, Stockwell & Austin, 1988), *Verticillium daliae* (Mussell *et al.*, 1987), *Erysiphe graminis hordei* (Walters & Kingham, 1990), *Helminthosporium maydis* and *H. carbonum*, *Ceratocystis minor*, *C. ulmi* and *Fusarium oxysporum* (Khan & Minocha, 1989a,b), Botrytis cinerea (Smith *et al.*, 1990), and *Puccinia hordei, Botrytis fabae, Uromyces viciae-fabae* and *Pyrenophora avenae* (Walters *et al.*, 1992). These and simliar studies will be discussed in detail in Chapter 3.

Polyamine metabolism can also be perturbed by polyamine analogues which are efficiently taken up into the cell by the polyamine transport system. Polyamine analogues fulfill some of the functions of polyamines and as such may occupy the same binding sites as natural polyamines and thus prevent their normal function by displacing them from these sites. It is also possible for some analogues to mimic the regulatory role of natural polyamines and thereby to repress biosynthetic enzyme activity and/or stimulate biodegradative enzymes leading to the depletion of intracellular polyamines and the cessation of growth.

Commercially available polyamine analogues have been investigated for the their effects on fungal growth. The putrescine analogue, keto-putrescine, was fungicidal as a pre- or post-inoculatory spray against the biotrophic fungi *E. graminis* f. sp. *hordei* and *U. viciae-fabae*, the hemibiotroph *Phytophthora infestans* and the necrotrophic pathogen *B. fabae* (Foster & Walters, 1993). The compound was less effective in

16

inhibiting fungal growth *in vitro*, corroborating other data (*e.g.* Smith *et al.*, 1992) and suggesting that fungal germination and/or development on the leaf surface are affected by the inhibitor.

Synthetic putrescine analogues and their derivatives have now been produced specifically for the control of fungal plant pathogens and have been patented for this use (Havis *et al.*, 1994a,b,c; Robins & Walters, 1992 a,b). Control of a range of economically important crop pathogens has been achieved under laboratory, glasshouse and field conditions with formulated compounds. Since post-inoculatory sprays of these compounds are most effective, it appears that control is related to the perturbation of polyamine biosynthesis in the developing germling on the plant surface.

Despite great interest in the use of polyamine biosynthesis inhibitors for the control of fungal diseases of plants, there is still a paucity of information regarding the effects of polyamine inhibition on fungi at a biochemical level, and virtually no information concerning polyamine synthesis in mycorrhizal fungi. Most work on the biosynthesis of polyamines in fungi has necessarily been focussed on a limited number of species. Consequently, many of the biochemical and genetic aspects of polyamine synthesis are well-understood in *Saccaromyces cerevisiae, Neurospora crassa* and *Aspergillus nidulans*, but the wide variation in the response of many fungi to polyamine inhibition indicates that general conclusions regarding polyamine metabolism in these organisms cannot be clearly drawn. In order to usefully expand our knowledge of polyamine metabolism in fungi, several criteria were chosen for a comprehensive study of polyamine metabolism in four species of EM fungi:

(1) Fungi are useful organisms to use for the study of polyamine biosynthesis. They are simple eucaryotes that are often amenable to laboratory culture. They exhibit observable changes in physiology at morphological, biochemical and genetic levels in response to experimental manipulation.

(2) Fungi are important in biotechnology. An understanding of the physiological processes linked to polyamine metabolism would clearly be valuable.

(3) It is important to assess the vulnerability of EM fungi to polyamine biosynthesis inhibitors that are presently being developed as fungicides since these organisms are highly important in terrestrial ecosystems.

(4) Agriculturally important phytopathogenic fungi were also examined.

2. MATERIALS AND METHODS

CULTURE MEDIA AND FUNGAL GROWTH.

The mycorrhizal fungi *L. proxima* (Boud.) Maire, *P. involutus* (Fr.) Fr., *H. mesophaeum* (Pers.) Quel., and *T. terrestris* (Ehrh.), were maintained for long periods in 500 ml Erlenmeyer flasks containing 250 ml of a 1:9 v/v peat/vermicultie mixture with the addition of 180 ml modified Melin-Norkrans' medium (Mason, 1980). All experiments were performed in modified Hagem's medium (Mason, 1980) from which organisms were subcultured (Table 3). ·

Table 3. Media used for the culture of EM fungi.

	Hagem's Medium	Modified Melin-Norkrans' Medium
$MgSO_4.7H_2O$	0.25 g	0.15 g
KH_2PO_4	0.25 g	0.5 g
NH_4Cl	0.25 g	
1 % $FeCl_3$ solution	0.25 ml	1.2 ml
Glucose	2.5 g	10 g
Thiamine HCl	25 µg	100 µg
NaCl		0.025 g
$(NH_4)_2HPO_4$		0.25 g

Distilled water to 1 l.

Agar (3 %) was added for solid medium only.

Pyrenophora. avenae Ito & Kuribayashi, was cultured on potato dextrose agar. Potato extract was prepared by boiling 200 g of washed, unpeeled potatoes in 1 l distilled water, and squeezing the pulp through muslin. This extract was made up to 1 l with distilled water and contained 20 g dextrose and, for solid medium only, 3 % agar.

Spores of *Uromyces. viciae-fabae* (Pers.) Schroet were obtained from infected broad bean plants and collected 20 days after inoculation by brushing them into a lysine decarboxylase extraction buffer described below (Ikeson, Bakhanashvilli & Apelbaum, 1986) from which they were removed by centrifugation.

Measurement of Fungal Growth on Solid Medium.

Initial experiments using a range of inhibitor concentrations showed that 2 mM was the lowest concentration of any of the inhibitors to affect fungal growth significantly in susceptible species. Filter-sterilised inhibitor (2 ml) was added to 100 ml of sterile medium at 40-45 °C to give a concentration of 2 mM. Medium was poured aseptically into 90 mm plastic Petri dishes and inoculated with a 5 mm^2 mycelial plug cut from the peripheral edge of stock culture plates. Mycelial growth was measured across two diameters and the mean diameter calculated.

ENZYME ASSAYS.

ODC and AdoMetDC Assays.

Crude enzyme extracts were prepared by grinding fungus (500 mg) in a mortar and pestle in 2 ml of a buffer described by Stevens *et al.* (1976), of the following composition: 10 mM potassium phosphate; 2 mM 1,4 dithiothreitol (DTT); 1 mM MgCl$_2$; 0.1 mM EDTA; and 0.1 mM pyridoxal phosphate, at pH 7.6. The resulting suspensions were sonicated (Soniprep 150) for 10 cycles of 10 sec on/ 20 sec off. Test tubes were kept on ice during sonication and during subsequent steps in the assay. Each sample was centrifuged at 24,000 g for 15 min at 0°C.

The supernatant was transfered to a dialysis sac (cellulose dialysis tubing, Sigma) and dialysed in 40 ml buffer for 24 hr in darkness at 4°C. The pellet was redissolved in the same volume of buffer and similarly dialysed. Following dialysis, enzyme activities were assayed by measuring $^{14}CO_2$ released following incubation of enzyme extracts in a reaction medium containing [U-^{14}C]ornithine and S-adenosyl[1-^{14}C]methionine for ODC and AdoMetDC assays, respectively.

The ODC reaction medium contained 50 mM tris-HCl; 0.05 mM ornithine; 0.031 mM pyridoxal phosphate and 0.125 μCi [U-^{14}C]ornithine (Amersham). The AdoMetDC reaction medium contained 0.1 M sodium phosphate buffer pH 7.4; 0.2 mM S-adenosylmethionine; 1mM putrescine and 0.025 μCi S-adenosyl[1-^{14}C]methionine (Amersham). Assays were done in 98 mm glass test tubes fitted with silicone rubber stoppers (Vacutainer) and 35 mm 22-gauge needles. To each needle was affixed a filter paper (5 mm^2) impregnated with 10 μl 2 M KOH to trap $^{14}CO_2$ released during the reaction. The reaction was initiated by the addition of 100 μl enzyme extract to 300 μl reaction medium. Test tubes were placed in a water bath at 37 °C for 30 min, after which time the reaction was stopped by the addition of 200 μl perchloric acid to each tube. The tubes were then incubated for a further 30 min to allow the entrapment of

19

remaining $^{14}CO_2$. Filter papers were placed in scintillation vials (Packard) containing 12 ml Emulsifier Safe scintillant (Packard) and counted for radioactivity in a 1900 TR liquid scintillation analyser (Packard). Enzyme activity was expressed as pmol $^{14}CO_2$ mg protein^{-1} hr^{-1}. Protein was assayed by the Lowry method using BSA as a standard.

ADC Assay.

ADC activity was expressed in terms of [^{14}C]putrescine formation following the decarboxylation of a [U-^{14}C]arginine substrate. Crude enzyme extract was prepared by grinding fungus in 100 mM potassium phosphate buffer (pH 7.4) containing 5.5 mM dithiothreitol; 1.5 mM EDTA and 50 µM pyridoxal phosphate, and using 250 mg fungus per ml buffer. Preparations were centrifuged at 14,000 g for 10 min at 0°C and the ADC extract retained in the supernatant. The reaction was initiated by the addition of a 200 µl aliquot of supernatant to a reaction mixture composed of 40 µl 3.88 mM unlabelled arginine and 1.6 µl L-[U-^{14}C]arginine monohydrochloride. Assays were performed in 98 mm glass test tubes incubated at 37 °C for 30 min. The reaction was stopped by the addition of 200 µl perchloric acid. A 100 µl aliquot of the reaction mixture was taken after completion of the assay for [^{14}C]putrescine analysis as described below for polyamine assays.

DAO and PAO Assays.

Assays of DAO and PAO were adapted from the method of Okuyama & Kubayashi (1961). Crude enzyme extract was prepared by grinding fungus in 100 mM potassium phosphate buffer (pH 8) containing 2 mM dithiothreitol and using 100 mg tissue per ml buffer. The homogenate was centrifuged at 20,000 g for 20 min at 4°C. For DAO assays, 0.5 ml supernatant was added to 0.5 ml of 100 mM phosphate buffer (pH 8) containing: 1 mM putrescine; 0.15 µCi [^{14}C]putrescine (108 mCi mmol^{-1}, Amersham) and 30 µg catalase. For PAO assays the buffer contained 1 mM spermidine, 0.15 µCi [^{14}C]spermidine (117 mCi mmol $^{-1}$, Amersham), and 30 µg catalase. Samples were incubated at 37°C for 30 min and the reaction then stopped by the addition of 1 ml of 4 M sodium hydroxide. Reaction products were extracted in 2 ml toluene, mixed for 20 sec and a 1 ml aliquot of the (upper) toluene phase transferred to 10 ml scintillant for radioactive counting as described above. Enzyme activity was expressed as pmol product mg $^{-1}$protein hr^{-1}.

POLYAMINE ANALYSIS.

Methods for polyamine assay were adapted from Slocum & Flores (1991). Fungus (300 mg) was macerated in 2 ml 10% (v/v) perchloric acid and the homogenate centrifuged at 24,000 g for 25 min at 0°C. Polyamines were retained in the supernatant. To 100 µl supernatant were added 200 µl saturated Na_2CO_3 and 400 µl dansyl chloride (10 mg ml^{-1} of acetone). This mixture was incubated in darkness for 20 min at 60 °C, after which 100 µl proline (100 mg ml) were added. After a 10 min incubation in darkness at room temperature, dansylated polyamines were extracted in 500 µl toluene and shaken for 20 sec. Aliquots of 25 µl of the toluene phase were spotted onto activated silica gel TLC plates (Whatman LK6D) and allowed to develop in chloroform/triethylamine (12:1 v/v). Polyamines were visualised under ultraviolet light, removed and placed in 4 ml ethyl acetate. Fluorescence was measured at an excitation wavelength of 365 nm and an emission wavelength of 506 nm.

Polyamine assays were performed individually for each treatment, together with a corresponding control for that treatment. This procedure was necessary since dansylated polyamines are unstable and must be processed rapidly. This could only be achieved by assaying treatments separately, thereby reducing sample numbers.

In vivo Incorporation of [U-^{14}C]lysine, [U-^{14}C]arginine and [U-^{14}C]ornithine into Polyamines.

Inhibitor treatments (2 mM) were made up in liquid culture medium with the addition of 0.2 µCi [U-^{14}C]lysine (specific activity 319 mCi $mmol^{-1}$, Amersham), 0.1 µCi [U-^{14}C]arginine (specific activity 342 mCi $mmol^{-1}$, Amersham) or [U-^{14}C]ornithine (specific activity 282 mCi $mmol^{-1}$, Amersham). Fungus (200 mg) was incubated, unshaken, in this medium for 24 hr at 20 °C and the samples then treated as described for polyamines. Spots corresponding to polyamines were removed to 10 ml scintillant (Emulsifier Safe, Packard) for radioactive counting. (2 mM) were made up in growth medium. Control treatments contained no inhibitor.

In vitro incorporation of [U-^{14}C]lysine into Polyamines.

The decarboxylation of a [U-^{14}C]lysine substrate, and subsequent reactions leading to the formation of cadaverine and polyamines, were allowed to proceed for 1 hour in the presence of a lysine decarboxylase extraction buffer prepared from each fungus according to Ikeson *et al.* (1986). This buffer contained 50 mM Tris-HCl , 0.5 mM EDTA and 5 mM dithiothreitol. Fungus (500 mg) was macerated in 1.5 ml buffer before centrifugation at 5000 *g* for 15 min at 4 °C. The lysine decarboxylase extract was

21

retained in the supernatant from which a protein determination was also made.Following the in vitro incubation, 100 µl aliquots of the reaction mixture were taken for polyamine analysis as described above.

In vivo incorporation of [U-^{14}C]aspartate into Polyamines.

Fungus was incubated, unshaken, in a buffer containing 10 mM potassium phosphate; 2 mM 1,4 dithiothreitol; 1 mM magnesium chloride; 0.1 mM EDTA; 0.1 mM pyridoxal phosphate; and 1 mM lysine with the addition of 0.2 µCi [U-^{14}C]aspartate (specific activity 216 mCi mmol^{-1}, Amersham), for various times at 20 °C. Following incubation, polyamines were extracted as described above.

Identification of N,N' *bis* (3 aminopropyl) cadaverine.

The presence of 3 APC was confirmed by nuclear magnetic resonance spectroscopy (NMR). Dansylated polyamines were extracted from reaction mixtures as described above. Spots corresponding to authentic 3 APC were eluted in 3 ml ethyl acetate and shaken. After 24 hours at 4 °C, the silica gel was removed by centrifugation at 3000 rpm for 5 min. The sample was evaporated to dryness at room temperature under nitrogen for subsequent NMR analysis.

EXPOSURE OF *P. INVOLUTUS* TO TOXIC METALS.

P. involutus was grown for 2 weeks in modified Hagem's medium to which the metal under examination had been added. Toxic metals were supplied as their salts (Table 4).

Table 4. Concentrations of metals commonly used in toxicity tolerance tests (Bradshaw & McNeilly, 1981).

Metal	Concentration in test solution (µg ml^{-1})	Salt	Salt concentration in stock solution (g l^{-1})
Copper	0.5	$CuSO_4.5H_2O$	0.393
Zinc	7.5	$ZnSO_4.7H_2O$	0.66
Lead	12.0	$Pb(NO_3)_2$	3.837
Nickel	2.0	$NiSO_4.7H_2O$	1.913

Aliquots of stock solution were added to growth medium to give the appropriate concentration of metal in the test solution. Subsequent assays were performed as described above. Measurements of metal

ions in fungal tissue were made by atomic absorption spectroscopy, following exposure of *P. involutus* to the metal for 2 weeks.

DATA ANALYSIS.

Results are the means of five replicates with standard errors and experiments were repeated several times. Error bars were ommitted from graphs when standard errors were too small to be clearly shown.

Statistical significance was assessed using Student's *t*-test and expressed as: * $P \geq 0.05$; ** $P \geq 0.01$; *** $P \geq 0.001$; ns = not statistically significant (i.e. $P < 0.05$); nd = not detected in assay.

3. EFFECTS OF POLYAMINE BIOSYNTHESIS INHIBITORS ON GROWTH, ENZYME ACTIVITIES AND POLYAMINE CONCENTRATIONS IN MYCORRHIZAL FUNGI.

INTRODUCTION.

Since plants are able to synthesise polyamines by either ornithine or arginine decarboxylation, whilst most fungi are capable only of ornithine decarboxylation, it has been shown that specific inhibition of fungal ODC can control fungal diseases without affecting the plant (Rajam *et al.*, 1985; Walters *et al.*, 1992). However, very little is known about the effects of polyamine inhibitiors, or indeed of conventional pesticides, on mycorrhizal fungi which are a necessary component of most plant systems. In view of the extremely important role of EM and VAM fungi in plant nutrition, litter decomposition and nutrient cycling, it is essential to be able to protect these organisms from harm. It is important to note that not all agrochemicals are necessarily harmful to mycorrhizas. Some herbicides (e.g. simazine) and fungicides, for example, stimulate mycorrhiza formation (Smith & Ferry, 1977; Spokes *et al.*, 1981), possibly by the suppresion of organisms normally antagonistic to mycorrhizal fungi. However, many agrochemicals have undoubtedly harmful effects on mycorrhizas and plant growth. Rhodes & Larson (1981) showed a suppression of mycorrhizal development and plant growth in creeping bent grass that had been treated with a variety of fungicides. Reduced plant growth was attributed to reduced nutrient uptake by the plant in the absence of the VAM fungus. The mechanism of action of the chemicals on the fungus was uncertain. *T. terrestris* and other EM Basidiomycetes were found to be susceptible to an experimental systemic fungicide developed for the control of pathogens of nursery *Pinus* seedlings (Kelley, 1978). Kelley & South (1978) found growth of the same Basidiomycetes to be inconsistently affected by a variety of hebicides *in vitro*. Again, the physiological mechanisms of toxicity could not be identified. It is of concern that a variety of agrochemicals are known to harm mycorrhizal fungi and furthermore, that some of these substances have remained in use a decade after their effects were first demonstrated. Consequently, there is a need to develop new fungicide targets which are specific to pathogenic fungi and non-damaging to other organisms. Polyamine metabolism meets these needs in many respects and several polyamine metabolic inhibitors have been studied for their fungicidal potential.

DFMO is a so-called mechanism-based, irreversible inhibitor of ODC that acts by binding to the enzyme such that binding of the natural substrate (ornithine), is prevented. It is of low toxicity to mammals and has been used extensivley as an experimental antifungal agent for plant disease control (see Chapter 1). A range of ODC and ADC inhibitors have now been evaluated for their effects on phytopathogenic fungi.

Fungal growth, and disease control in infected glasshouse plants have been most widely studied (e.g. Rajam et al., 1985; Mussell et al., 1987; West & Walters, 1988). Foliar application of DFMO delayed the appearance of symptoms of Verticillium wilt in tomato, and at a concentration of 5 mM, reduced the severity of the disease (Mussell et al., 1987). Substantial control of the powdery mildew fungus (Erysiphe graminis hordei) was acheived following treatment with DFMO applied as a drench at a concentration of 5 mM to the roots infected barley plants (Walters & Kingham, 1990). These authors showed that DFMO was taken up rapidly by the roots of barley seedlings and transported to the shoots. Uptake appeared to be an active process similar to the uptake of amino acids in several plant systems. Indeed, amino acid-based ODC inhibitors were found to be more readily taken up than amine-based inhibitors in Botrytis cinerea (Smith et al., 1990). DFMO also possesses some phloem mobility since it was transported from the upper leaves to the lower leaves and roots, although at very low rates (Walters & Kingham, 1990). Although many workers report the anti-fungal and fungicidal activity of DFMO, and other polyamine biosynthesis inhibitors, it was not known whether the inhibitory effects observed were in fact due to the inhibition of polyamine biosynthesis. An important limitation to the use of DFMO as a fungicide is its inability to deplete the cell of spermine. This may be due to incomplete inhibition of the pathway since AdoMetDC activity will be unaffected..

Foster & Walters (1990), working on the oat-stripe pathogen Pyrenophora avenae, showed that DFMO inhibited fungal growth and reduced ODC activity and spermidine levels in fungal tissue. Moreover, combined use of DFMO and MGBG, to inhibit both ODC and AdoMetDC, was very effective in reducing fungal growth. This treatment led to substantial reductions in the activity of ODC and AdoMetDC and spermidine levels.

