THE EFFECTS OF AGROCHEMICALS ON LITTER FUNGI

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S U M M A R Y

The effects of the fungicides CAPTAN and VERDASAN, and the herbicides, MAZIDE, PARAQUAT, and 2,4,5-trichlorophenoxyacetic acid (2,4,5T) on the occurrence and physiology of fungi inhabiting the litter layer were studied.

When applied at normal field rates, the fungicides slightly reduced the total fungal numbers and species numbers isolated from leaf litter of Agrostis tenuis, although there were little or no effects with the herbicides at either normal field rate or elevated rates. When applied at a rate three times greater than normal field rate the fungicides drastically reduced the number of species isolated, although only CAPTAN markedly reduced the total number of isolations.

The species composition of the leaf litter mycoflora was also altered: Alternaria alternata and Ulocladium botrytis showed increased incidence from fungicide-treated leaves, although Epicoccum purpurascens and Fusarium spp. were reduced. U. botrytis was recorded as the major component of VERDASAN-treated leaf litter. Its numbers increased dramatically when the fungicide was applied at the higher rate.

An attempt was made to isolate Basidiomycetes from a number of cellulosic substrates, and one which was successfully isolated was used in subsequent physiological studies.

The growth of a number of mycorrhizal and non-mycorrhizal Basidiomycetes was studied. The non-mycorrhizal fungi showed higher growth rates than the mycorrhizal forms in media, both with and without agrochemical addition. The results obtained with Phallus impudicus indicated that it was more comparable with the mycorrhizal species.

The effects of PARAQUAT and VERDASAN on the leakage of potassium, phosphate and amino acids from mycorrhizal and non-mycorrhizal Basidiomycetes were also studied. The mycorrhizal species generally leaked cellular metabolites faster and more copiously than the non-mycorrhizal species in the presence of agrochemicals.

The organo-mercurial fungicide VERDASAN was shown to inhibit cellulase activity of a Basidiomycete found inhabiting leaf litter.

AGROCHEMICALS/LEAF LITTER/BASIDIOMYCETES/CELLULASE/MEMBRANE PERMEABILITY

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To my parents, my family, and my friends.

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General Introduction

The importance of studying the leaf litter layer of soil was strongly emphasised by Chesters in 1960. He stressed that "most mycologists interested in soil fungi neglect the surface detritus in favour of that apparently entrancing and most elusive quality - the soil".

The surface litter layer, by its microbiological decay, contributes minerals and soluble organic compounds to the water descending from the surface into the soil, a complex series of processes involving not only fungi, but also bacteria, actinomycetes, and a vast array of macro- and microfauna. The efficient functioning of litter decomposition is a result of a delicate equilibrium between these organisms and is, in turn, mediated by many environmental factors.

Agrochemicals are considered to be indispensable aids in agricultural, horticultural and pastoral practices, and a vast array of these compounds is applied directly to the soil, as run off from treated aerial systems or from drifting sprays. In such situations an environmental disturbance of the soil population is created, as described by Pugh (1980). Some of the more persistent chemicals will remain on the leaves after leaf fall and be subsequently incorporated into the litter layers. In addition, the litter layers produce a 'filter' effect whereby agrochemicals which are applied in suspension may be partially withheld at the surface.

The extent to which agrochemicals are used in modern agriculture is illustrated by the striking increase in their production in recent years. Total sales of herbicides increased from

£30 million in 1970 to £50 million in 1974 in the United Kingdom alone; by 1974, world sales of mercurial fungicides amounted to £23m, while the dithiocarbamates (e.g. Maneb, Thiram) and phthalimides (e.g. Captan, Captafol) had achieved world sales of £225m and £187m respectively (Bent, 1979).

Corden and Young (1965) defined a perfect fungicide as being non-persistent and selectively killing or inhibiting the growth of pathogenic fungi when used at concentrations non-toxic to other soil micro-organisms. Equally, a desirable herbicide is designed to control or eliminate unwanted vegetation, at the same time having no undesirable effects on other non-target forms of life.