DFMO was also found to be effective against powdery mildew in spring barley in field trials, when it gave early season control at least as good as that obtained with a commercial fungicide (Early Impact) (Havis & Walters, 1992). Two synthetic putrescine analogues have also been shown to control mildew in barley in two trials (Havis et al., 1994a,b).

Post-inoculatory sprays of DFMO and MGBG were more effective than pre-inoculatory sprays against Erysiphe graminis f.sp. hordei on barley. Pre-inoculatory sprays may be less effective due to loss of inhibitor by binding to ODC within the plant, made possible by the rapid rate of turnover of the enzyme. Mixtures of DFMO and MGBG also gave significant mildew control (West & Walters, 1988).

Species-dependent variation in the response of fungi to polyamine biosynthetic inhibitors is apparent. Spore germination and germ tube elongation were susceptible to both DFMO and DFMA in

25

oli (Rajam *et al.*, 1989) but were unaffected in *Tilletia caries* and *T. controversa* (Trione *et* ... ...though DFMO reduced ODC activity in both *Septoria tritici* and *Ustilago maydis*, growth of ... was relatively unaffected and spermidine concentrations were maintained (Smith *et al.*, ...the interspecific differences in fungal polyamine metabolism correlate with the evolutionary ...the organisms. In most fungi, spermidine is the most abundant polyamine, and spermine the least ... slime moulds however, putrescine is the predominant amine (Stevens, 1981). AdoMetDC ...ed by putrescine in the higher fungi, but not in the slime moulds, whose polyamine distribution ...sembles that of the Protozoa. However, variation in polyamine metabolism persists within phylogenetically-related groups of fungi. For example, whilst it has been assumed that fungi do not possess ADC activity, evidence is available that suggests the contrary. Biosynthetic and biodegredative forms of ADC have been described in bacteria and have been distinguished on the basis of their kinetic properties. The biosynthetic enzyme is constitutive and always present in the cell. The biodegredative form is inducible in the presence of excess arginine (Tabor & Tabor, 1985). Khan & Minocha (1989a,b) reported significant biosynthetic ADC activity, together with low ODC activity, in the phytopathogenic fungi *Ceratocystis minor, C. ulmi, Verticillium daliae, Helminthosporium maydis, H. carbonum*, and *Fusarium oxysporum* f. sp. *lycopersici* , after measuring the release of $^{14}CO_2$ from DL-$[1-^{14}C]$arginine and L-$[1-^{14}C]$ornithine, respectively. This has been corroborated by Biondi, Polgrossi & Bagni (1992) who detected ADC activity in *C. ulmi* (*Ophiostoma ulmi*) using similar techniques.

However, fungicidal performance in the glasshouse is not always matched by similar performance in the field, since experimental compounds are not *formulated*. Formulation of a pesticide involves the addition of chemical adjuvants to the pesticide medium to enhance its physical and chemical qualities. Detergent adjuvants, for example, are used to improve the distribution and adhesion of the pesticide to the plant surface. Formulation also involves chemical modifications of the active compound to increase its biocidal efficacy. Thus, formulation of a synthetic putrescine analogue has greatly improved both glasshouse and field trial performance (Havis & Walters, unpublished).

Given the present interest in the use inhibitors of polyamine biosynthesis as fungicides, and our limited knowledge of polyamine metabolism in mycorrhizal fungi, work was undertaken to examine the efects of DFMA, DFMO, MGBG and CHA on growth, enzyme activities and polyamine concentrations in the four EM fungi using techniques described in Chapter 2. The four species, described below, were chosen for ease of axenic culture and their diverse morphology and ecology.

Plates 1 - 4 reproduced by kind permission of Mr. Roger Phillips.

Plate 1. *Laccaria proxima* ×1.

Plate 2. *Paxillus involutus* ×1.

Plate 3. *Hebeloma mesophaeum* ×1.

Plate 4. *Thelephora terrestris* ×1.

Laccaria proxima (Boud.) *syn. L. laccata* var *proxima* (Boud.) **Maire. Habitat** on poor heath and bog soils, most frequent in autumn. Mycorrhizal with birch (Plate 1).

Paxillus involutus (Fr.) **Fr.** Brown Roll-Rim. **Habitat** in broad-leaved woodland, especially with birch, most frequent in autumn (Plate 2).

Hebeloma mesophaeum (Pers.) **Quel. Habitat** in damp woodland, often on burnt ground, from late summer to early winter (Plate 3).

Thelephora terrestris (Ehrh.) **Fr.** Earth Fan. **Fruiting body** fan-shaped 30-60 mm diameter, in clusters. **Habitat** in conifer woods or heaths, usually on sandy soil from late summer to early winter (Plate 4).

RESULTS.

L. proxima.

Growth on solid medium Radial growth of *L. proxima* was increased by DFMA and retarded by DFMO. Although these changes were small, they were significantly different from the control (Fig 4).

ODC and AdoMetDC activities All treatments were associated with increased soluble AdoMetDC activity and unchanged soluble ODC activity. The bound activities of each enzyme fraction were increased by DFMA and decreased by DFMO. Whereas bound ODC activity was decreased by the inhibitor combination, bound AdoMetDC activity was increased by this treatment (Figs. 5 & 6).

DAO and PAO activities Apparent but statistically non-significant increases in DAO activity were observed with all treatments. PAO activity was depressed by DFMO and increased by both DFMA and the inhibitor combination (Fig. 7).

ADC activity ADC activity, measured by following the release of $^{14}CO_2$ and [^{14}C]putrescine from [U-^{14}C]arginine, was detected in *L. proxima*. Although the release of $^{14}CO_2$ was not affected by DFMA, formation of [^{14}C]putrescine was eliminated by exposure to DFMA (Table 5).

Polyamine concentrations DFMO treatment was associated with increased concentrations of all polyamines, although the increase in putrescine concentration was not statistically significant. Putrescine and spermidine concentrations were decreased by DFMA, but the decrease in spermidine was not

31

statistically significant. Spermine concentration was not significantly affected by DFMA. The inhibitor combination was associated with increased concentrations of all three polyamines (Figs. 8, 9 & 10).

Incorporation of [U-^{14}C]arginine and [U-^{14}C]ornithine into polyamines DFMA completely inhibited the conversion of [U-^{14}C]arginine into polyamines, whilst DFMO did not affect putrescine synthesis from [U-^{14}C]arginine. Spermidine and spermine could not be detected following incubation with [U-^{14}C]arginine (Fig. 11).

DFMA significantly enhanced the synthesis of spermidine and spermine from [U-^{14}C]ornithine, but did not significantly affect putrescine synthesis. DFMO inhibited putrescine and spermidine synthesis and significantly enhanced spermine synthesis from [U-^{14}C]ornithine (Fig. 12).

P. involutus.

Growth on solid medium Radial growth of *P. involutus* was significantly inhibited by both DFMA and DFMO (Fig. 13).

ODC and AdoMetDC activities All treatments significantly inhibited ODC activity. The activity of the soluble fraction was abolished by DFMA. Soluble AdoMetDC activity was also reduced by all treatments, with the inhibitor combination being most effective and DFMA least effective. Activity of the bound fraction was reduced only by DFMO, with DFMA and the inhibitor combination having no significant effect (Figs. 14 & 15).

DAO and PAO activities Whilst exposure to DFMA was associated with increased DAO activity, both DFMO and the inhibitor combination caused significant reductions in enzyme activity. Significant reductions in PAO activity were associated with DFMA treatment and the inhibitor combination but DFMO had no significant effect on enzyme activity (Fig. 16).

Polyamine concentrations Exposure to DFMO was not associated with reduced polyamine concentrations. Thus Pputrescine and spermidine concentrations were unaffected by DFMO treatment, whilst spermidine concentrations were substantially increased. DFMA treatment led to reductions in putrescine and spermidine, and complete depletion of spermine. Apparent but statistically non-significant increases in the

32

concentrations of spermidine and spermine were associated with the inhibitor combination, although putrescine concentration was substantially decreased by this treatment (Figs. 17, 18 & 19).

T. terrestris.

Growth on solid medium DFMA did not affect radial growth of *T. terrestris*, while growth was substantially reduced by DFMO (Fig. 20).

ODC and AdoMetDC activities DFMA, DFMO and the inhibitor combination were associated with successively greater increases in the bound activities of both ODC and AdoMetDC. Neither DFMA nor DFMO substantially reduced the activities of the soluble fractions of these enzymes but the inhibitor combination produced a significant reduction in soluble ODC activity and an abolition of soluble AdoMetDC activity (Figs. 21 & 22).

DAO and PAO activities Whilst treatment with DFMA and DFMO led to increased DAO activity, these inhibitors had no effect on PAO activity. The inhibitor combination however, was associated with substantially increased DAO, and an abolition of PAO activity (Fig. 23).

Polyamine concentrations Although putrescine concentration was apparently reduced by DFMA treatment and increased by DFMO and the inhibitor combination, these effects were not statistically significant. Spermidine concentrations were reduced, and spermine entirely depleted by both DFMA and the inhibitor combination. DFMO treatment did not affect spermidine concentration but significantly reduced that of spermine (Fig. 24).

H. mesophaeum.

Growth on soild medium Radial growth of *H. mesophaeum* was unaffected by DFMO but significantly increased by DFMA (Fig. 25).

ODC and AdoMetDC activities Inhibition of ODC was not achieved by any treatment. Activity of the bound fraction was substantially increased by the inhibitor combination, but unaffected by either DFMA or DFMO. Activity of the soluble fraction was not significantly affected by any treatment (Fig. 26). Apparent but statistically non-significant increases in bound AdoMetDC activity were associated with all three

33

Statistical differences shown as: * $P \geq 0.05$; ** $P \geq 0.01$; *** $P \geq 0.001$; nd = not detected in assay; ns = not statistically significant *i.e.* $P < 0.05$.
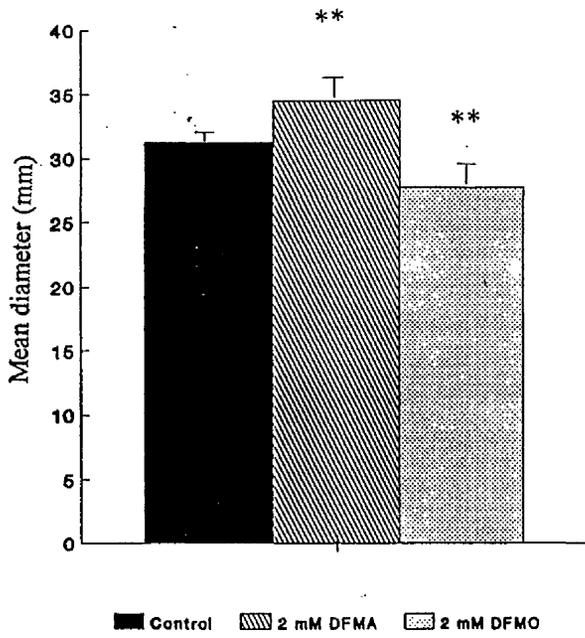
Figure 4. Effect of DFMA and DFMO on growth of *L. proxima*.
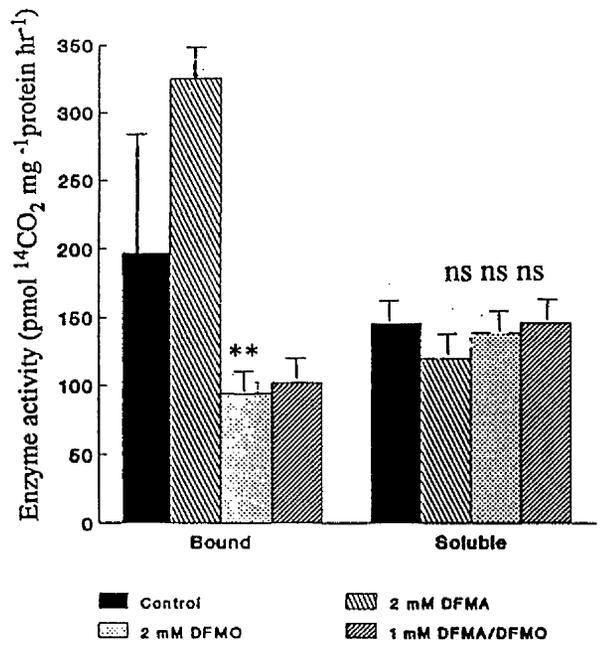

Figure 5. Effect of DFMA and DFMO on ODC activity in *L. proxima*.
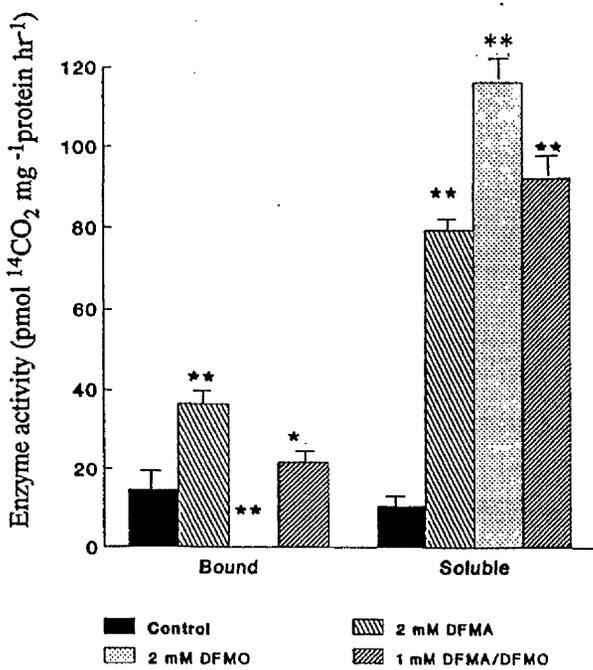
Figure 6 . Effect of DFMA and DFMO on AdoMetDC activity in *L. proxima*.
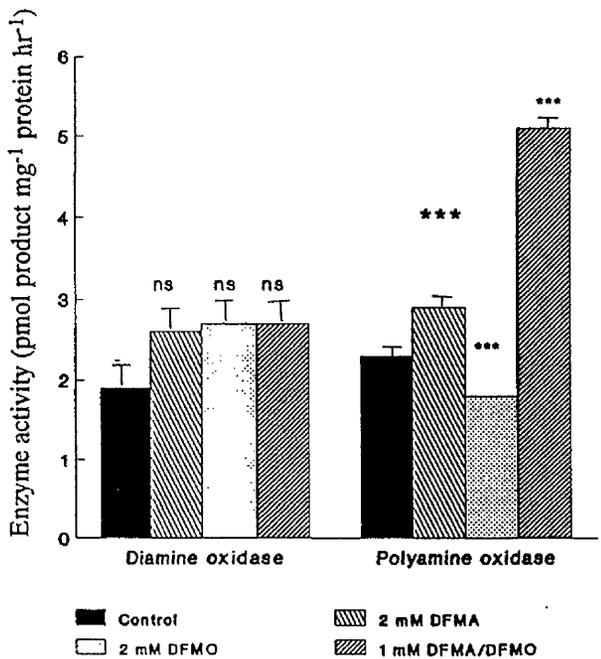
Figure .7 . Effect of DFMA and DFMO on DAO and PAO activities in *L. proxima*.

34
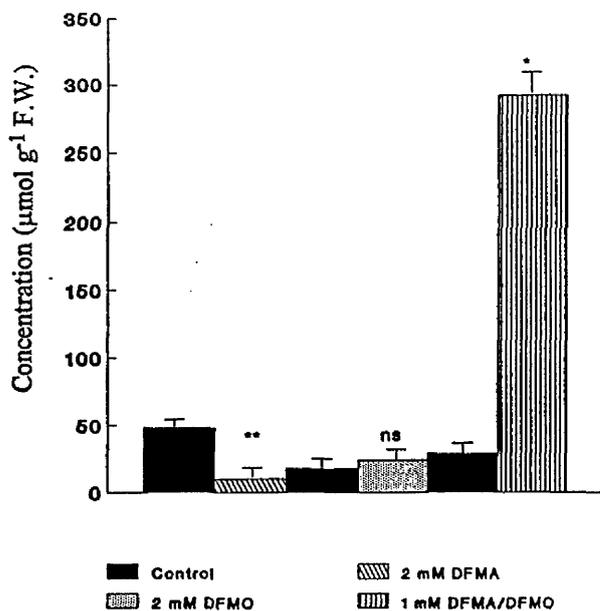
Figure 8. Effect of DFMA and DFMO on putrescine concentration in *L. proxima*.
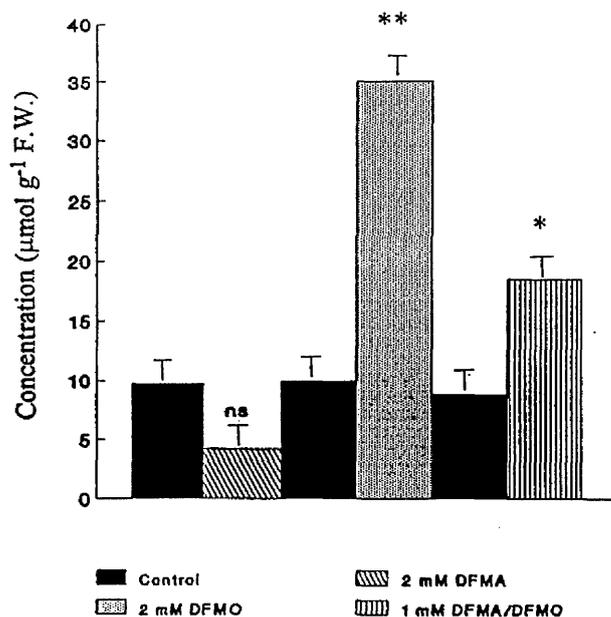
Figure 9. Effect of DFMA and DFMO on spermidine concentration in *L. proxima*.
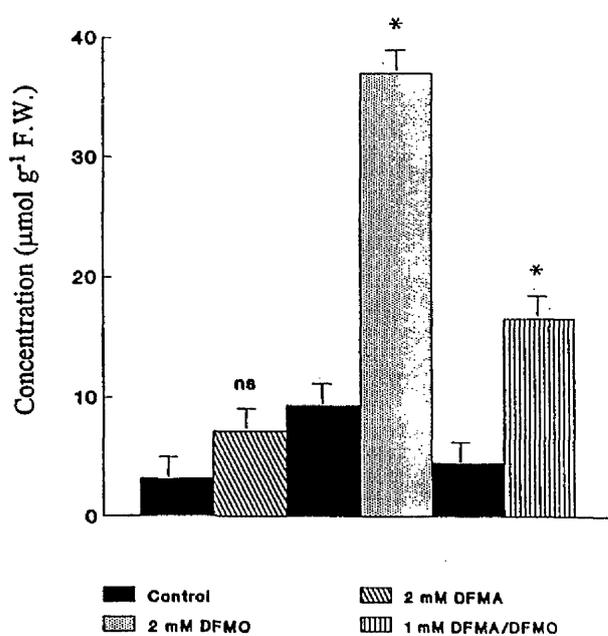
Figure 10. Effect of DFMA and DFMO on spermine concentration in *L. proxima*.
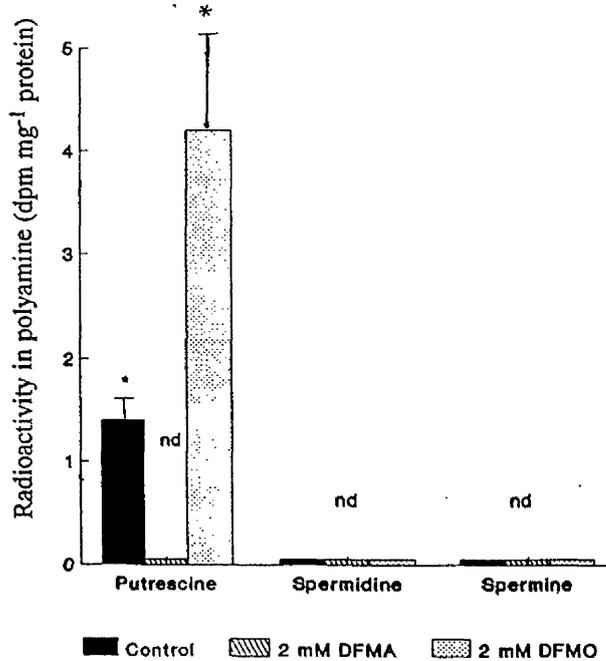
Figure 11. Effect of DFMA and DFMO on the incorporation of [U-^{14}C]arginine into polyamines in *L. proxima*.
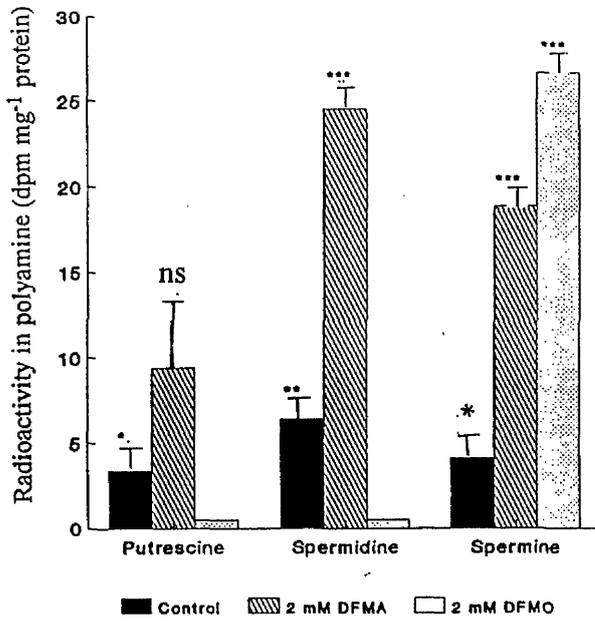
Figure 12. Effect of DFMA and DFMO on the incorporation of [U-^{14}C]ornithine into polyamines in *L. proxima.*
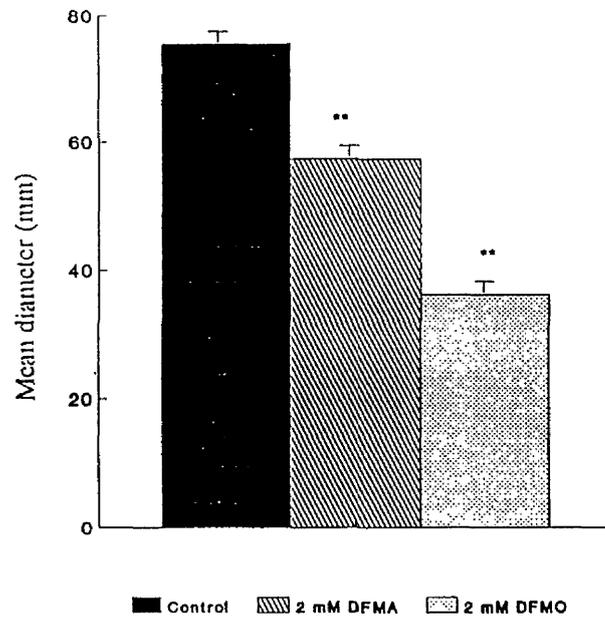
Figure 13. Effect of DFMA and DFMO on growth in *P. involutus.*
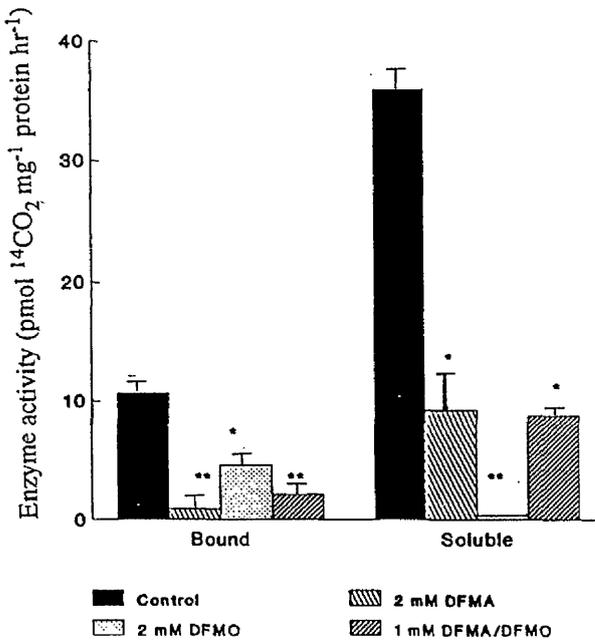
Figure 14. Effect of DFMA and DFMO on ODC activity in *P. involutus.*
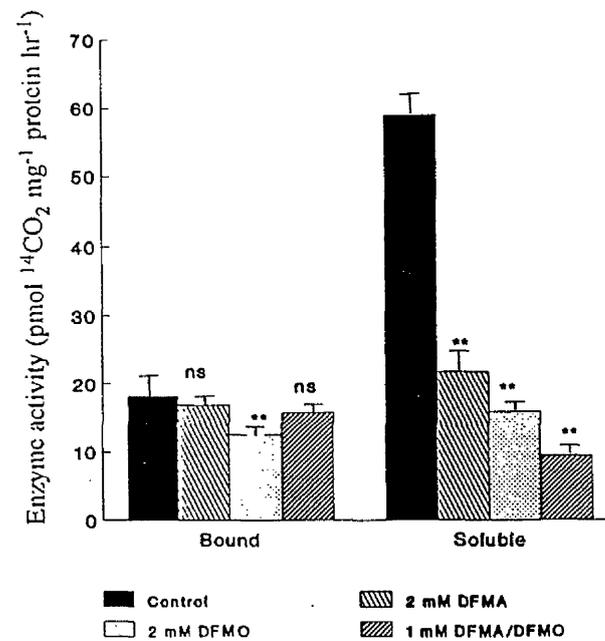
Figure 15. Effect of DFMA and DFMO on AdoMetDC activity in *P. involutus.*