However, since these chemicals, and other biocides, are designed to inhibit cellular processes common to many organisms, such specificity is rarely achieved. A highly specific systemic fungicide, Benomyl, has been shown to reduce earthworm numbers in soil (Stringer and Wright, 1973).

Fungi are the major degraders of plant material in soils (Alexander, 1961), and their elimination may have serious effects on the rate at which organic matter is broken down (Pugh and Williams, 1971; Williams, 1973). Estimates of the total number of fungal species vary between as few as 100,000 (Ordish and Mitchell, 1967) to perhaps as many as 240,000 (Ainsworth, 1971); of these about 100 species are common and serious plant pathogens. Thus, considerably more than 99% of known fungal species are not harmful to plants, and through their decomposition activities are beneficial in nature (Pugh, 1976). Yet they may be as susceptible to fungicides as are the plant pathogenic species.

The first portion of this study is devoted to an investigation of the mycoflora of leaf litter after repeated monthly applications of agrochemicals. While Wainwright and Pugh (1975) used single applications of fungicides, and Kuthubutheen and Pugh(1979) used monthly applications of fungicides, these studies were mainly on the effects on soil fungal populations. Pugh and Williams (1971) in their study of the golf course 'thatch' problem carried out work on fungi in the undecomposed plant remains which had accumulated over many years. This study can be regarded as forming the earliest stage in the study of the processes which can lead to thatch formation.

To complement field studies, it was decided to concentrate the later work with agrochemicals on those important, but often neglected members of the litter layer - the Basidiomycetes. Although a great number of these organisms are known to be litter decomposers or mycorrhiza formers, the role of the Basidiomycetes as a whole in the ecology of the soil is still obscure. Chesters (1949) referred to them as the"missing link in soil mycology"; relatively little work has been carried out on them in the intervening 30 years.

In any study of the occurrence and activities of micro-organisms in the presence of agrochemicals which have been designed to control or eliminate part of the living biomass, it is essential that the effects of these compounds on the saprophytic mycoflora of soil be continually monitored. As Daubenmire (1968) stated; "Man must disturb ecosystems but he should recognise that there are limits to the safety in so doing for disturbance becomes dangerous when individual conditions exceed the range of fluctuating natural

conditions to which the organisms have become inured".

CHAPTER I

EFFECT OF AGROCHEMICALS ON LEAF LITTER FUNGI

EFFECT OF AGROCHEMICALS ON LEAF LITTER FUNGI

Introduction

The process of decomposition of leaves has been shown to begin even before the leaf buds burst open. Pugh and Buckley (1971) found that freshly opened sycamore leaves already possessed a well defined mycoflora: the two most common fungi were *Aureobasidium pullulans*, which occurred predominantly along the veins and could be isolated from within them, and *Sporobolomyces roseus*, which also occurred mainly on the veins.

The presence of actively growing fungi on green living leaves has also been cited by Dickinson (1965), Friend (1965) and by Pugh and Mulder (1971). Dickinson (1965) showed that, besides the fungi present only as detachable propagules on the leaf surface, others may be classified as those species, such as *Cladosporium*, which grew and sporulated immediately, and those such as *Ascochytula* which grew as sterile mycelium and sporulated after leaf senescence.

The fact that the primary leaf colonists play more than a passive role on the leaf surface has never been disputed. Last and Deighton (1965) indicated that "leaf saprophytes may act as scavengers 'mopping up' energy sources such as amino acids and sugars which otherwise might stimulate the growth of plant parasites". Also, di Menna (1962) suggested that the balance between micro-organisms, whether populations of different saprophytes or mixtures of parasites and saprophytes, may be maintained by antagonism or more probably by the competition for nutrients between the various species. Micro-organisms on the leaf surface

are in a state of dynamic equilibrium, and this balancing mechanism not only lies between the major groups but also within them (Last and Warren, 1972). In a study of the phylloplane of wheat leaves by Last (1955), Sporobolomyces, having previously been isolated from the entire leaf surface, was suddenly reduced to the margins, while Tilletiopsis, also a member of the Sporobolomycetae, appeared on the remainder of the lamina.

Many phylloplane micro-organisms produce antimicrobial metabolites when grown in vitro. Crosse (1967) concluded that the overall effect of saprophytes was to reduce the effective pathogen-inoculum dose and that they did this either by competing with the pathogen in the early stages of intravascular growth, by secreting metabolites which impaired virulence in the pathogen, or by stimulating resistance in the host.

Auxins, and other growth regulating substances can be produced by some phylloplane micro-organisms in vitro. Aureobasidium pullulans, Cladosporium herbarum and Epicoccum purpurascens are able to produce indole-acetic-acid (IAA) and indole-acetic-nitrile (IAN)(Buckley and Pugh, 1971).

Abdalla (1970) showed that culture filtrates from a wide range of fungi, including C. herbarum and E. purpurascens both inhibited and, at lower concentrations, stimulated germination of barley grains. It was also speculated by Last and Warren (1972) that gibberellins affecting plant growth may be produced by leaf surface micro-organisms. Valadon and Lodge (1970) have shown that both IAA and IAN can be produced by Cladosporium in vitro. Although, to date, the production of plant growth regulating subst-

ances by phylloplane fungi has only been shown in laboratory experiments, the fact that these compounds have been found in plant leachates, and that leachates can be absorbed by both roots and leaves (Tukey, 1971), emphasises the potential ecological significance of phylloplane micro-organisms.

It has been suggested that the prior colonisation of leaf surfaces by saprophytes may trigger the release of phytoalexins capable of decreasing the germination and attack by pathogens (Cruickshank and Perrin, 1963; Bailey, 1971). Cruickshank (1966) suggested a form of biological control by stimulating phytoalexin production.

The degradation of plant waxes on the surface of leaves by phylloplane micro-organisms has been discussed by Last and Deighton (1965) who suggested that populations of Aureobasidium pullulans and Cryptococcus sp., which are able to decompose pectin (Smit and Weiringa, 1953) and lipids (Ruinen, 1963), may render leaf surfaces more wettable, and in doing so may increase the availability of nutrients to parasites. However, no evidence of pectolytic activity has been found by Hering (1967) in A. pullulans isolated from leaf litter of oak.

Ruinen (1956, 1961, 1971) discussed the exchange of metabolites between phylloplane flora and leaves and she suggested (Ruinen, 1956) that nitrogen fixing micro-organisms, acting as a 'factory' for organic nitrogen on the leaf surface, may contribute to the nitrogen economy of the plant in exchange for nutrients excreted by the leaves. A second cycle, supporting the first, may occur in the soil and decomposing leaf litter (Ruinen, 1956).

Thus, apart from being ideally situated to exploit the underlying tissues at the earliest opportunity, the primary leaf colonists have been shown, under laboratory conditions, to have the potential to perform other important functions on the leaf surface. The application of chemicals, either as fungicides to alleviate or control plant diseases, or herbicides to control or eradicate unwanted vegetation, which are to some extent non-specific in the variety of organisms they affect, may alter the dynamic microbial balance of both the phylloplane and soil microflora. In doing so, they may upset the many important interactions contributing to the decay of plant material.

Attention has mostly been directed towards the effects of fungicides on leaf surface micro-organisms in recent years. Fungicides may delay senescence or they may upset the balanced micro-ecosystems on the leaf surface allowing the prolific development of harmful pathogens or non-beneficial fungi (Hislop, 1976).

Rayner (1957) and Price (1969) reported a prolonged leaf life following fungicide spraying which was, in the latter case, attributed to a reduction in the activity of phylloplane saprophytes. However, Bainbridge and Dickinson (1972) did not find any close correlation between the rate of leaf senescence in potatoes, and fungicide application.