36
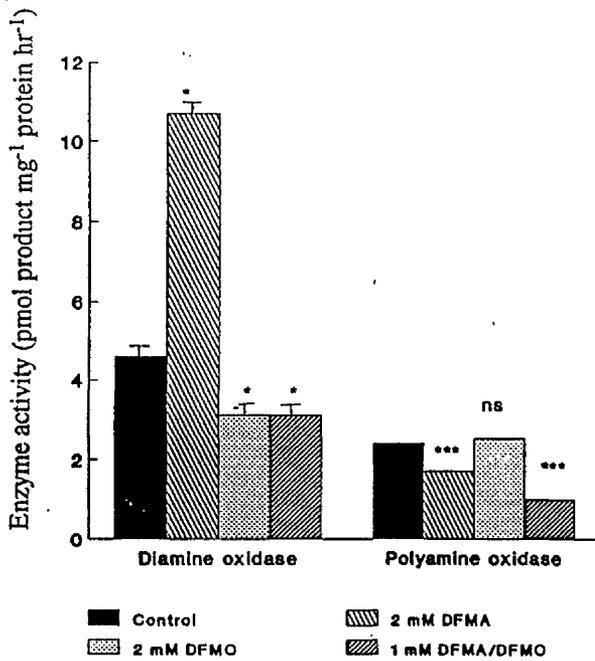
Figure 16 Effect of DFMA and DFMO on

DAO and PAO activities in *P. involutus*.
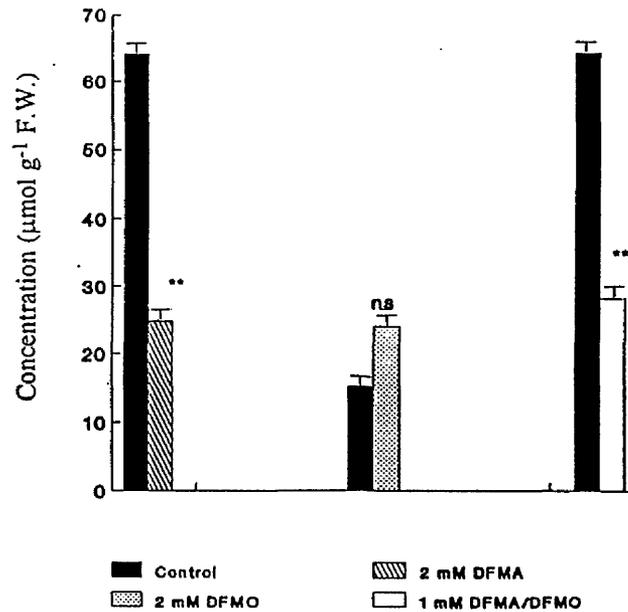
Figure 17. Effect of DFMA and DFMO on

putrescine concentration in *P. involutus*.
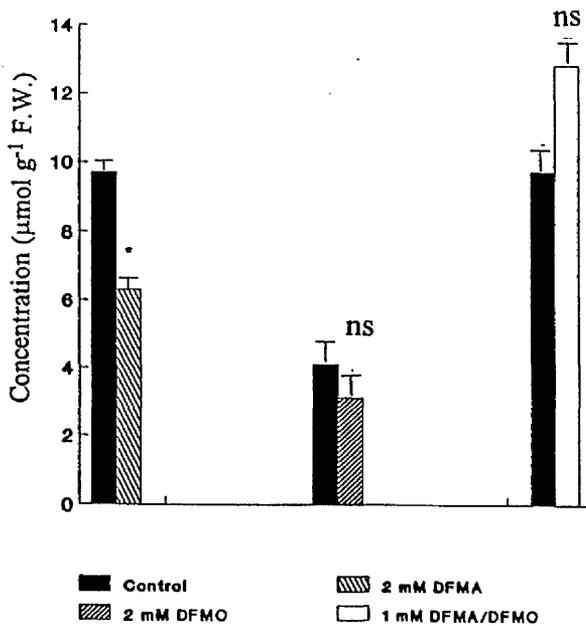
Figure 18. Effect of DFMA and DFMO on

spermidine concentration in *P. involutus*.
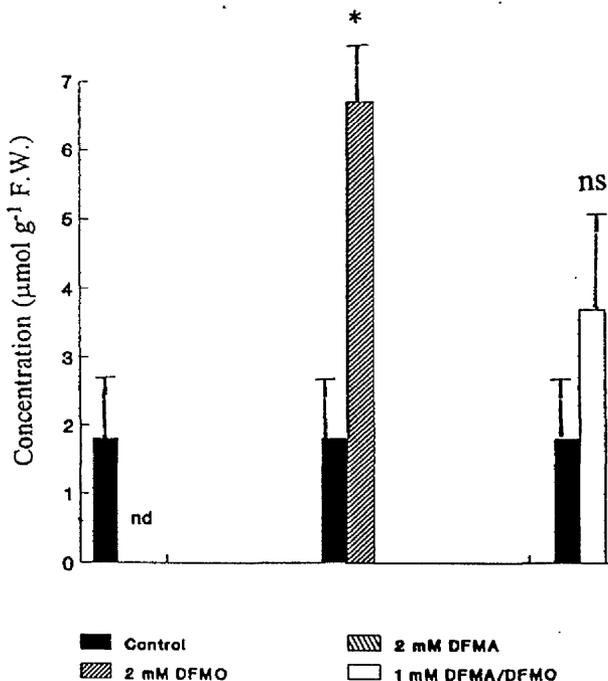
Figure 19. Effect of DFMA and DFMO on

spermine concentration in *P. involutus*.

37
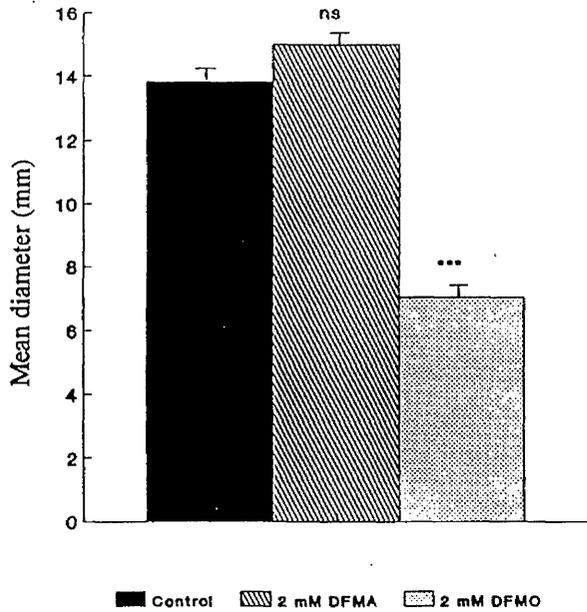
Figure 20. Effect of DFMA and DFMO on growth of *T. terrestris*.
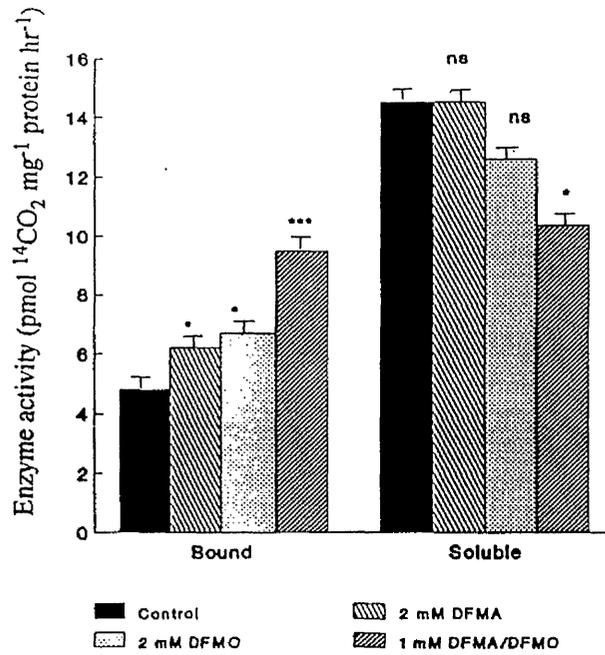
Figure 21. Effect of DFMA and DFMO on ODC activity in *T. terrestris*.
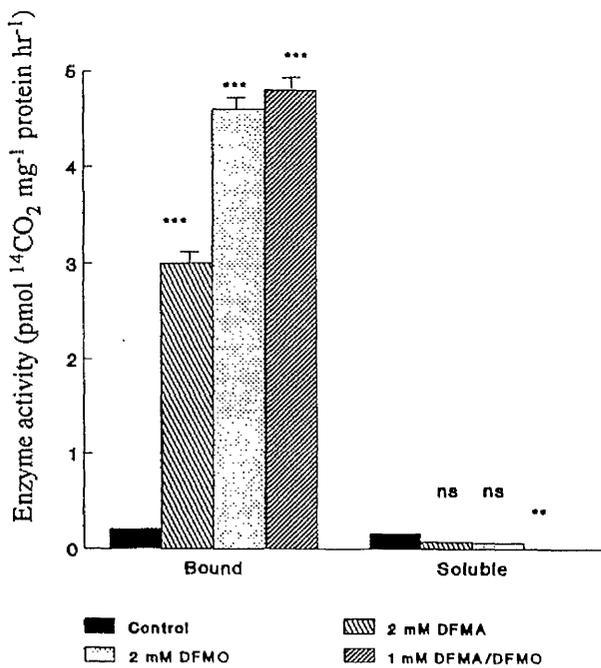
Figure 22. Effect of DFMA and DFMO on AdoMetDC activity in *T. terrestris*.
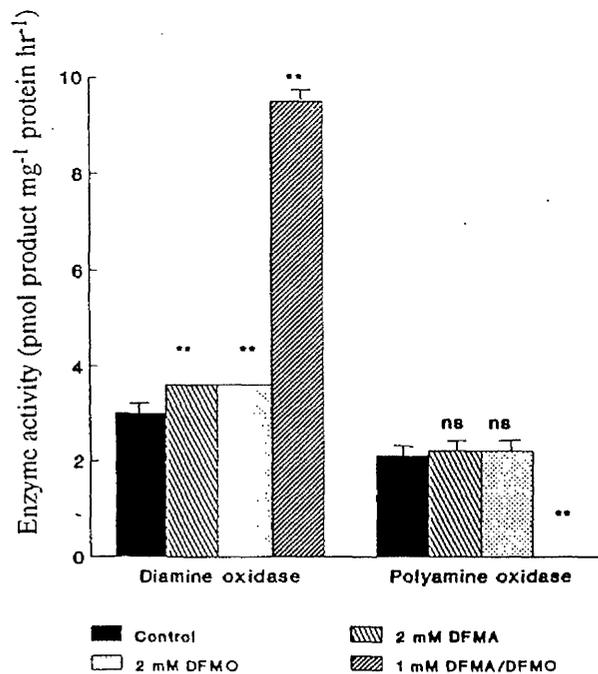
Figure 23. Effect of DFMA and DFMO on DAO and PAO activities in *T. terrestris*.
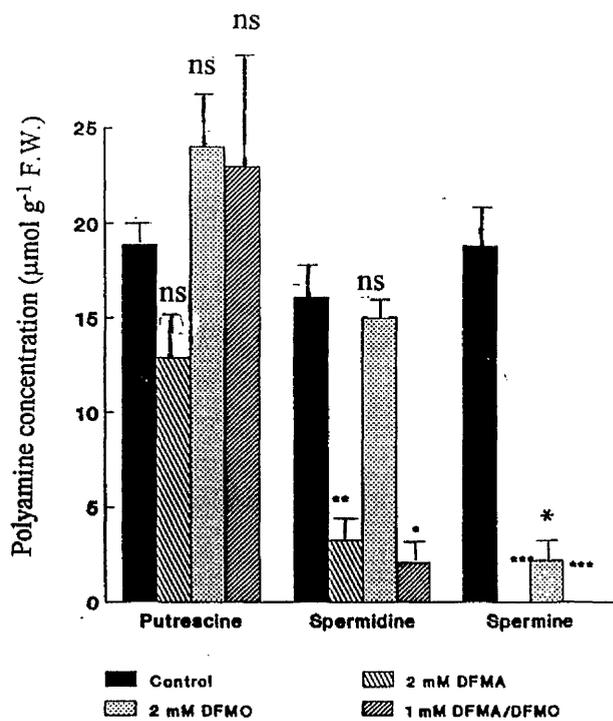
Figure 24. Effect of DFMA and DFMO on
polyamine concentrations in *T. terrestris*.
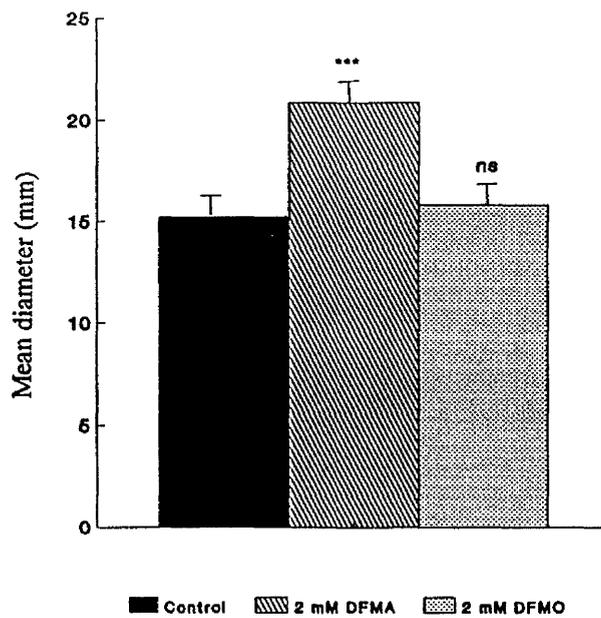
Figure 25. Effect of DFMA and DFMO on
growth of *H. mesophaeum*.
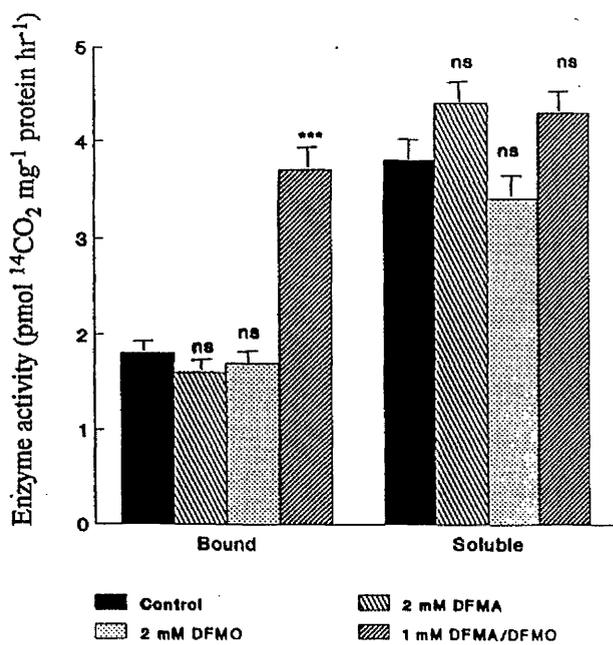
Figure 26. Effect of DFMA and DFMO on
ODC activity in *H. mesophaeum*.
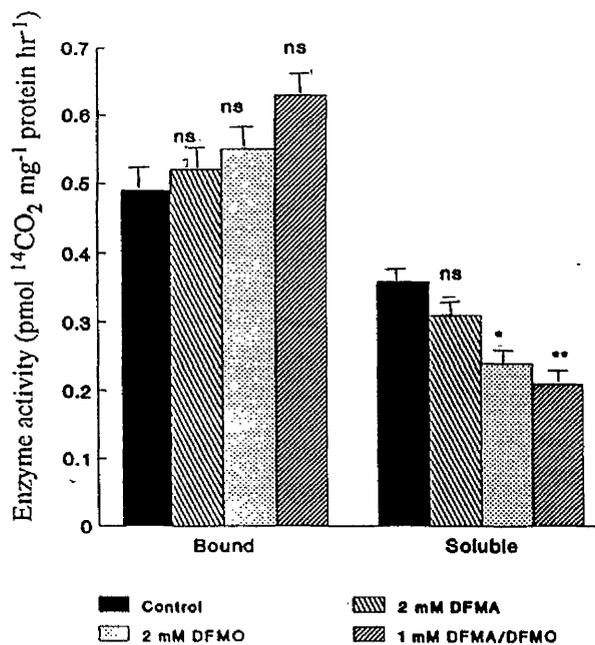
Figure 27. Effect of DFMA and DFMO on
AdoMetDC activity in *H. mesophaeum*.

39
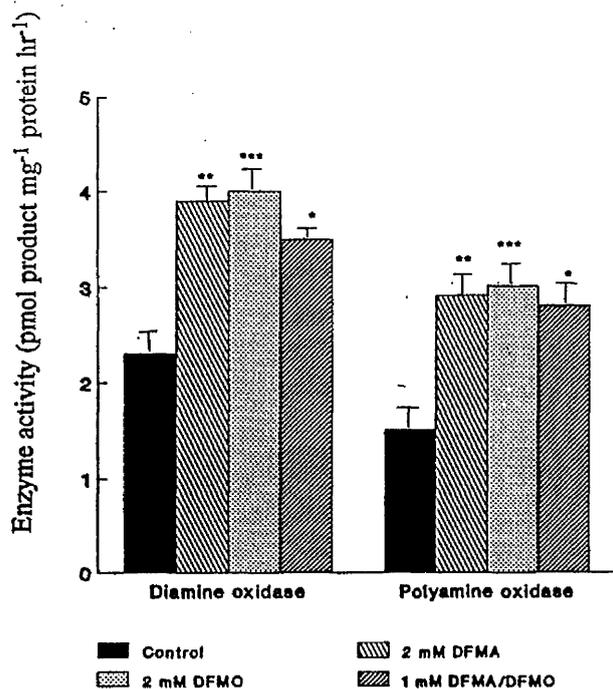
Figure 28. Effect of DFMA and DFMO on
DAO and PAO activities in *H. mesophaeum*.
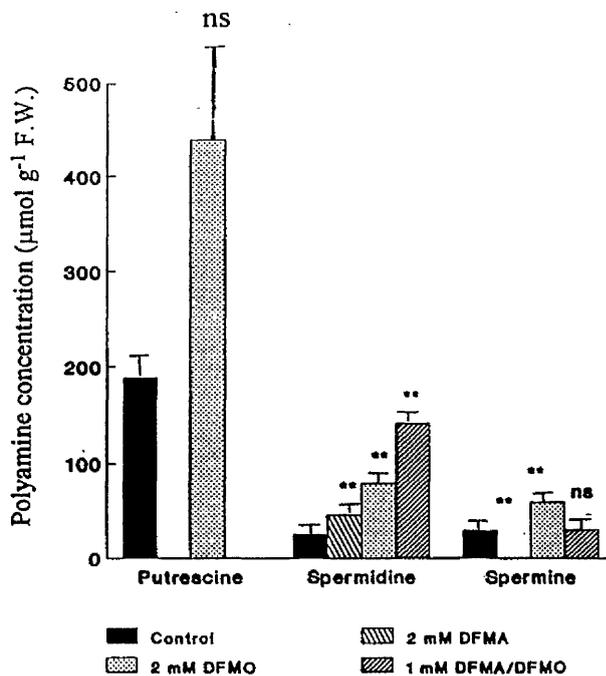
Figure 29. Effect of DFMA and DFMO on
polyamine concentrations in *H. mesophaeum*.

Table 5. Effect of DFMA on ADC activity in *L. proxima*.

Treatment	ADC activity (pmol $^{14}CO_2$ mg^{-1} protein hr^{-1})	Radioactivity in putrescine (dpm mg^{-1} protein)
Control	0.02 ± 0.004	34.1 ± 1.2
2 mM DFMA	0.02 ± 0.005 ns	nd **

Statistical differences shown as: ** $P \geq 0.01$; ns = not statistically significant; nd = not detected in assay.

40

treatments, with DFMA producing the least, and the inhibitor combination, the largest increase in enzyme activity. These treatments had the converse effect on the activity of the soluble fraction *i.e.* an apparent but statistically non-significant decrease in enzyme activity associated with DFMA, and significant, larger decreases in activity associated with DFMO and the inhibitor combination, respectively (Figs. 26 & 27).

DAO and PAO activities All treatments were associated with increased activity of both DAO and PAO. DFMO treatment was associated with the greatest increase , and the inhibitor combination with the smallest increase in enzyme activity (Fig. 28).

Polyamine concentrations All treatments substantially increased spermidine concentration. The greatest increase in spermidine concentration was associated with the inhibitor combination treatment, and the least with DFMA. Putrescine was completely depleted in mycelium grown in the presence of either DFMA or the inhbitor combination. However, DFMO treatment led to an apparent but statistically non-significant increase in putrescine concentration. Similarly, spermine was entirely depleted in DFMA-treated mycelia but its concentration was unaffected by the combined inhibitors, and significantly increased by DFMO treatment (Fig. 29).

DISCUSSION.

It is evident from these data that EM fungi respond differently to inhibitors of polyamine biosynthesis. For example, exposure of *P. involutus* to DFMO was not associated with reduced polyamine concentrations, despite the fact that mycelial growth and biosynthetic enzyme activity were reduced by this treatment. In fact, putrescine and spermidine concentrations were unaffected by DFMO treatment, whilst spermine concentration was substantially increased. This contrasts with the well-documented effects of DFMO on both animal and fungal cells, which exhibit substantial depletions in putrescine and spermine pools (Foster & Walters, 1990; Porter & Janne, 1987). In animal systems (Pegg, 1988) and in the fungal plant pathogen *Pyrenophora avenae* (Foster & Walters, 1990), DFMO caused a marked increase in AdoMetDC activity. This would allow spermine concentrations to be sustained. In *P. involutus* exposed to DFMO, spermine concentration was increased in spite of decreased AdoMetDC activity. However, this may be attributable to bound AdoMetDC activity, which remained unaffected by DFMO, and to sustained

41

putrescine synthesis, since putrescine concentration, too, was not affected by DFMO. These data suggest that a mechanism exists in *P. involutus* to maintain intracellular concentrations of free polyamines.

Mechanisms which effectively ensure a supply of polyamines to the cell have been described in bacterial and animal cells (Kallio & McCann, 1981; Alhonen-Hongisto *et al.*, 1982). Cadaverine, for example, can serve as a substrate for spermidine and spermine synthases and can therefore be converted to its higher homologues which can fulfill the requirement for putrescine and its derivatives. Although the rate of this reaction is less than 10% that for putrescine, the reaction can still proceed in Ehrlich ascites carcinoma cells in the presence of DFMO. It is possible that the growth reduction seen in *P. involutus* exposed to DFMO was due to diversion of essential polyamines from growth to sustain vital processes such as nucleic acid or protein syntheis in which polyamines have been implicated.

Quite different mechanisms appear to exist in *L. proxima* for the maintenance of polyamine biosynthesis. It is very significant that biosynthetic ADC activity was demonstrated in this organism. The detection of [^{14}C]putrescine from a [U-^{14}C]arginine substrate is itself good evidence for this assertion. However, supporting data include the depletion of putrescine, and the absence of any effect on ODC by DFMA. Moreover, polyamine synthesis from [U-^{14}C]arginine was inhibited by exposure of the fungus to DFMA. Arginine is a substrate for both ADC and arginase. The former reaction yields putrescine, the latter ornithine, and both reactions liberate CO_2. The $^{14}CO_2$ detected in the *L. proxima* ADC assay may be attributed to ADC activity, rather than arginase activity, because of the subsequent detection of [^{14}C]putrescine in the assay medium and the elimination of [^{14}C]putrescine formation in the presence of DFMA. It would have been useful to have utilised an arginase inhibitor in the ADC assay as a futher control. It is probably biosynthetic ADC activity that exists in *L. proxima* since micromolar concentrations of [U-^{14}C]arginine still led to putrescine synthesis. To verify this it would be necessary to further characterise the enzyme.

A further interesting proposition arising from these data is that since DFMA depleted putrescine in *L. proxima*, and DFMO inhibited both growth and bound ODC activity in this organism, it appears that this species possesses both ODC and ADC activities. The fact that DFMO inhibited both putrescine and spermidine formation from [U-^{14}C]ornithine supports this view. A further example of a mechanism designed to sustain polyamine synthesis may be inferred from these data. Inhibition of *E. coli* ADC results in increased ODC activity. Conversly, if ODC is inhibited in this organism, there is a concommitant rise in ADC activity (Kallio & McCann, 1981; Bitonti *et al.*, 1987). The presence of both ODC and ADC in *L. proxima* would explain why DFMA treatment (causing ADC inhibition) was associated with a large rise in

42

bound ODC activity. However, this rise in ODC activity was still unable to sustain putrescine synthesis, perhaps due to a concommitant rise in DAO activity, which would tend to deplete putrescine. Such a system operating in *L. proxima* would also help to explain how polyamine synthesis could be sustained in the presence of DFMO when bound ODC activity was reduced.

The application of both DFMA and DFMO to an organism supposedly possessing both ODC and ADC activities should, in theory, cause cessation of polyamine biosynthesis. This was not the case in *L. proxima*. There was a rise in AdoMetDC activity, increased polyamine concentrations and no effect on growth in the presence of both inhibitors. The inhibitor combination behaved, in effect, as DFMO alone. The concentration of DFMA and DFMO used here (1 mM) may have been too low to inhibit ADC activity effectivley.

Following the incorporation of $[U-^{14}C]$arginine and $[U-^{14}C]$ornithine into polyamines not only provided evidence for the existence of both ADC and ODC in *L. proxima*, but also revealed differences in the response of polyamine biosynthesis to short-term and long-term inhibition. Over 24 hours, the inhibition of ADC by DFMA, and ODC by DFMO, was rapid and absolute, with reference to putrescine synthesis. After DFMO treatment, the synthesis of spermine from $[U-^{14}C]$ornithine may have been possible from putrescine and spermidine. This would help to account for their depletion. Exposure of this organism to labelled substrates for longer periods, however, may not be a simple or reliable method for tracing polyamine metabolism, because the further metabolism of labelled polyamines would result in the loss of ^{14}C to other compounds not detectable in the polyamine assay.

Over longer periods, when the organism was grown in the presence of an inhibitor, the effects of inhibition could be overcome in virtually all cases, so that enzyme activity and polyamine concentrations were maintained. This feature was common to all the organisms studied and may be due, for example, to rapid enzyme protein turnover, or to the substitution of the common polyamines by close analogues, a phenomenon that will be discussed in Chapter 4. This ability to maintain polyamine synthesis and growth under long-term exposure to DFMO might allow the survival of organisms under environmental conditions where DFMO compounds were used fungicidally.

After supplying $[^{14}C]$ornithine to *L. proxima* for 24 hours, ^{14}C appeared in putrescine, spermidine and spermine. However, after exposure to $[^{14}C]$arginine, label was detected only in putrescine. It is possible that ADC and ODC in this organism are compartmentalised, with each enzyme producing a distinct supply of putrescine with a different function. Therefore putrescine derived from arginine would not necessarily be destined for the synthesis of spermidine and spermine. Such a system was described by Hiatt (1989) in

43

maize cell lines, in which putrescine derived from arginine was initially sequestered from ornithine-derived putrescine and destined for conjugation to a hydrocinnamic acid amide. Thus, distict populations of polyamine were identified that gave rise to separate end products and which probably had separate functions.

Although growth of *T. terrestris* was substantially reduced following treatment with DFMO, this was not accompanied by reductions in putrescine and spermidine concentrations. Spermine, however, was significantly depleted by this treatment, but neither biosynthetic nor oxidative enzyme activities were reduced. In fact, the activities of the bound fractions of both ODC and AdoMetDC, and the activity of DAO were all significantly increased by DFMO, DFMA and the inhibitor combination. This contrasts with the effects of DFMO on *P. involutus* in which polyamine concentrations were sustained but ODC and AdoMetDC activities were markedly decreased. Two possibilities exist which may explain the effects of the inhibitors on *T. terrestris*. First, DAO activity was increased, and PAO activity unaffected by DFMO (and DFMA) treatment. Oxidation of putrescine and spermidine would have generated hydrogen peroxide and free radicals, thereby inducing membrane damage in the fungus. Such a mechanism has been reported to account for putrescine-induced damage in maize seedlings (DiTomaso *et al.*, 1992). Second, all treatments led to substantial depletions in spermine. Although there is little information on the importance of spermine to fungi, studies of polyamine-defficient mutants of *Saccharomyces cerevisiae* show that this organism has an absolute requirement for putrescine, spermidine and spermine for growth (Tabor, 1981).

The lack of enzyme inhibition by any treatment in *H. mesopheum* is notable. Only soluble AdoMetDC activity was reduced in the fungus, by DFMO and by the inhibitor combination. DAO and PAO activities were markedly increased by all treatments. This observation goes some way towards explaining the complete depletion of putrescine associated with DFMA and the inhibitor combination treatments, and the similar depletion of spermine in mycelium exposed to DFMA alone. Depletion of putrescine may be a consequence of its utilisation for spermidine and spermine synthesis required to sustain growth or other vital processes. The increased activity of the oxidative enzymes may also help to account for DFMA-induced putrescine depletion but not for the affects associated with the other inhibitors.

There are several possibilities that may account for these phenomena. Firstly, it would be interesting to examine levels of peroxidase activity in fungi demonstrating increased DAO and PAO activities, since high peroxidase activity would tend to ameliorate the harmful effects of hydrogen peroxide and free radicals that are produced as a consequnce of polyamine oxidation. Secondly, the lack of any substantial enzeyme inhibition in *H. mesopheum* may indicate that the inhibitors are not readily taken up by

44

the organism. It has been mentioned earlier that polyamine inhibitor uptake appears to rely on mechanisms similar to those used for the uptake of amino acids. The type of uptake mechanisms present in *H. mesopheum* may simply preclude DFMA and DFMO uptake. The observations that DFMA treatment is associated with a growth increase, a depletion of putrescine and that it does not affect biosynthetic enzyme activity, suggest that ADC activity is present in *H. mesopheum*. If conjugated forms of putrescine had been synthesised by ADC activity in this organism then they would not have been detected in the polyamine assay. The presence of conjugated forms of putrescine of unique biosynthetic origin would account for the apparent lack of free putrescine associated with DFMA and the inhibitor combination treatments.

This work has shown that mycorrhizal fungi respond differently to inhibitors of polyamine biosynthesis. At least one of the species studied, *L. proxima*, possesses ADC activity, which may produce a distinct pool of putrescine with a specific function. A comparable situation may exist in *H. mesophaeum*, with ADC synthesising putrescine destined for conjugation, but able to support growth. All four fungi demonstrated an ability to overcome the inhibitory effects of DFMA and DFMO to a greater or lesser extent, possibly by rapid enzyme protein turnover or metabolism of the inhibitor. DFMO is readily taken up by the fungal cell (Smith, Barker & Jung, 1990; West & Walters, 1990). It can be metabolised by arginase, ornithine transcarbamoylase, and other enzymes (Slocum *et al.*, 1988); and it may have some effect on membrane transport systems (Smith *et al.*, 1990). DFMA is also metabolised in fungal, plant and animal cells (Mussell *et al.*, 1987; Slocum *et al.*, 1988; Smith, Barker & Owens, 1992). Since little is known of polyamine biosynthesis in relation to broader aspects of cellular metabolism, it is possible that DFMA and DFMO exert effects on processes other than those studied in this report, such as polyamine acetylation, transmethylation reactions or amino acid synthesis, for example. What is clear is that mechanisms exist which tend to maintain the supply of polyamines for vital functions under adverse conditions. One aspect of this phenomenon will be addressed in the following chapter.

4. THE FORMATION OF CADAVERINE, AMINOPROPYLCADAVERINE AND N,N' *bis* (3 AMINOPROPYL)CADAVERINE IN MYCORRHIZAL AND PHYTOPATHOGENIC FUNGI.

INTRODUCTION.

Cadaverine ($NH_2(CH_2)_5NH_2$) is a diamine formed by the decarboxylation of lysine. It is a homologue of putrescine and occurs sporadically in plants, animals and micro-organisms. The aminopropylation of cadaverine leads to the formation of the higher homologues aminopropylcadaverine (APC, $NH_2(CH_2)_3NH(CH_2)_5NH_2$) and N,N' *bis* (3 aminopropyl)cadaverine (3 APC, $NH_2(CH_2)_3NH(CH_2)_5NH(CH_2)_3NH_2$). These cadaverine homolgues have been identified only in several bacteria (*e.g.* Hamana *et al.*, 1988, 1990), in human tumour cells (Alhonen-Hongisto & Janne, 1980), and in *Neurospora crassa* (Paulus *et al.*, 1982). Several species of Halococcus were shown to be able to synthesise both these compounds, and they have also been identified in *Paracoccus denitrificans* (Hamana *et al.*, 1988, 1990). When this organism was cultured in a growth medium containing cadaverine, it was found to accumulate both APC and the tetra-amine aminopentylnorspermidine. Although *Agrobacterium tumifaciens* and *Halococcus acetoinfaciens* have not been found to synthesise cadaverine, both could produce aminopropyl derivatives of cadaverine, including 3 APC, when cultured in media containing cadaverine.

In many systems, cadaverine synthesis is prompted when putrescine synthesis via ornithine decarboxylation becomes limiting. Under these circumstances, cadaverine and its homologues are able to fulfill the roles of the usual polyamines in supporting growth and other processes, at least for short periods. When polyamine synthesis in Erlich ascites carcinoma cells was inhibited by DFMO, cells became rapidly depleted of both putrescine and spermidine. This was accompanied by the appearence of cadaverine and APC (Alhonen-Hongisto & Janne, 1980). Cadaverine can act as a substrate for spermidine synthase, albeit at a reduced rate, and can therefore be converted to APC and 3 APC. In subsequent work using tumour cells (Alhonen-Hongisto *et al.*, 1982), it was shown that cadaverine was able to compensate for the DFMO-induced loss of putrescine and spermidine in supporting growth. However, cell growth was slow and cells continued to exhibit phenomena typical

of polyamine depletion. Furthermore, the cadaverine derivatives were unable to fulfill the regulatory role of the natural polyamines, for example their role in enzyme repression, so that tumour cells continued to overproduce ODC.

A refinement of this mechanism was noted in polyamine-deprived mycelia of *Neurospora crassa*, which were found to have elevated ODC activity (Paulus, Kiyono & Davis, 1982). Cadaverine and APC appeared in cells which lacked ornithine and which had high ODC activity. ODC-deficient mutants of *N. crassa* were unable to decarboxylate lysine, and ODC appeared to be the only enzyme capable of achieving this reaction.

Starvation of arginase-limited, polyamine-dependant Chinese hamster ovary cells for ornithine or ornithine-derived polyamines also resulted in the formation of cadaverine, APC and 3 APC (Höltta & Pohjanpelto, 1983). When these cells were exposed to DFMO however, cadaverine synthesis was depressed. This contrasts with the observation in Erlich ascites tumour cells where cadaverine synthesis continued in the presence of DFMO. Although ornithine is the normal substrate for ODC, it appears from this work that , as in *N. crassa*, ODC could catalyse the decarboxylation of lysine to cadaverine.

Studies of polyamine-dependant mutants of *E. coli* grown in the absence of polyamines suggested that APC was important for cell growth under these conditions and that it was synthesised preferentially from cadaverine (Igarishi *et al.*, 1986). Again, cadaverine was synthesised by the ODC-mediated decarboxylation of lysine. The formation of APC was inhibited by the ODC inhibitor monofluoromethylputrescine. Episodes of polyamine deprivation can thus be countered by the ODC-mediated conversion of lysine to cadaverine in a variety of cell types.