Dickinson (1973a,b) has discussed the interactions of fungicides and leaf surface fungi. In addition, Dickinson and Walpole (1975) and Dickinson and Wallace (1976) studied the effects of fungicides on the yield of winter wheat, and on the activity of micro-organisms on winter wheat leaves, respectively.

The effects of fungicides and herbicides on soil micro-organisms have been extensively reviewed (Domsch, 1964; Wainwright, 1978, for fungicides; Tu and Bollen, 1968; Greaves *et al*. 1976, for herbicides). However, the effects of these compounds on the more specific processes of plant litter decomposition have received little attention. Most work in this area has been concentrated on successional studies on decaying plant material (Hering, 1965; Frankland, 1966). It was decided, therefore, to study the effects of both fungicides and herbicides on leaf litter fungi of *Agrostis tenuis*.

Materials and Methods

a. Description of Field Plots

The experimental plots used in this study were situated at Handsworth Village Student Campus, Handsworth, Birmingham (grid reference: SP 055 906). The site contained mainly *Agrostis tenuis*, although *Taraxacum officinale*, *Plantago major* and seedlings of *Acer pseudoplatanus* were found occasionally. Individual plots had been sprayed with fungicides or herbicides between May 1977 and July 1977 in an earlier study (Smith and Pugh, 1979). In the present study, the plots were sprayed at the rates shown in Table 1.

b. Experimental Design and Field Study

The site consisted of a rectangle 5.5 m x 11 m, laid out using tent pegs and nylon string and divided into 12 experimental plots, each measuring 2 m x 1 m (Fig. 1). The treatment of the plots was randomised and each plot was bounded by a path 0.5 m wide, to aid spraying and to reduce the risk of cross-contamination by drifting and by leaching. From December 1977 to April 1978 inclusive, the agrochemicals were applied at normal field application rate (FR), and from May 1978 to November 1978 inclusive, at three times their field application rate (3 x FR), to the same plots.

The fungicides, CAPTAN and VERDASAN, and the herbicides MAZIDE, PARAQUAT and 2,4,5T were applied in solution or in suspension, in tap water. The CONTROL was sprayed with tap water of similar volume to that used on treatment plots. The grass on all plots was cut after sampling on Day 0 between April 1978 and

Table 1. Rates of Application of Fungicides and Herbicides.

	RATE OF APPLICATION	
	NORMAL FIELD RATE $g/l/m^2$	3 x NORMAL FIELD RATE $g/l/m^2$
CAPTAN	0.90	2.70
VERDASAN	1.25	3.75
MAZIDE	1.9×10^{-3}	5.7×10^{-3}
PARAQUAT	3.67×10^{-4}	1.10×10^{-3}
2,4,5T	3.97×10^{-4}	1.19×10^{-3}

Fig.1. The experimental plots.

CON	CONTROL
C	CAPTAN
V	VERDASAN
M	MAZIDE
P	PARAQUAT
T	2,4,5T

< 0.5m >< 2m >

< 1m >

P

M

V

T

CON

C

October 1978 inclusive, and the cuttings allowed to remain on the surface. The agrochemicals, their uses and chemistry are summarised in Appendix 1. The meteorological conditions prevailing during the course of this study are shown in Appendix 2.