Since several cell types are able to synthesise a variety of tri- and tetra-amines, such as derivatives of cadaverine, it is likely that the biosynthetic pathways involved have broad substrate specificity for the aminopropylation of diamines and triamines. Spermidine and spermine are normally formed by the addition of an aminopropyl group from decarboxylated SAM to putrescine (Fig. 3a). The aminopropyltransferase enzymes, spermidine synthase and spermine synthase, have been shown to be capable of aminopropylating cadaverine to form the tri- and tetra-amine derivatives (Fig. 37b). However, an alternative pathway for the addition of an aminopropyl moiety to a primary amine has been demonstrated in several bacteria , and in seedlings of the plant *Lathyrus sativa,* in

which L-aspartic β-semialhehyde is the aminopropyl group donor to spermidine. In this scheme, putrescine and L-aspartic β-semialdehyde condense to form a Schiff base complex which is reduced to form the basic amino acid carboxyspermidine. Carboxyspermidine is decarboxylated to form spermidine (Fig. 37b). A Schiff base is formed when a primary amine and an aldehyde condense with an accompanying dehydration.

Cultures of *Micrococcus denitrificans* and *Rhodotobacter sphaeroides* (*Rhodopseudomonas sphaeroides*) were able to produce ^{14}C-labelled spermidine when supplied with ^{14}C-labelled arginine, ornithine, aspartate and putrescine, but not when supplied with ^{14}C-labelled methionine and unlabelled putrescine. Enzyme extracts did not form radioactive spermidine when incubated with [^{14}C]putrescine and SAM. Tait (1976) therefore inferred that a metabolic product of L-aspartate, possibly L-aspartic β-semialdehyde, was the aminopropyl group donor to spermidine. Tait (1985) has subsequently shown the Schiff base pathway to be used for spermidine synthesis in several species of purple photosynthetic bacteria and in *Agrobacterium tumifaciens*.

The triamine norspermidine, a spermidine analogue, is synthesised in *Vibrio alginolyticus* and *V. parahaemolyticus* from L-aspartic β-semialdehyde and diaminopropane, which condense to form a Schiff base (Yamamoto *et al.*, 1985). The Schiff base is subsequently reduced to form carboxynorspermidine, which in turn is decarboxylated to yield norspermidine. Again, a novel pathway for polyamine biosynthesis was demonstrated, and since the activity of an aminopropyltransferase utilising decaboxylated SAM could not be detected, this pathway appeared the primary route for polyamine biosynthesis in these species.

Interestingly, the co-existence of two pathways to spermidine synthesis has been observed in *Lathyrus sativa* (Srivenugopal & Adiga, 1980). By supplying [^{14}C]aspartate and [^{14}C]methionine to *L. sativa* seedlings, and measuring the subsequent incorporation of radioactivity into putrescine, spermidine and spermine, these authors were able to show that methionine was the most efficient precursor of spermine, and that the aspartate pathway, operating concurrently, was restricted largely to spermidine synthesis.

As a logical extension of the foregoing, the possibility of APC and 3 APC formation *via* Schiff base formation was investigated in mycorrhizal and phytopathogenic fungi. Evidence presented below suggests that (1) cadaverine derivatives are synthesised from L-aspartic β-

48

semialdehyde and cadaverine; and (2) cadaverine, APC and 3 APC are synthesised routinely in the fungi examined, and that the activities of AdoMetDC and spermidine synthase are involved in their formation.

Details of all experimental procedures are given in Chapter 2.

RESULTS.

When extracts of all the fungi used were fed [U-^{14}C]lysine, label appeared in APC and 3 APC (Figs. 30 - 35). In these experiments, exposure of extracts to MGBG and CHA resulted in significant reductions in the synthesis of cadaverine and cadaverine derivatives in the phytopathogenic fungi (Figs. 32 & 33). This contrasts with the response of the EM fungi to the inhibitors in that cadaverine synthesis was unaffected in these fungi, or even increased in the case of *T. terrestris* (Figs. 30, 34 & 35). Furthermore, APC synthesis was apparently increased in all three EM fungi, although only the increase in *P. involutus* was statistically significant (Figs. 30, 31 & 33). Although 3 APC synthesis in *T. terrestris* was unaffected by MGBG and CHA, all other species showed a marked decline in the flux of radiolabel into this compound.

Although the synthesis of cadaverine and 3 APC was abolished in *L. proxima* exposed to DFMA, the flux of radiolabel into APC was increased ten-fold. On exposure to DFMO, cadaverine and APC synthesis was abolished, but the synthesis of 3 APC was more than doubled (Fig. 35).

In *in vivo* experiments with *L. proxima*, little formation of the cadaverine derivatives occured within 10 hours following exposure of the fungus to [U-^{14}C]lysine. After this time, formation of the compounds increased substantially, with most label appearing in 3 APC (Fig. 36a). Similarly, the incorporation of [U^{14}C]aspartate progressed slowly up to 10 hours, and then increased subsequently in the same pattern as that observed for [U-^{14}C]lysine, with most label appearing this time in APC (Fig. 36b).

Statistical differences shown as: * P ≥ 0.05; ** P ≥ 0.01; *** P ≥ 0.001; nd = not detected in assay;
ns = not statistically significant *i.e.* P < 0.05.
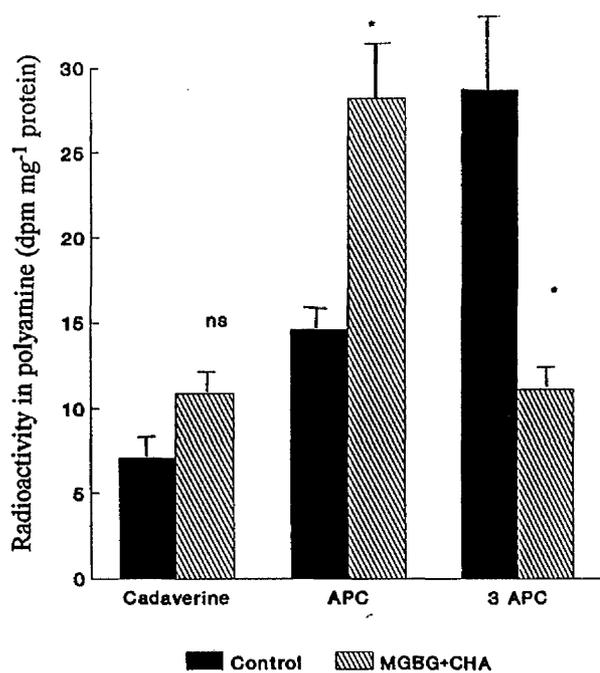
Figure 30. Effect of MGBG & CHA on the incorporation
of [U-^{14}C]lysine into cadaverine derivatives
in *P. involutus*.
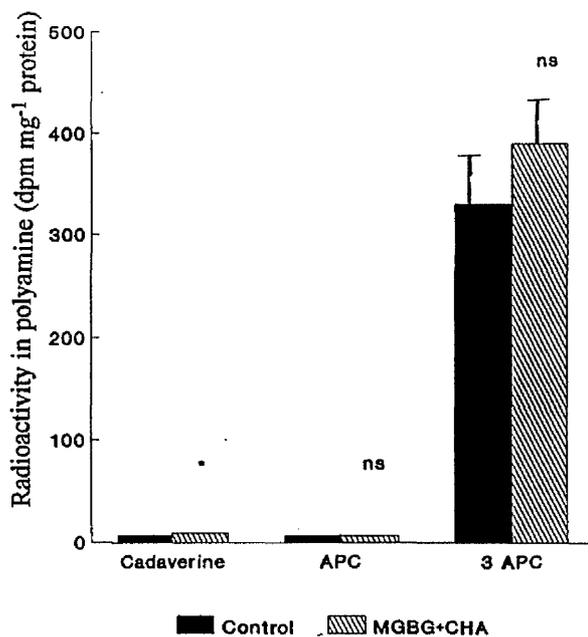
Figure 31. Effect of MGBG & CHA on the incorporation
of [U-^{14}C]lysine into cadaverine derivatives
in *T. terrestris*.
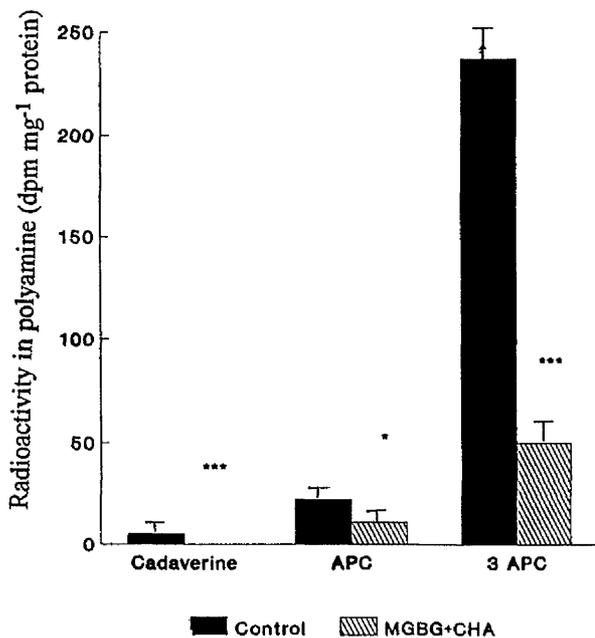
Figure 32. Effect of MGBG & CHA on the incorporation
of [U-^{14}C]lysine into cadaverine derivatives
in *Pyr. avenae*.
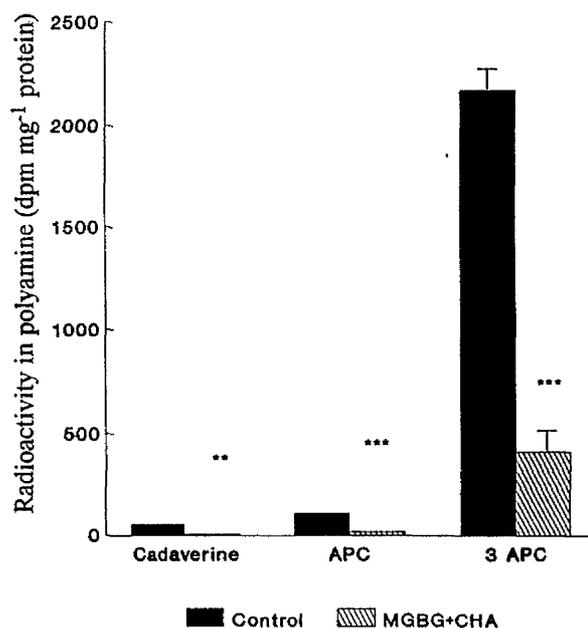
Figure 33. Effect of MGBG & CHA on the incorporation
of [U-^{14}C]lysine into cadaverine derivatives
in *U. viciae-fabae*.

50
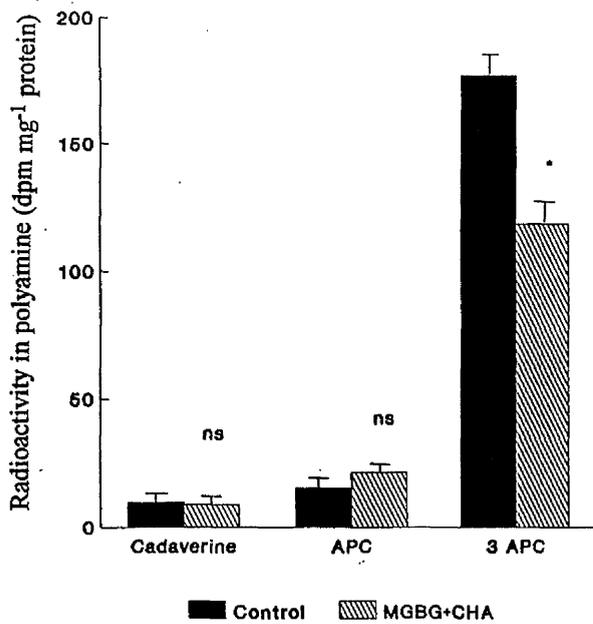
Figure 34 Effect of MGBG & CHA on the incorporation
of [U-^{14}C]lysine into cadaverine derivatives
in *L. proxima*.
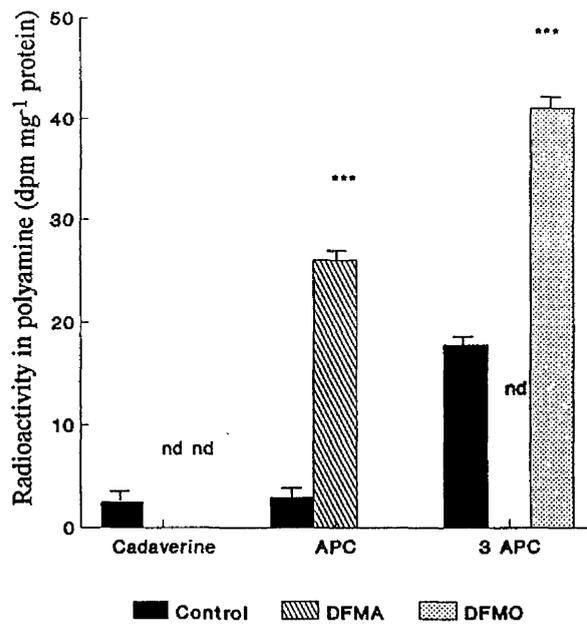
Figure 35 Effect of DFMA & DFMO
on the *in vivo* incorporation
of [U-^{14}C]lysine into cadaverine derivatives
in *L. proxima*.

Figure 36 a. *In vivo* incorporation of [U-^{14}C]lysine into cadaverine derivatives in *L. proxima* over a 24 hour period. —— CAD   —+— APC   —*— 3 APC
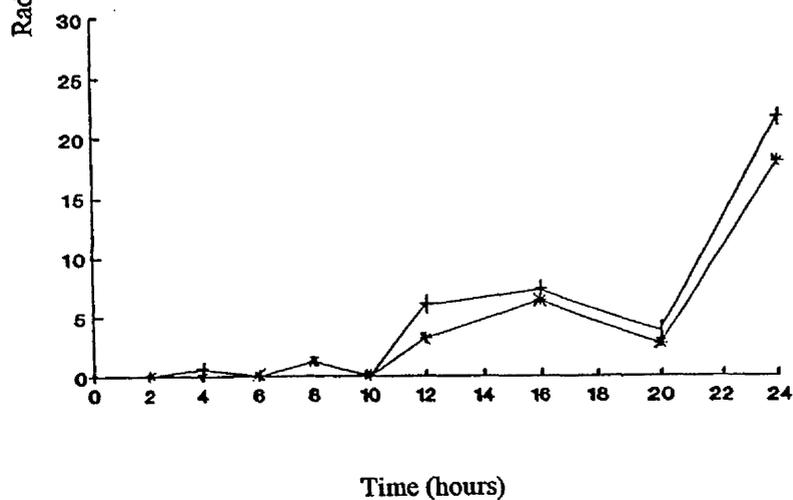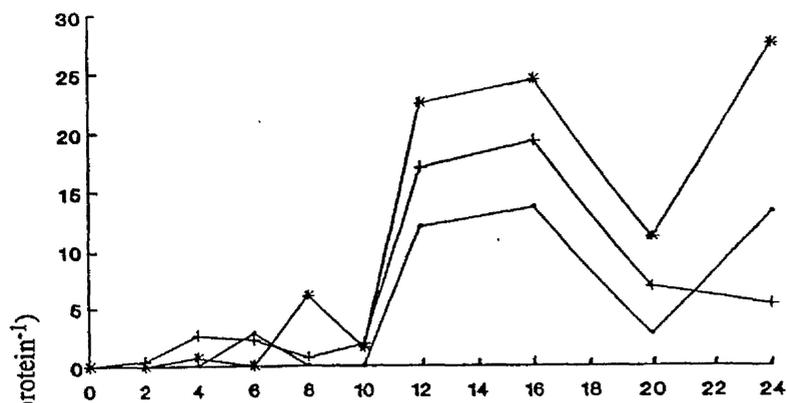

Figure 36 b. *In vivo* incorporation of [U-^{14}C]aspartate + unlabelled lysine into cadaverine derivatives in *L. proxima* over a 24 hour period.   —+— APC   —*— 3 APC

DISCUSSION.

Radiolabelled compounds that co-chromatographed with authentic standards of cadaverine, APC and 3 APC were isolated from reaction mixtures after the decarboxylation of [U-^{14}C]lysine by fungal extracts. This provides strong evidence for the biosynthesis of these compounds in the fungi examined. The identity of 3 APC was confirmed subsequently by NMR spectroscopy and was found to be present in all the fungi examined.

The biosynthesis of higher homologues of cadaverine is thought to occur in the same way as that of spermidine and spermine *i.e.* by the addition of an aminopropyl group to cadaverine and APC, to form APC and 3 APC, respectively. These reactions would be catalysed by AdoMetDC (for the formation of decarboxylated AdoMet) and the aminopropyltransferases, spermidine and spermine synthase. Consequently, the inhibition of both AdoMetDC and spermidine synthase should prevent the flux of [U-^{14}C]lysine into APC and 3 APC. Indeed, in *in vitro* studies with several fungi, the incorporation of [U-^{14}C]lysine into APC and 3 APC was significantly reduced by MGBG and CHA, with the greatest reduction occurring in extracts of the two pathogenic fungi, *P. avenae* and *U. viciae-fabae*. In these fungi, exposure to the inhibitors also reduced the flux of radiolabel to cadaverine and APC, whereas in the two EM fungi *L. proxima* and *P. involutus*, only the flux to 3 APC was reduced. These data suggest that APC and 3 APC can be formed in these fungi using AdoMetDC and spermidine and spermine synthases.

Although treatment with MGBG and/or CHA reduced the flux of [U-^{14}C]lysine through to 3 APC, this flux was not completely abolished. This suggests two possibilities: (1) the inhibition of enzyme activity by MGBG and CHA was incomplete, or (2) that APC and 3 APC continued to be synthesised by another route. The aminopropyltransferases, spermidine synthase and spermine synthase, are not rapidly turned over and are less responsive to the regulatory changes associated with polyamine depletion (Pegg, 1988). This would theoretically render them more amenable to inhibition, and indeed, substantial inhibition of both spermidine and spermine synthase has been acheived with CHA in plants and micro-organisms (Smith, 1985; Slocum & Flores, 1991). In view of the continued synthesis of APC and 3 APC in the presence of MGBG and CHA, and the subsequent incorporation of [U-^{14}C]aspartate into these amines in *L. proxima*, it is proposed that

53

Figure 37 a. Route for the formation of cadaverine derivatives from lysine with S-adenosylmethionine as aminopropyl donor.

LDC   *ADOMETDC*

LYSINE ———▶ CADAVERINE   DECARBOXYLATED ◀——— ADOMET
                                              ADOMET

                              APC   *SPD SYN*

                              3 APC
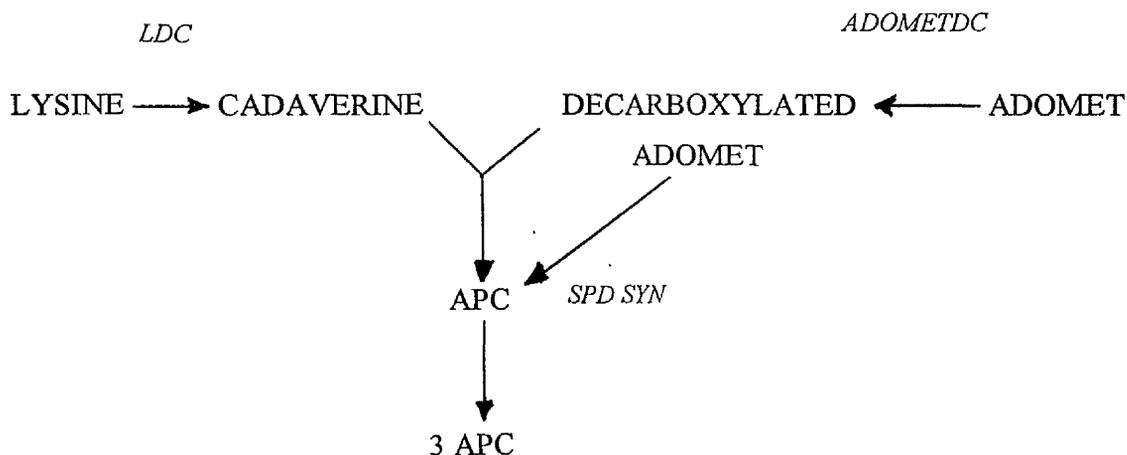
Figure 37 b. Proposed route for the formation of cadaverine derivatives from lysine with L-aspartic-β-semialdehyde as aminopropyl donor.

ASPARTATE ———▶ L-ASPARTIC-β-PHOSPHATE   HOMOSERINE

CADAVERINE   L-ASPARTIC-β-SEMIALDEHYDE

              SCHIFF BASE COMPLEX

              CARBOXY APC

              APC
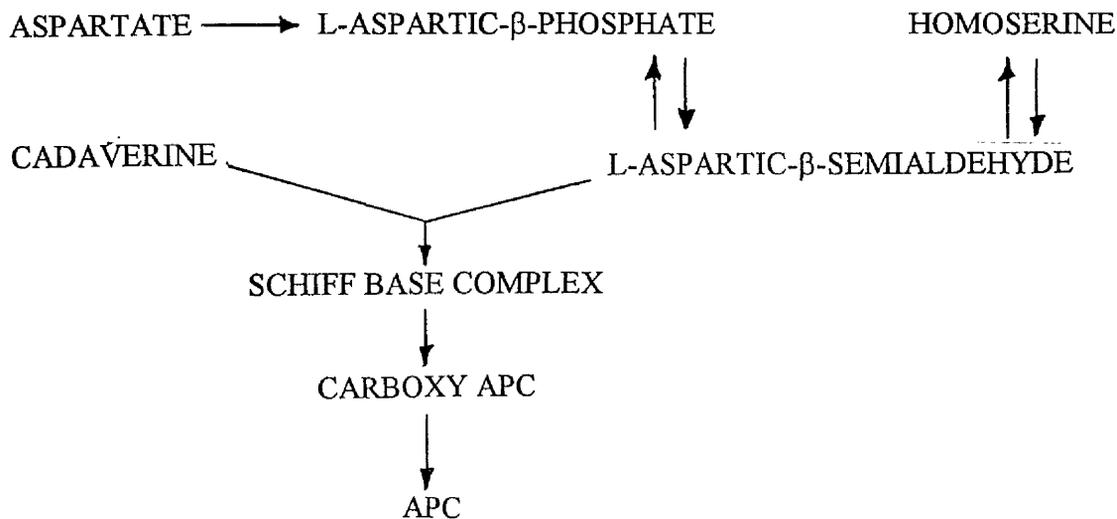
Abbreviations used: **LDC**, lysine decarboxylase; **AdoMetDC**, adenosylmethionine decarboxylase; **Spd Syn**, spermidine synthase; **AdoMet**, S-adenosylmethionine; **APC**, aminopropylcadaverine; **3 APC**, N,N' bis (3 aminopropyl)cadaverine.

54

APC and 3 APC are also synthesised in these fungi *via* the formation of a Schiff base between L-aspartic-β-semialdehyde and cadaverine (Fig. 37b).

The time courses for the incorporation of [U-^{14}C]lysine and [U-^{14}C]aspartate into APC and 3 APC in *L. proxima* followed a similar pattern, with distinct phases of ^{14}C incorporation being apparent (Figs. 36a & b). Peaks of lysine and aspartate incorporation into 3 APC occurred after eight hours and then diminished. Subsequent peaks occured after 10 hours, whereupon ^{14}C incorporation into APC and 3 APC was sustained for a six hour period. After this time, the incorporation of radiolabel into the cadaverine homologues declined. After exposure to the radiolabelled substrates for 20 hours, the pattern of uptake of [U-^{14}C]lysine ceased to mirror that of [U-^{14}C]aspartate. Whereas [U-^{14}C]lysine incorporation into 3 APC increased once more, its incorporation into APC continued to decline. However, increases in the incorporation of [U-^{14}C]aspartate into both APC and 3 APC were notable. These data lend further credence to the operation of a Schiff base mechanism for the biosynthesis of APC and 3 APC from cadaverine, and furthermore, that this mechanism occurs concurrently with the operation of the AdoMetDC and aminopropyltransferase system.

Alhonen-Hongisto & Janne (1980) have shown that depletion of intracellular polyamines in tumour cells can lead to the accumulation of APC and 3 APC. Subsequent work showed that in tumour cells grown in the presence of DFMO, putrescine, spermidine and spermine were eventually replaced by cadaverine, APC and 3 APC (Alhonen-Hongisto *et al.*, 1982). Data presented in Chapter 3 show *L. proxima* to possess both ODC and ADC activities. When this fungus was grown in the presence of DFMA, there was an accumulation of APC. Growth of *L. proxima* in the presence of DFMO resulted in 3 APC accumulation. These results are consistent with those of Alhonen-Hongisto *et al.* (1982), and support the idea of a compensatory role between complementary polyamines.

These data show that a number of EM and plant pathogenic fungi are able to synthesise the higher homologues of cadaverine, APC and 3 APC. It appears that these compounds are formed in these fungi *via* the action of AdoMetDC, spermidine synthase and spermine synthase, and that they may also be produced *via* a Schiff base formed between cadaverine and L-aspartic-β-semialdehyde, which is subsequently reduced and decarboxylated to yield APC. Since the synthesis of cadaverine,

APC and 3 APC in *T. terrestris* was unaffected by MGBG and CHA, it suggests that Schiff base

formation is the primary route for APC and 3 APC formation in this organism.

5. THE EFFECTS OF TOXIC METALS ON ENZYME ACTIVITIES AND POLYAMINE BIOSYNTHESIS IN THE EM FUNGUS *PAXILLUS INVOLUTUS.*

INTRODUCTION.

Soil and water pollution by toxic metals are major environmental problems. Toxic metals originate from various industrial operations including mining and smelting, and from the soils and rocks in which they are naturally present. Toxic metals affect the fungal population as a whole by selecting for resistant and tolerant species, and by generally reducing species diversity.

Resistance and Tolerance Mechanisms.

A range of EM fungi are frequently encountered on industrially-polluted soils. Metal-tolerant fungi possess innate properties, such as the ability to bind toxic metals outside the cell, or to achieve chemical transformations of metals, which enable them to inhabit polluted sites. The concept of resistance is exemplified in mechanisms which are mobilised in response to toxic agents. Resistance responses to toxic metals in fungi may be strain-specific (Gadd, 1993).

GENERAL INTERACTIONS BETWEEN FUNGI AND TOXIC METALS.

Metal Binding at Cell Walls.

The fungal cell wall may sequester metal species at various charged sites including carboxyl, amine, hydroxyl, phosphate and sulphydryl groups. Gadd (1993) and Bell & Wheeler (1986), note the significance of chitin and melanin as metal-binding components of some fungal cell walls. Since melanins contain peptides, carbohydrates, and aliphatic hydrocarbons, they possess many potential binding sites, and can adsorb significant amounts of metal. Mowll & Gadd (1989) were able to relate cadmium tolerance, and the cadmium binding capacity of chlamydospores of *Aureobasidium pullalans*, to the the thickness of melanisation of the chlamydospore.

Extracellular Metal Precipitation.

Some extracellular fungal products are able to complex or precipitate metal ions, thereby preventing interaction between the metal ion and the cell. Murphy & Levi (1983) demonstrated the formation of copper oxalate crystals around the cell walls of *Aspergillus niger, Penicillium spinulosum* and *Verticillium psaliotae*, which had been grown in the presence of copper. The exudation of acids as metal chelators *e.g.* citric acid (Gadd, 1993), may be important in some fungi. *P. involutus* produces organic acids thought to be responsible for antibiosis (Rasanayagam & Jeffries, 1992). Such acids may also have some value as metal complexing agents.

Metal Transformations.

Fungi, together with a range of soil microbes, are capable of achieving chemical transformations of metals by oxidation, reduction, and methylation, each of which may render the metal less toxic. For example, *Candida albicans* and *Saccharomyces cerevisiae* can reduce Hg^{2+} to Hg^0 (Yannai, Berdicevsky & Duek, 1991). Methylation of mercury is also effected by some fungi, and since the methylated form is more volatile, its toxicity is diminished to the fungus by its loss from the soil.

Environmental Influences on Metal Toxicity.

The mechanisms by which toxic metals interact with fungi may be modulated by a number of environmental factors. For example, soil organic matter, and clay minerals, and soils with a high cation exchange capacity, will tend to chelate metal ions, and consequently reduce their toxicity by making them less easily available to cells. The pH of the environment may influence fungal cell metabolism, and metal ion speciation, uptake, and toxicity. Nickel toxicity, for example, may decrease as pH increases, due to to the formation of less toxic hydroxylated species, and to pH effects on nickel uptake mechanisms (Gadd, 1983). As pH decreases, hydrogen ions may compete with metal ions for cellular binding sites, so that toxic ions are displaced from the fungus. The more electronegative metals such as copper, mercury and silver, have greater affinities for sulphydryl, imino, and amino groups (Ross, 1983). These can occur as important structural and reactive

components of cellular proteins. Clearly therefore, cell metabolism, including polyamine biosynthesis, is potentially vulnerable to toxic metal ions.

Polyamine Metabolism in the Presence of Toxic Metals.

Weinstein *et al.* (1986) demonstrated an accumulation of the polyamine, putrescine, in oat and bean leaves exposed to cadmium at concentrations typical of mildly polluted soils. This concurs with the general observation that plants subjected to environmental stresses show increased polyamine biosynthetic activity (Galston & Kaur-Sawhney, 1990), and establishes a link between polyamine metabolism and interactions with toxic metals.

EM Fungi and Toxic Metals.

The tolerance of many EM fungi to toxic metals is of great interest, since the fungus can confer tolerance to toxic metal pollution to the plant symbiont. This has important implications for the restoration of industrially polluted sites. In studies of the zinc tolerance of mycorrhizal *Betula*, for example, Brown & Wilkins (1985) showed that the EM fungi *P. involutus* and *Amanita muscaria* increased the tolerance to zinc of both Zn-tolerant and non-tolerant plants. Furthermore, translocation of zinc to the plant shoot was found to be reduced, and zinc was accumulated in the fungi. Denny & Wilkins (1987) proposed a mechanism for this phenomenon. As the fungal mycelium colonises fresh soil, zinc is adsorbed to the hyphal surface, thereby lowering the zinc concentration in the soil solution surrounding the plant root. Thus, less zinc is taken up by the plant, and the onset of toxicity alleviated. These workers also found that the metal was adsorbed at electronegative sites of the hyphal cell wall, especially in the extra-matrical hyphae, and in extrahyphal polysaccharide slime.

There is little information on the interaction between toxic metals and polyamines in fungi. In view of the importance of mycorrhizal fungi in the reclamation of toxic soils, and the essential and versatile functions of polyamines in fungi, some effects of toxic metals on polyamine metabolism in *P. involutus* were investigated. Biosynthetic enzyme activity, polyamine concentrations, and the flux of polyamines from [U-^{14}C]lysine through cadaverine were measured in *P. involutus* which had been

grown in the presence of copper, nickel, lead and zinc, at concentrations typical of mildly-polluted soils.

All experimental procedures are detailed in Chapter 2.

RESULTS.

Exposure of P. involutus to each of copper, zinc, nickel and lead led to dissimilar effects on polyamine biosynthetic enzyme activity, polyamine concentrations and on the in vitro conversion of lysine into cadaverine. Metal ions were accumulated by the fungus, and relative accumulation was in the order: Pb > Ni > Zn > Cu (Tables 6 - 9). Indeed, P. involutus accumulated lead to a concentration three-hundred times that of a control not exposed to the metal (Table 6). The regions of distribution of the metals in the fungus were not investigated.

Table 6. Concentration of lead in fungal tissues following exposure of P. involutus for 2 weeks. Values represent a single measurement.

Lead treatment concentration (μg ml^{-1})	Concentration of lead in fungus (μg g^{-1} dry weight)
Control	2.7
6.0	374
Control	0.8
12.0	113
Control	0.8
24.0	220

The overall effects on P. involutus of exposure to toxic metals could not be generalised since the effects of the metals on the parameters measured varied considerably. However, fungal fresh weight was unaffected by any treatment. With regard to the highest treatment concentrations used, although nickel and lead were associated with similar decreases in soluble ODC activity (Figs. 37 & 41), their corresponding effects on polyamine concentrations varied. Thus nickel treatment led

60

to decreased spermidine, and increased spermine concentrations, although the increase in spermine was not statistically significant (Fig. 43).

Table 7. Concentration of nickel in fungal tissues following exposure of *P. involutus* for 2 weeks. Values represent a single measurement.

Nickel treatment concentration (μg ml^{-1})	Concentration of nickel in fungus (μg g^{-1} dry weight)
Control	2.7
1.0	26.1
Control	0.8
2.0	20.4
Control	0.8
4.0	220

Table 8. Concentration of zinc in fungal tissues following exposure of *P. involutus* for 2 weeks. Values represent a single measurement.

Zinc treatment concentration (μg ml^{-1})	Concentration of zinc in fungus (μg g^{-1} dry weight)
Control	13.6
3.75	30.4
Control	6.5
7.5	25.3
Control	4
15.0	21.2

Putrescine concentration in the fungus was unaffected by nickel. However, exposure to lead was associated with greatly increased putrescine and spermidine concentrations, together with decreased spermidine concentration (Fig. 39). Exposure of P. involutus to lead or nickel resulted in increased bound AdoMetDC activity (Figs. 38 & 42). Interestingly, soluble AdoMetDC activity was little affected by these metals.

Table 9. Concentration of copper in fungal tissue following exposure of P. involutus for 2 weeks. Values represent a single measurement.

Copper treatment concentration (μg ml^{-1})	Concentration of copper in fungus (μg g^{-1} dry weight)
Control	26
0.25	83.9
Control	3.3
0.5	9.1
Control	3.2
1.0	12.3

The flux of lysine into cadaverine, APC and 3 APC was not significantly affected by nickel treatment (Fig. 44). Exposure of the fungus to 24 g μg ml^{-1} lead depressed APC and 3 APC synthesis, whereas exposure to 6 μg ml^{-1} lead was associated with increased synthesis of cadaverine and its homologues (Fig. 40).

Following exposure of P. involutus to zinc, putrescine and spermidine concentrations remained unaffected, despite a substantial reduction in ODC activity (Figs. 51 & 49). Furthermore, spermine concentration was doubled, and large reductions in the flux of [U-^{14}C]lysine into cadaverine, APC and 3 APC were observed (Fig. 52). Bound AdoMetDC activity was substantially

Statistical differences shown as: * $P \geq 0.05$; ** $P \geq 0.01$; *** $P \geq 0.001$; nd = not detected in assay; ns = not statistically significant *i.e.* $P < 0.05$.
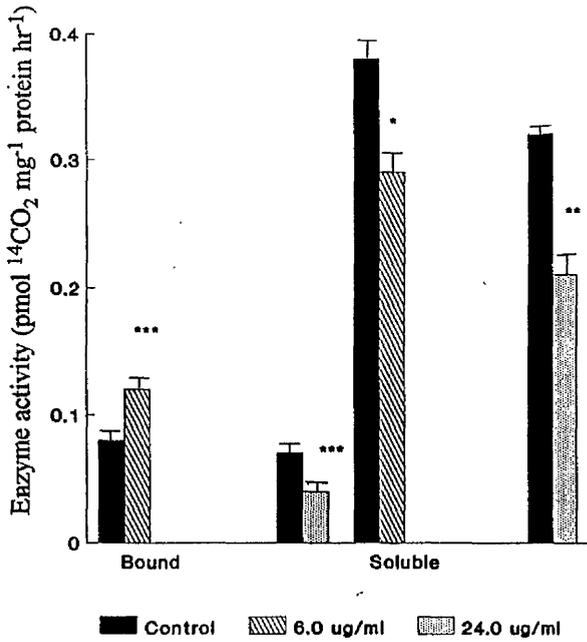

Figure 37. ODC activity in *P. involutus* exposed to lead.
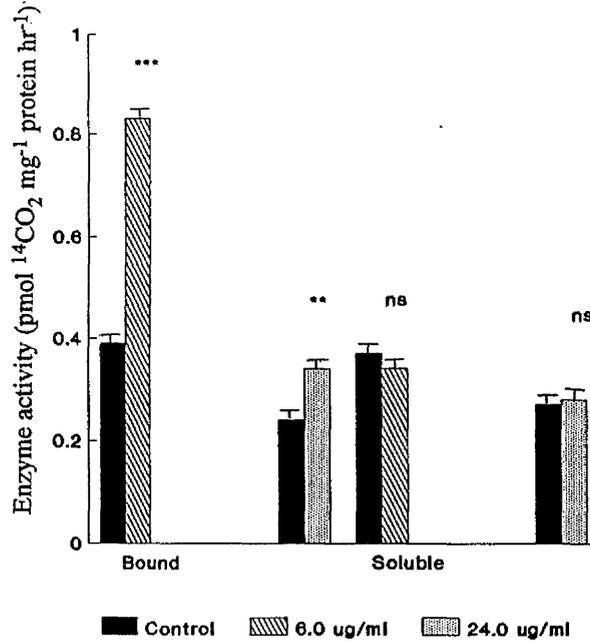
Figure 38. AdoMetDC activity in *P. involutus* exposed to lead.
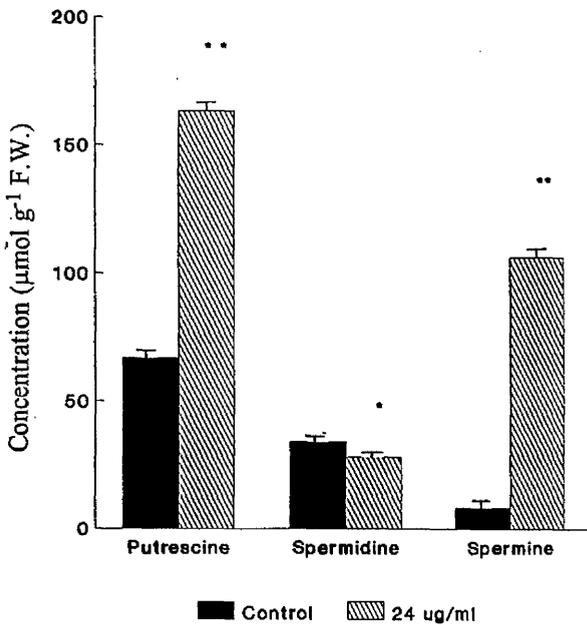
Figure 39. Polyamine concentrations in *P. involutus* exposed to lead.
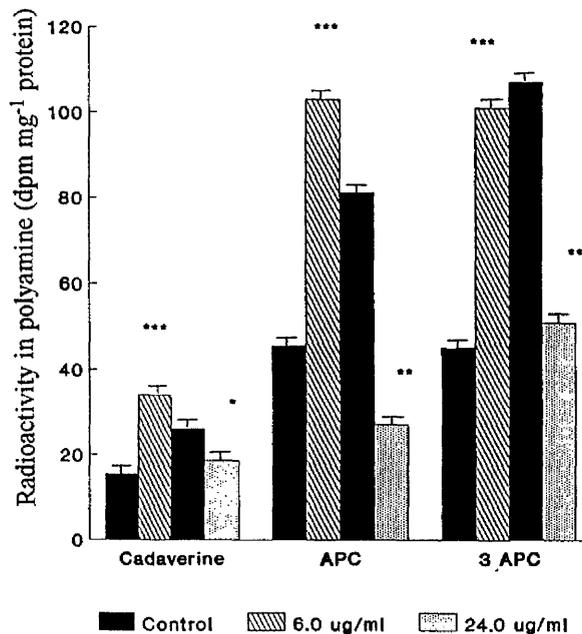
Figure 40. Effect of lead on *in vivo* incorporation of [U-^{14}C]lysine into cadaverine derivatives in *P. involutus*.