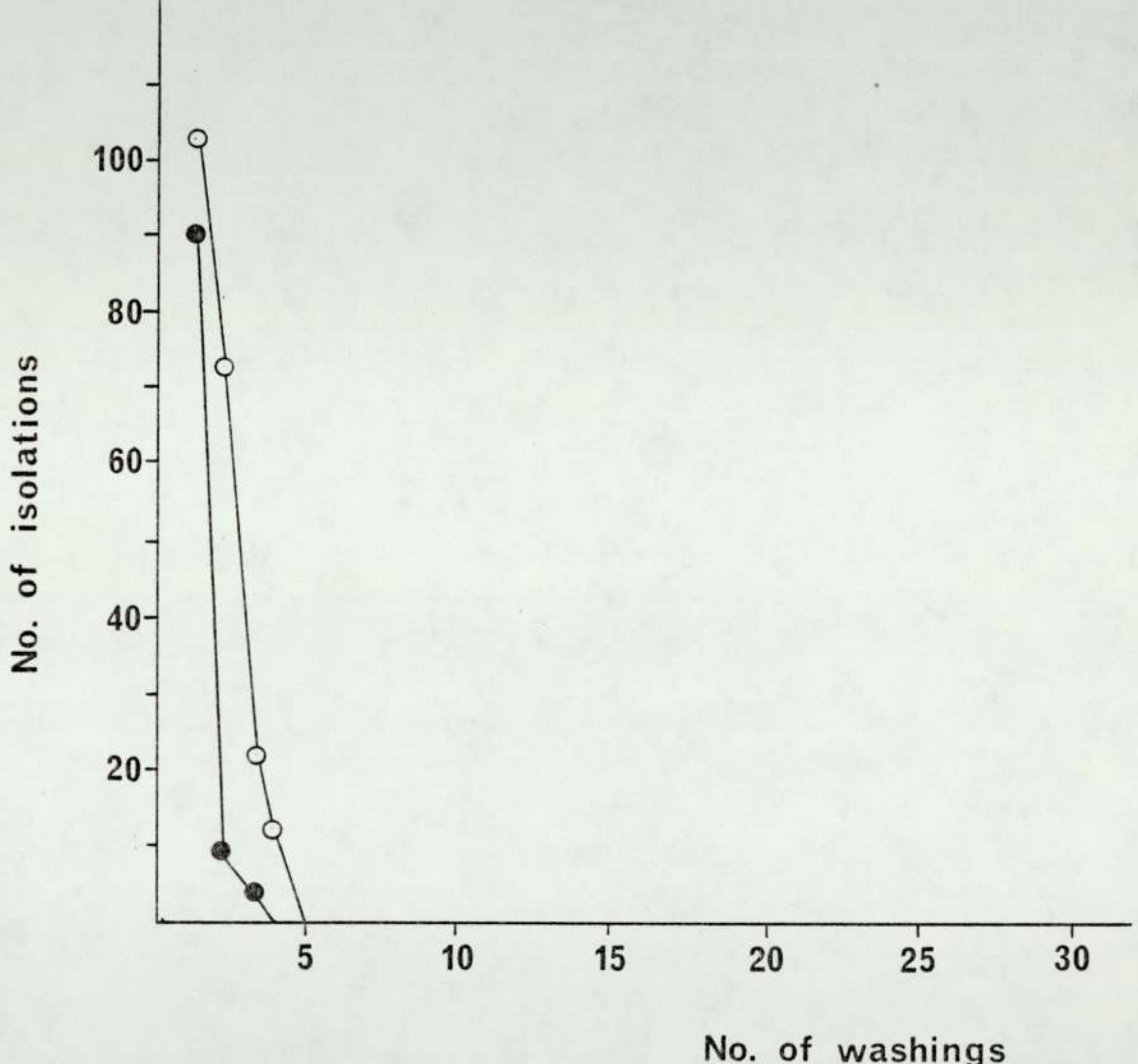
Leaf litter samples of A. tenuis were collected from the fungicide and herbicide treated plots immediately after spraying (Day 0) and subsequently on Days 7, 14 and 28. After the collection of samples on Day 28 the plots were sprayed and considered as Day 0 for the next month. Twenty-five cores of soil, 1 cm deep, were sampled from each plot using a sterile 2 cm soil borer, and were mixed together in a sterile polythene bag. Only when the ice layer was so thick on three occasions in Feruary 1978 was sampling prevented. The samples were transported immediately to the laboratory, air dried overnight and then sieved to separate green leaves and litter from the soil particles. From the sieve trays, 25 pieces of fresh leaf litter were removed. Any pieces which showed signs of disintegration or skeletonisation were ignored. Leaf litter segments of uniform length (3 mm) were cut using a sterile parallel blade cutter. In order to remove loosely adhering spores, mycelial fragments and soil, the litter segments were washed serially. The application of such a technique to beech roots and litter (Harley and Waid, 1955) and Pinus needles (Kendrick and Burges, 1962) has been successfully used to isolate actively growing fungi. To determine the efficiency of the washing process, 25 litter segments were shaken vigorously in 10 ml of sterile distilled water in McCartney bottles in thirty changes of sterile water. An aliquot of each wash was plated out with tap water agar.