63
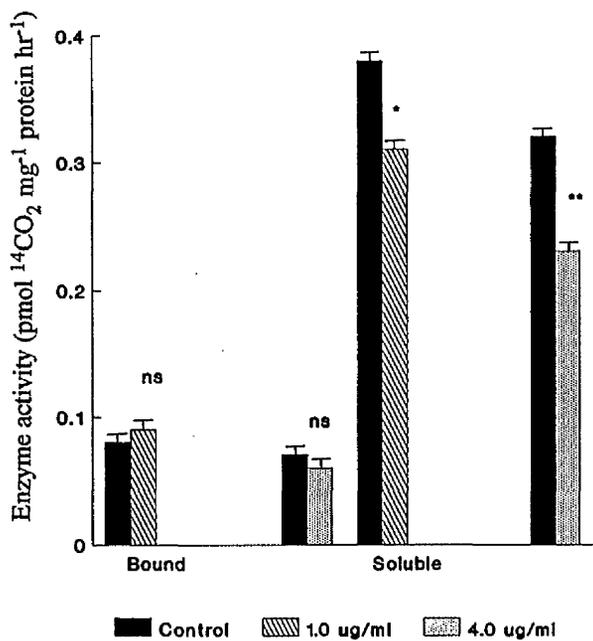
Figure 41. ODC actvity in *P. involutus* exposed to nickel.
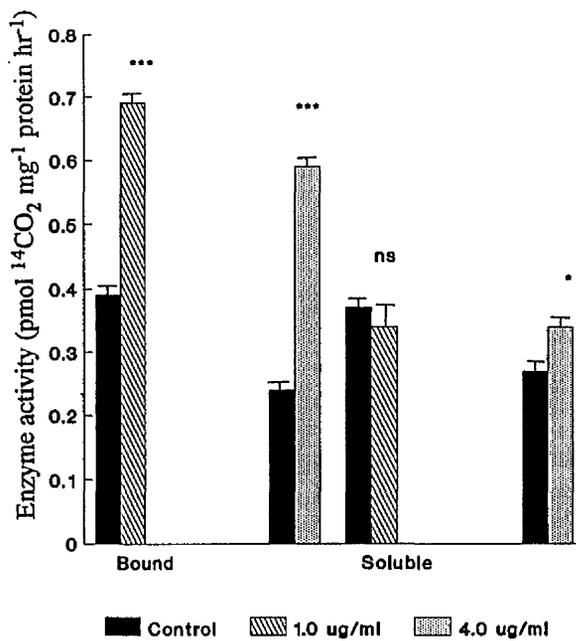
Figure 42. AdoMetDC activity in *P. involutus* exposed to nickel.
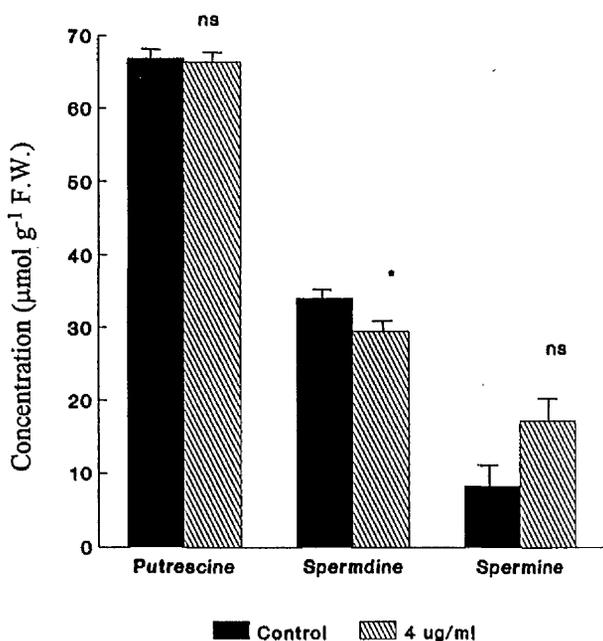
Figure 43. Polyamine concentrations in *P. involutus* exposed to nickel.
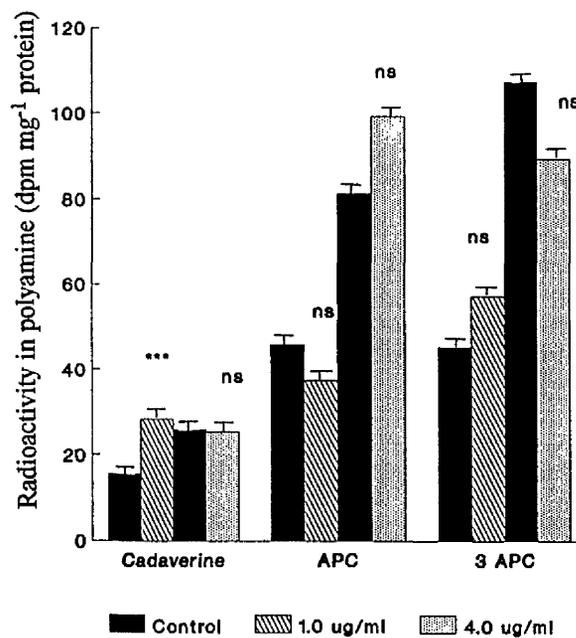

Figure 44. Effect of nickel on *in vivo* incorporation of [U-^{14}C]lysine into cadaverine derivatives in *P. involutus*.
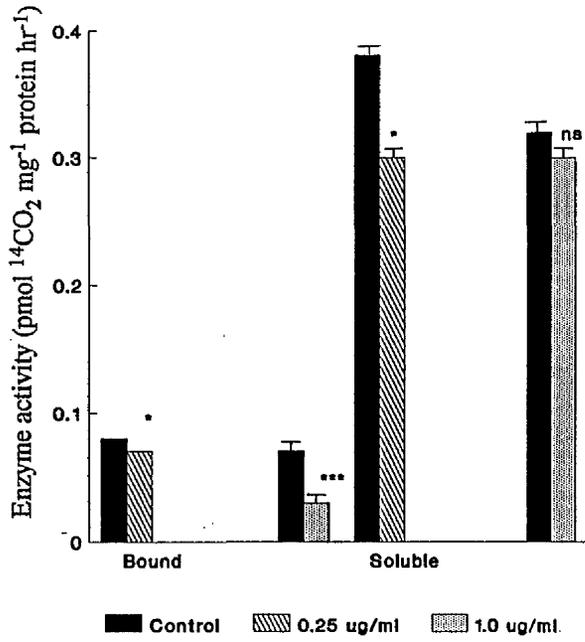
Figure 45. ODC activity in *P. involutus* exposed to copper.
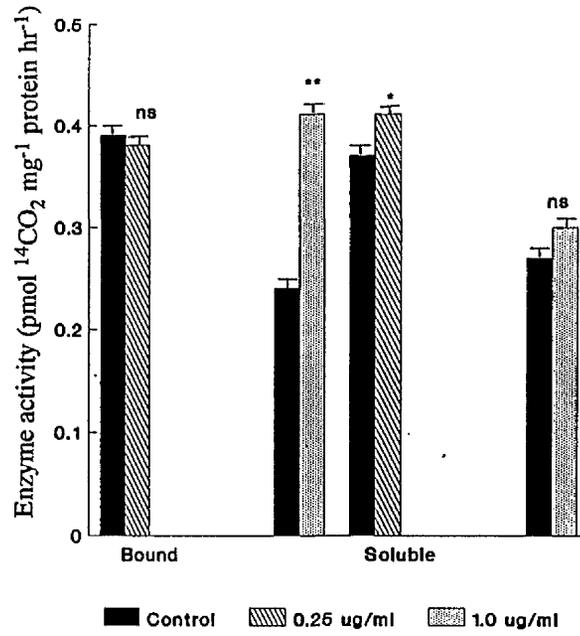
Figure 46. AdoMetDC activity in *P. involutus* exposed to copper.
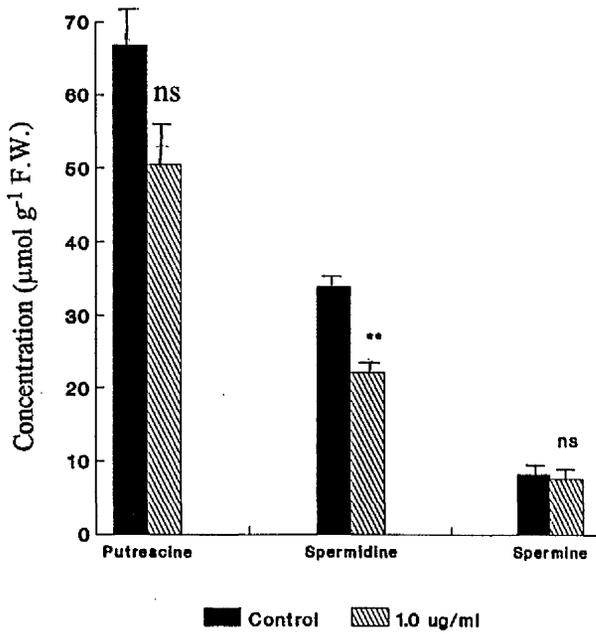
Figure 47. Polyamine concentrations in *P. involutus* exposed to copper.
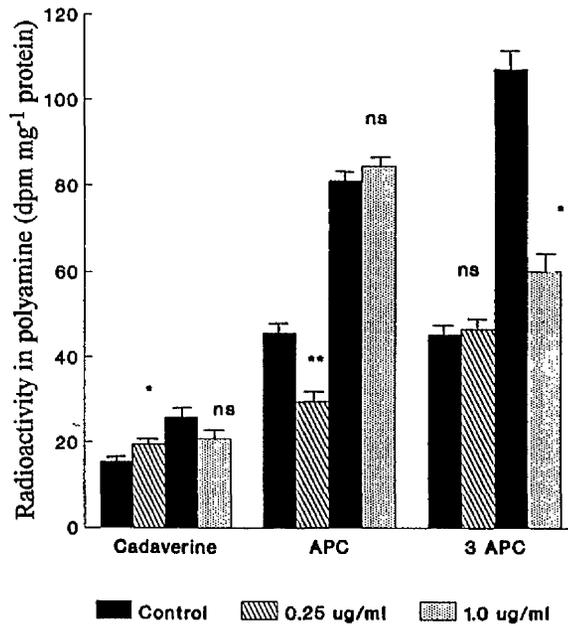
Figure 48. Effect of copper on *in vivo* incorporation of [U-^{14}C]lysine into cadaverine derivatives in *P. involutus*.
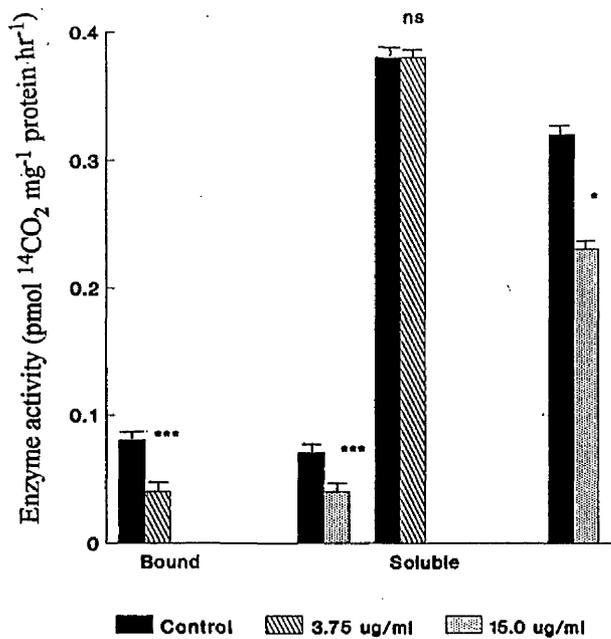
Figure 49. ODC activity in *P. involutus* exposed to zinc.
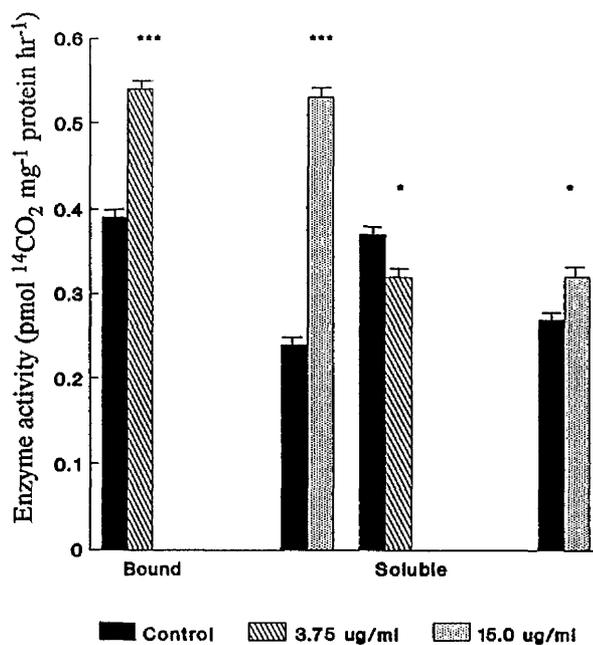
Figure 50. AdoMetDC activity in *P. involutus* exposed to zinc.
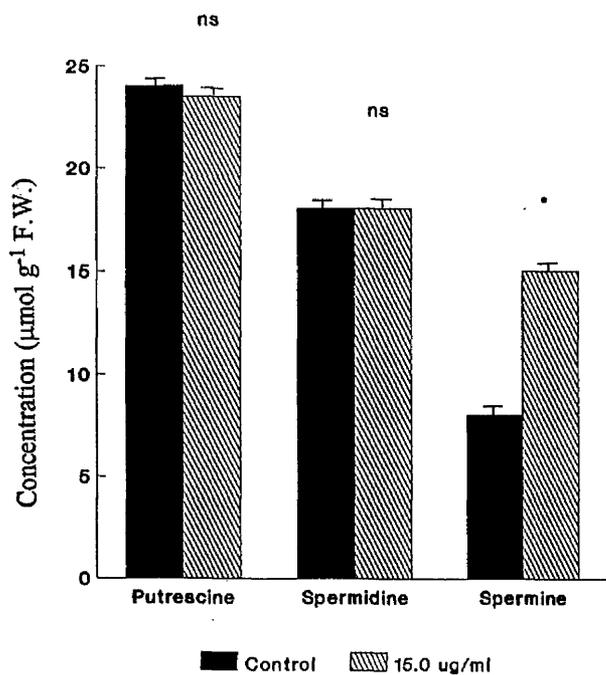
Figure 51. Polyamine concentrations in *P. involutus* exposed to zinc.
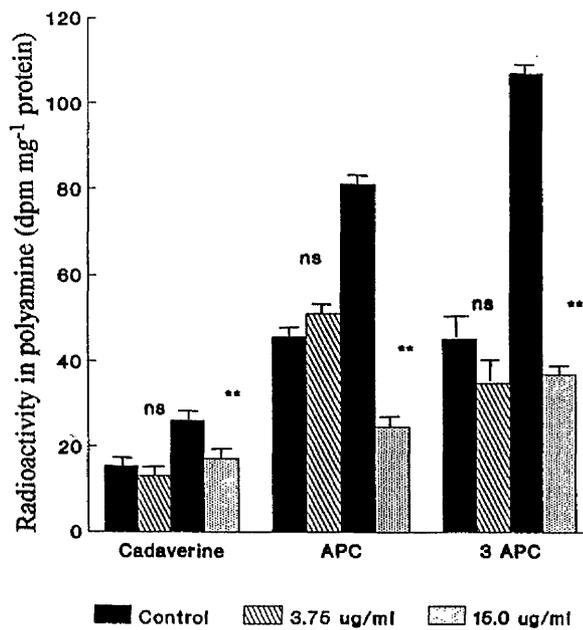


Figure 52. Effect of zinc on *in vivo* incorporation of [U-^{14}C]lysine into cadaverine derivatives in *P. involutus*.

66

increased in the presence of zinc. The activity of the soluble fraction was depressed by 3.75 μg ml⁻¹ zinc, but increased in the presence of 15 μg ml⁻¹ of the metal (Fig. 50).

Copper treatment significantly depressed bound ODC activity, and although the activity of the soluble fraction was reduced by exposure to 0.25 μg ml⁻¹ copper, soluble ODC activity was unaffected by exposure to 1 μg ml⁻¹ copper (Fig. 45). Concentrations of putrescine, spermidine and spermine were correspondingly reduced, but only the reduction in spermidine was statistically significant (Fig. 47). The *in vitro* incorporation of [U-¹⁴C]lysine into cadaverine was not significantly affected by exposure of the fungus to copper. However, the flux of ¹⁴C into APC was reduced by exposure to 0.25 μg ml⁻¹ copper, and the flux of 3 APC was substantially reduced by 1 μg ml⁻¹ (Fig. 48). Bound AdoMetDC activity was increased following exposure of *P. involutus* to 1 μg ml⁻¹ copper. Soluble activity was increased subsequently on exposure of the fungus to 0.25 μg ml⁻¹ copper (Fig. 46).

DISCUSSION.

In view of the range of functions and possible physico-chemical interactions of metal ions in the cell, the apparent disparity between the effects of toxic metals on polyamine metabolism is to be expected. Although the concentrations of putrescine, spermidine and spermine were affected to some extent, with copper and nickel treatments being associated with reductions in spermidine, the variations in polyamine concentration did not significantly affect fungal fresh weight.

Some of the largest perturbations in polyamine biosynthesis were those leading to the *in vitro* formation of cadaverine homologues from [U-¹⁴C]lysine in fungal extracts. Several reports describe an accumulation of cadaverine, and its derivatives, which can sustain growth in animal, bacterial and fungal cells defficient in putrescine. For example, polyamine-dependent Chinese hamster ovary cells deprived of ornithine, or ornithine-derived polyamines, were shown to synthesise cadaverine, APC and 3 APC. Synthesis of these compounds was inhibited by exposure of the cells to DFMO, indicating that the formation of cadaverine in the absence of ornithine and polyamines was catalysed by ODC (Holtta & Pohjanpelto, 1983). Similarly, Igarishi *et al.* (1986) demonstrated the ODC-mediated synthesis of APC from cadaverine in polyamine-dependant strains of *E. coli* grown in the absence of polyamines. In certain *Neurospora crassa* mutants, ODC was believed to be the

sole enzyme available for the decarboxylation of lysine to cadaverine (Paulus *et al.*, 1982). Polyamine-deprived mycelia showed greatly enhanced ODC activity, and cadaverine and its derivatives appeared to be synthesised only in cells with high ODC activity and which lacked ornithine.

In *P. involutus* exposed to toxic metals, polyamine concentrations were not greatly depleted. Indeed, in some cases, following exposure to lead, for example, putrescine and spermidine concentrations were greatly increased. These changes were associated with an increased flux of [U^{14}-C]lysine through to cadaverine, APC and 3 APC when the fungus was exposed to 6 µg ml^{-1} lead, but with a decrease in this flux on exposure to 24 µg ml^{-1} lead. It seems unlikely that the doubling of putrescine concentration observed here (Fig. 39) was due to increased biosynthesis, since ODC activity was either reduced or only slightly increased (Fig. 37). Rather, it is more probable that this elevated free putrescine is the result of displacement of the diamine from binding sites by the divalent metal ion. Although this would also account, in part, for the increased concentration of spermidine, increased biosynthesis was probably only a contributory factor. Thus, the activity of bound AdoMetDC was increased in *P. involutus* exposed to lead (Fig. 38), and moreover, the small reduction in spermidine concentration is consistent with its use as a substrate for increased spermine formation. The increased bound AdoMetDC activity could also account for the increased formation of APC and 3 APC at 6 µg ml^{-1} lead. However, the reasons for the decreased formation of these compounds at 24 µg ml^{-1} lead remains unknown. It is possible that at this lead concentration, the formation of cadaverine homologues was reduced in favour of spermine formation. Spermine accumulation under these circumstances may be important in maintaining the structural integrity of certain proteins and nucleic acids. Although displacement could also partly explain the similar increases in spermine observed following exposure to zinc and nickel, it is also likely that increased synthesis *via* elevated bound AdoMetDC was an important contributory factor. It is of interest, and worthy of further investigation, that all of the metals examined increased bound AdoMetDC activity to some extent.

Each of the metals to which *P. involutus* was exposed was probably immobilised in fungal tissues. Zinc accumulation has been reported by several authors (Brown & Wilkins, 1985; Denny & Wilkins, 1987). Zinc appears to be excluded from mycorrhizal plants by immobilsation in the cell

wall of extra-matrical hyphae, and in extra-hyphal polymeric compounds, where it is bound at electronegative sites. This would presumably prevent it from interacting with cytoplasmic processes. Data presented here support this premise, since soluble (cytoplasmic) ODC and AdoMetDC activities and polyamine biosynthesis in *P. involutus* were minimally affected by exposure of the organism to zinc. Extra-hyphal polymeric compounds may be more important binding site for metal ions than the cell wall itself since the latter will have only a finite number of saturable binding sites for cations. Extra-hyphal excretions may well be replaced more rapidly than cell wall material. It is possible that polyamines bound to the cell wall could be displaced by cationic metal ions. This could account for the small increases in the concentration of free polyamines associated with exposure of the fungus to some metal ions in *P. involutus*, for example, increases in spermine following exposure to nickel and zinc (Figs. 43 & 51).