This process was repeated in the summer months to compensate for annual fluctuations in air-borne spora and soil inoculum. In view of the results (Fig. 2), it was decided to wash serially the litter segments six times in both summer and winter months. The segments were plated out, some with their adaxial surface down and some with their abaxial surface down, onto cellulose agar (Eggins and Pugh, 1962) (Appendix 3). The plates were incubated at 25°C. The plates were examined after 10 days and subsequently at regular intervals until no new species were recorded. Each species occurring or growing from a leaf litter segment was considered as one isolate. Analysis of variance was performed on the resulting data to see if the application of fungicides or herbicides had a significant effect on the numbers of fungi growing in or on the leaf litter.

Fig.2. Number of washings required to remove fungal propagules from leaf litter surfaces.

●——● winter washings

○——○ summer washings


No. of isolations
100
80
60
40
20
5
10
15
20
25
30
No. of washings

Results

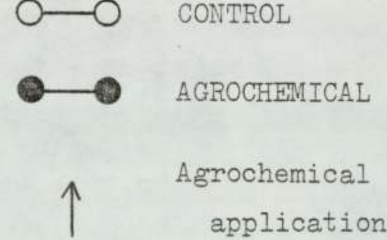
CONTROL plots showed a smaller spring peak in numbers of isolations, and a larger summer-early autumn peak. In general, lower numbers of fungi were isolated in the winter months (Figures 3, 4 and 5, and Appendix 4).

With both CAPTAN and VERDASAN there was a general reduction in the frequency of isolations, which tended to become more marked when the 3 x FR treatment was applied. However, in November and December the VERDASAN plots showed an increase over the CONTROL. All three herbicide plots showed only minor variations in the levels of isolation, compared with CONTROL, throughout the year.

The numbers of isolations and numbers of species of leaf litter fungi growing on or from CONTROL and treated leaves are shown in Tables 2 and 3 respectively. Overall, application of the agrochemicals at their field rate did not significantly alter the number of isolations and neither was the variation in numbers significant on any of the sampling days (Table 2). At 3 x FR the variation in the number of isolations between treatments was significant over the whole period ($F = 7.56$; $P < 0.01$). Also, numbers were significantly different between treatments on Day 0 ($F = 5.3$, $P < 0.05$). Day 14 ($F = 6.5$; $P < 0.01$) and Day 28 ($F = 7.56$; $P < 0.01$). Numbers on CAPTAN-treated leaves were significantly lower than on CONTROL, MAZIDE, PARAQUAT and VERDASAN-treated leaves on Days 0, 14 and 28, and on 2,4,5T-treated leaves on Days 4 and 28. Numbers on VERDASAN-treated leaves sprayed at the higher rate were slightly increased compared with CONTROL.

The effects of agrochemicals on the number of species

Fig.3. The effect of CAPTAN and MAZIDE on leaf litter fungal numbers.