The speciation and solubility of metal ions in solution are greatly influenced by pH. *P. involutus* can reduce the pH of the growth medium from pH 6.5 to pH 3 after 2-3 weeks. This has been reported previously (Rasanayagam & Jeffries, 1992), and is due to the exudation of organic acids, including oxalic acid, into the growth medium. This phenomenon occurs in nature and can ameliorate the toxic effects of metal ions by reducing metal uptake by the fungus, even though copper shows increased solublility and availability to fungi at low pH (Gruhn & Miller, 1991). In *Aureobasidium pullalans*, for example, copper toxicity is reduced in both copper-sensitive and copper-tolerant strains of the fungus when the pH of the medium is reduced from 4.5 to 2.2 (Gadd & Griffiths, 1980). This amelioration of copper toxicity at low pH is attributable to a reduction in the uptake of the metal by the fungus, and is due to the effect of pH alone, since the same effect could be acheived using sulphuric, nitric or hydrochloric acids to reduce the pH of the medium. The reduced uptake of copper in *P. involutus* correlates with these findings.

Copper has been found to affect polyamine concentrations in some fungi, and to have a concommitant effect on fungal tyrosinase activity, which cataylses steps in the synthesis of the dark pigment, melanin (Gruhn & Miller, 1991). Melanisation has been linked to increased tolerance to copper, and other toxic metals, in some pigmented fungi. These authors consider that tyrosinase activity in some fungi can be stimulated by copper, possibly because the enzyme is a copper-protein complex, thereby increasing melanin pigmentation of the hyphae. Cell wall melanisation then limits

69

the entry of copper, and other ions, into the cell. Furthermore, tyrosinase itself may chelate and detoxify copper. It is therefore possible that pigmentation in *P. involutus* plays a role in restricting copper ion ingress to the cells.

It was notable that metal accumulation occured in *P. involutus* with no significant effect on fungal fresh weight. Therefore polyamine synthesis was maintained sufficiently to sustain growth, possibly by the exclusion of metal ions from the fungal cell by sequestration at extra-cellular binding sites. *P. involutus* appears to have at least two major mechanisms that would limit metal toxicity in mycorrhizal plants. One involves the accumulation of metals, such as lead, in fungal tissues. The other involves the exclusion of metals, such as copper, from the the fungal cell by sequestering it at extra-cellular locations. These mechanisms do not appear to significantly affect total polyamine biosynthesis, or growth of the fungus. Cell wall pigmentation in *P. involutus* may be important in limiting the entry of metals into the cell.

REFERENCES.

Alhonen-Hongisto L, Janne J. 1980. Polyamine depletion induces enhanced synthesis and accumulation of cadaverine in cultured Ehrlich ascites carcinoma cells. *Biochemical and Biophysical Research Communications* **93**: 1005-1013.

Alhonen-Hongisto L, Seppanen P, Holta E, Janne J. 1982. Replacement of natural polyamines by cadaverine and its aminopropyl derivatives in Ehrlich ascites carcinoma cells. *Biochemical and Biophysical Research Communications* **106**: 291-297.

Barnet GR, Seyfzadeh M, Davis RH. 1988. Putrescine and spermidine control the degradation and synthesis of ornithine decarboxylase in *Neurospora crassa*. *Journal of Biological Chemistry* **263**: 10005-10008.

Bell AA, Wheeler MH. 1986. Biosynthesis and functions of fungal melanins. *Annual Review of Phytopathology* **24**: 411-451.

Biondi S, Polgrossi I, Bagni N. 1993. Effect of polyamine biosynthesis inhibitors on mycelial growth and polyamine levels in *Ophiostoma ulmi*. *New Phytologist* **123**: 415-419.

Bitonti AJ, McCann PP. 1987. Inhibition of polyamine biosynthesis in micro-organisms. In: McCann PP, Pegg AE, Sjoerdsma A, eds. *Inhibition of Polyamine Metabolism*. Orlando: Academic Press, 259-274.

Bloss HE, Pfeiffer CM. 1984. Latex content and biomas increase in mycorrhizal guayle (*Parthenium argentatum*) under field conditions. *Annals of Applied Biology* **104**: 175-183.

Bradshaw AD, McNeilly T. 1981. Evolution and Pollution. Studies in Biology 130. London: Edward Arnold.

Brown MT, Wilkins DA. 1985. Zinc tolerance of *Amanita* and *Paxillus*. *Transactions of the British Mycological Society* **84**: 367-369.

Cohen AS, Popovic RB, Zalik S. 1979. Effects of polyamines on chlorophyll and protein content, photochemical activity, and chloroplast ultrastructure of barley leaf discs during senescence. *Plant Physiology* **64**: 717-720.

Cooper KM, Grandison GS. 1986. Interaction of VAM fungi and root knot nematode on cultivars of tomato and white clover susceptible to *Meloidogyne hapla. Annals of Applied Biology* **108**: 555-565.

Davis RH, Krasner GN, DiGangi JJ, Ristow JL. 1985. Distinct roles of putrescine and spermidine in the regulation of ornithine decarboxylase in *Neurospora crassa. Proceedings of the National Academy of Science USA* **82**: 4105-4109.

Dehne HW. 1982. Interactions between vesicular-arbuscular mycorrhizal fungi and plant pathogens. *Phytopathology* **72**: 1115-1118.

Denny HJ, Wilkins DA. 1987. Zinc tolerance in *Betula* spp. IV. The mechanism of ectomycorrhizal amelioration of zinc toxicity. *New Phytologist* **106**: 545-553.

DiTomaso JM, Hart JJ, Kochain LV. 1992. Transport kinetics and metabolism of exogenously applied putrescine in roots of intact maize seedlings. *Plant Physiology* **98**: 611- 620.

Foster SA, Walters DR. 1990. The effects of polyamine biosynthesis inhibitors on the oat-infecting fungus *Pyrenophora avenae. Journal of General Microbiology* **136**: 233-239.

Foster SA, Walters DR. 1993. Fungicidal activity of the polyamine analogue, keto-putrescine. *Pesticide Science* **37**: 267-272.

Friedman R, Altman A, Bachrach U. 1985. Polyamines and root formation in Mung bean hypocotyl cuttings. *Plant Physiology* **79**: 80-83.

Friedman R, Levin N, Altman A. 1986. Presence and identification of polyamines in xylem and phloem exudates of plants. *Plant Physiology* **82**: 1154-1157.

Gadd GM. 1993. Interactions of fungi with toxic metals. *New Phytologist* **124**: 25-60.

Gadd GM, Griffiths AJ. 1980. Influence of pH on copper uptake and toxicity in *Aureobasidium pullulans. Transactions of the British Mycological Society* **75**: 91-96.

Gadd GM,

Galston AW, Kaur-Sawhney R. 1990. Polyamines in plant physiology. *Plant Physiology* **94**: 406-410.

Geiger LE, Morris DR. 1980. Stimulation of deoxyribonucleic acid replication fork movement by spermidine analogues in polyamine-deficient *Escherichia coli. Journal of Bacteriology* **141**: 1192-1198.

72

Gruhn CM, Miller OK. 1991. Effect of copper on tyrosinase activity and polyamine content of some ectomycorrhizal fungi. *Mycological Research* **95**: 268-272.

Haas JH, Bar Tal A, Krikun J. 1986. Nutrient availability effects on VAM bell pepper (*Capiscum annuum*) seedlings and transplants. *Annals of Applied Biology* **108**: 171-179.

Hamana K, Niitsu M, Samejima K, Matsuzaki S. 1988. Occurence of aminopropylcadaverine and its aminopropyl derivatives aminopentylnorspermidine and N,N' *bis* (3 aminopropyl)cadaverine in *Halococcus acetoinfasciens. FEMS Microbiology Letters* **50**: 79-83.

Hamana K, Matsuzaki S, Niitsu M, Samejima K. 1990. Synthesis of novel polyamines in *Paracoccus, Rhodobacter* and *Micrococcus. FEMS Microbiology Letters* **67**: 267-274.

Havis ND, Walters DR. 1992. Evaluation of difluoromethylornithine against powdery mildew on spring barley. *Tests of Agrochemicals and Cultivars 13, Annals of Applied Biology* **120**: (Supplement), 16-17.

Havis ND, Walters DR, Foster SA, Martin WP, Cook FM, Robins DJ. 1994a. Fungicidal activity of the synthetic putrescine analogue, (E)-1,4-diaminobut-2-ene, and derivatives. *Pesticide Science* **41**: 61-69.

Havis ND, Walters DR, Martin WP, Cook FM, Robins DJ. 1994b. Evaluation of the putrescine analogue (E)-1,4-diaminobut-2-ene against powdery mildew on spring barley. *Tests of Agrochemicals and Cultivars 15, Annals of Applied Biology* **124**: (supplement), 14-15.

Havis ND, Walters DR, Martin WP, Cook FM, Robins DJ. 1994c. Fungicidal activity of three putrescine analogues. *Pesticide Science* **41**: 71-76.

Herbst EJ, Snell EE. 1948. Putrescine as a growth factor for *Hemophilus parainfluenzae. Journal of Biological Chemistry* **176**: 989-990.

Hiatt A. 1989. Polyamine synthesis in maize cell lines. *Plant Physiology* **94**: 1378-1381.

Hölttä E, Pohjanpelto P. 1983. Polyamine starvation causes accumulation of cadaverine and its derivatives in a polyamine-dependent strain of Chinese-hamster ovary cells. *Biochemical Journal* **210**: 945-948.

Ickeson I, Bakhanashvili M, Apelbaum A. 1986. Inhibition by ethylene of polyamine biosynthetic enzymes enhanced lysine decarboxylase activity and cadaverine accumulation in pea seedlings. *Plant Physiology* **82**: 607.

Igarishi K, Kashiwagi K, Hamasaki H, Miura A, Kakegawa T, Hirose S, Matsuzaki S. 1986. Formation of a compensatory polyamine by *Escherichia coli* polyamine-requiring mutants during growth in the absence of polyamines. *Journal of Bacteriology* 166: 128-134.

Jorstad CM, Harada JJ, Morris DR. 1980. Structural specificity of the spermidine requirement of an *Escherichia coli* auxotroph. *Journal of Bacteriology* 141: 456-463.

Kallio A, McCann PP. 1981. Difluoromethylornithine irreversibly inactivates ornithine decarboxylase of *Pseudomonas aeruginosa*, but does not inhibit the enzymes of *Escherichia coli*. *Biochemical Journal* 200: 69-75.

Kelley WD. 1978. Control of *Cronartium fusiforme* on Loblolly pine seedlings with the experimental systemic fungicide Benodanyl. *Plant Disease Reporter* 62: 595-598.

Kelley WD, South DB. 1978. Effects of herbicides on in vitro growth of mycorrhizae of pine (*Pinus spp*). *Weed Science* 28: 599.

Khan AJ, Minocha SC. 1989a. Biosynthetic arginine decarboxylase in phytopathogenic fungi. *Life Sciences* 44: 1215-1222.

Khan AJ, Minocha SC. 1989b. Polyamine biosynthetic enzymes and the effect of their inhibition on the growth of some pathogenic fungi. *Plant Cell Physiology* 30: 655-663.

Large PJ. 1992. Enzymes and pathways of polyamine breakdown in micro-organisms. *FEMS Micobiology Reviews* 88: 249-262.

Lehto T. 1992a. Mycorrhizas and drought resistance of *Picea sitchensis* (Bong.) Carr. I. In conditions of nutrient defficiency. *New Phytologist* 122: 661-668.

Lehto T. 1992b. Mycorrhizas and drought resistance of *Picea sitchenis* (Bong.) Carr. II. In conditions of adequate nutrition. *New Phytologist* 122: 669-673.

Lewis DH. 1973. Concepts in fungal nutrition and the origin of biotrophy. *Biological Reviews* 48: 261-278.

Mason PA. 1980. Aseptic synthesis of sheathing (ecto-) mycorrhizas. In: Ingram DS, Helgeson JP, eds. *Tissue culture methods for plant pathologists*. London: Blackwell Scientific Press, 173-178.

Mowll JL, Gadd GM. 1983. Zinc uptake and toxicity in the yeasts *Sporobolomyces roseus* and *Saccharomyces cereviciae*. *Journal of General Microbiology* 129: 3421-3425.

Murphy RJ, Levy JF. 1983. Production of copper oxalate by some copper-tolerant fungi. *Transactions of the British Mycological Society* 81: 165-168.

Mussell H, Osmeloski J, Wettlaufer SH, Weinstein L. 1987. Suppression of Verticillium wilt of tomato by difluoromethylornithine, a suicidal inhibitor of polyamine biosynthesis. *Plant Disease* 71: 313-316.

Okuyama T, Kubayashi Y. 1961. Determination of diamine oxidase activity by liquid scintillation counting. *Archives of Biochemistry and Biophysics* 95: 242.

Parke JF, Linderman RG, Black CH. 1983. The role of ectomycorrhizas in drought tolerance of Douglas fir seedlings. *New Phytologist* 95: 83-95.

Peat HJ, Fitter AH. 1993. The distribution of arbuscular mycorrhizas in the British flora. *New Phytologist* 125: 845-854.

Paulus TJ, Kiyonu P, Davis RH. 1982. Polyamine deficient *Neurospora crassa* mutants and synthesis of cadaverine. *Journal of Bacteriology* 152: 291-297.

Pegg AE. 1988. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Research* 48: 759-774.

Pegg AE, Williams-Ashman HG. 1987. Pharmacologic interference with enzymes of polyamine biosynthesis and of 5'-methylthioadenosine metabolism. In: McCann PP, Pegg AE, Sjoerdsma A, eds. *Inhibition of Polyamine Metabolism*. Orlando: Academic Press, 33-46.

Porter CW, Sufrin JR. 1986. Interference with polyamine biosynthesis and/or function by analogues of polyamines or methionine as a potential anticancer chemotherapeutic strategy. *Anticancer Research* 6: 525-542.

Porter CW, Janne J. 1987. Modulation of antineoplastic drug action by inhibitors of polyamine biosynthesis. In: McCann PP, Pegg AE, Sjoerdsma A, eds. *Inhibition of polyamine metabolism*. Orlando, Florida: Academic Press, 203-243.

Rajam MV, Galston AW. 1985. The effect of some polyamine biosynthetic inhibitors on growth and morphology of phytopathogenic fungi. *Plant Cell Physiology* 26: 683-692.

Rajam MV, Weinstein LH, Galston AW. 1985. Prevention of a plant disease by specific inhibition of fungal polyamine biosynthesis. *Proceedings of the National Academy of Science USA* 82: 6874-6878.

Rajam MV, Weinstein LH, Galston AW. 1989. Inhibition of uredospore germination and germ tube growth by inhibitors of polyamine metabolism in *Uromyces phaseoli* L. *Plant Cell Physiology* **30**: 37-41.

Rasanayagam S, Jeffries P. 1992. Production of acid is responsible for antibiosis by some ectomycorrhizal fungi. *Mycological Research* **96**: 971-976.

Rhodes LH, Larson PO. 1981. Effects of fungicides on mycorrhizal development of creeping bentgrass. *Plant Disease* **65**: 145-147.

Robins DJ, Walters DR. 1992a. Antifungal diamine. *UK Patent Application No.* GB 2 256 141 A.

Robins DJ, Waters DR. 1992b. Antifungal diaminobutane derivatives. *UK Patent Application No.* GB 2 256 142 A.

Ross, IS. 1975. Some effects of heavy metals on fungal cells. *Transactions of the British Mycological Society* **64**: 175-193.

Schreiner RP, Koide RT. 1993. Antifungal compounds from the roots of mycotrophic and non-mycotrophic plant species. *New Phytologist* **123**: 99-105.

Slocum RD, Bitonti AJ, McCann PP, Feirer RP. 1988. D-L-α-difluoromethyl[3,4-^{3}H]arginine metabolism in tobacco and mammalian cells. *Biochemical Journal* **255**: 197-202.

Slocum RD, Flores HE. 1991. *Biochemistry and physiology of polyamines in plants.* Boca Raton, Florida: C.R.C. Press.

Smith GS. 1988. The role of phosphorous nutrition in interactions of vesicular-arbuscular mycorrhizal fungi with soilborne nematodes and fungi. *Phytopathology* **78**: 371-374.

Smith JR, Ferry BW. 1977. The effects of simazine applied for weed control on the mycorrhizal development of Pinus seedlings. *Annals of Botany* **43**: 93-99.

Smith TA. 1985. Polyamines. *Annual Review of Plant Physiology* **36**: 117-143.

Smith TA, Barker JHA, Jung M. 1990. Growth inhibition of *Botrytis cinerea* by compounds interfering with polyamine metabolism. *Journal of General Microbiology* **136**: 985-992.

Smith TA, Barker JHA, Owens WJ. 1992. Insensitivity of *Septoria tritici* and *Ustilago maydis* to inhibitors of ornithine decarboxylase. *Mycological Research* **95**: 395-400.

Spokes JR, Macdonald DRM, Hayman S. 1981. Effects of plant protection chemicals on vesicular arbuscular mycorrhizas. *Pesticide Science* **12**: 346-350.

Srivenugopal K, Adiga S. 1980. Coexistence of two pathways of spermidine biosynthesis in *Lathyrus sativus* seedlings. *FEBS Letters* 112: 260-264.

Stevens L. 1981. Regulation of the biosynthesis of putrescine, spermidine and spermine in fungi. *Medical Biology* 59: 308-313.

Stevens L, McKinnon IR, Winther M. 1976. Polyamine and ornithine metabolism during the germination of conidia of *Aspergillus nidulans*. *Biochemical Journal* 158: 235-241.

Stevens L, Stevens E. 1981. Regulation of ornithine decarboxylase activity during the germination of conidia of *Aspergillus nidulans*. *FEMS Microbiology Letters* 11: 229-232.

Tabor CW. 1981. Mutants of *Saccharomyces cerevisiae* deficient in polyamine biosynthesis: studies on the regulation of ornithine decarboxylase. *Medical Biology* 59: 272-278.

Tabor CW, Tabor H. 1985. Polyamines in micro-organisms. *Microbiological Reviews* 49: 81-99.

Tait GH. 1976. A new pathway for the biosynthesis of spermidine. *Biochemical Society Transactions* 4: 610-612.

Tait GH. 1985. Bacterial polyamines, structures and biosynthesis. *Biochemical Society Transactions* 13: 316-318.

Thompson BD, Grove TS, Malajczuk N, StJ-Hardy GE. 1994. The effectiveness of ectomycorrhizal fungi in increasing the growth of *Eucalyptus globus* Labill. in relation to root colonisation and hyphal development in soil. *New Phytologist* 126: 517-524.

Trione EJ, Stockwell VO, Austin HA. 1988. The effects of polyamines on the growth and development of the wheat bunt fungi. *Botannical Gazette* 149: 173-178.

Walters DR, Kingham G. 1990. Uptake and translocation of α-difluoromethylornithine, a polyamine biosynthesis inhibitor, by barley seedlings: effects on mildew infection. *New Phytologist* 114: 659-665.

Walters DR, Havis ND, Foster SA, Robins DJ. 1992. Control of fungal diseases of arable crops using inhibitors of polyamine biosynthesis. In: *Brighton Crop Protection Conference - Pests and Diseases 1992*, 645-650.

Weinstein LH, Kaur-Sawhney R, Rajam MV, Wettlaufer SH, Galston AW. 1986. Cadmium-induced accumulation of putrescine in oat and bean leaves. *Plant Physiology* 82: 641-645.

West HM, Walters DR. 1988. The effects of polyamine biosynthesis inhibitors on infection of *Hordeum vulgare* L. by *Erysiphe graminis* f.sp. *hordei* Marchal. *New Phytologist* **110**: 193-200.

Yamamoto S, Hamanaka K, Suemoto Y, Ono B, Shinoda S. 1985. Evidence for the presence of a novel biosynthetic pathway for norspermidine in *Vibrio. Canadian Journal of Microbiology* **32**: 99-103.

Yannai S, Berdicevsky I, Duek, L. 1991. Transformations of inorganic mercury by *Candida albicans* and *Saccharomyces cereviciae. Applied and Environmental Microbiology* **57**: 245-247.