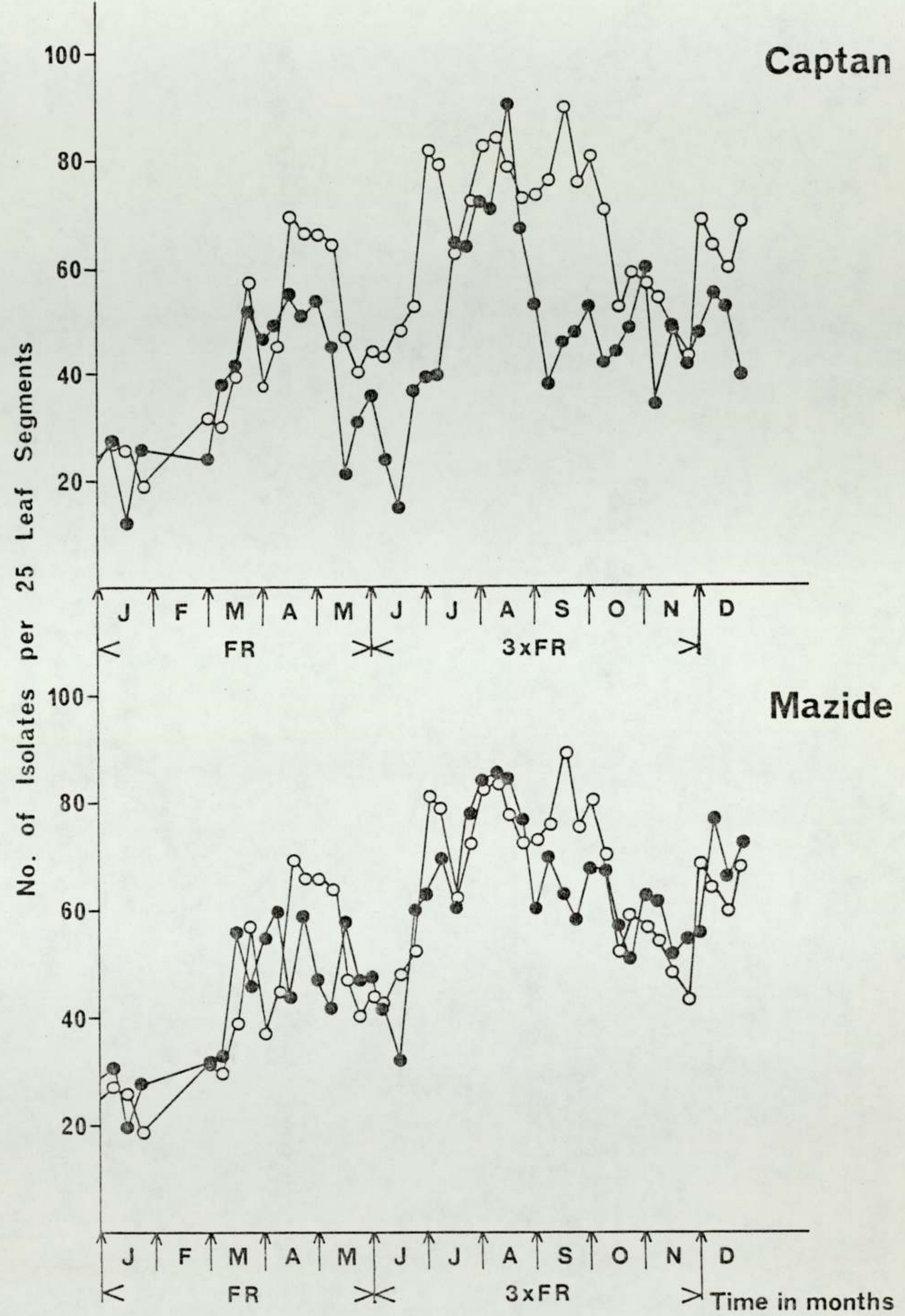

Captan
Mazide
No. of Isolates per 25 Leaf Segments
100
80
60
40
20
J
F
M
A
M
J
J
A
S
O
N
D
FR
3xFR
Time in months

Fig.4. The effect of PARAQUAT and 2,4,5T on leaf litter fungal numbers.

O—O CONTROL

●—● AGROCHEMICAL

↑ Agrochemical application

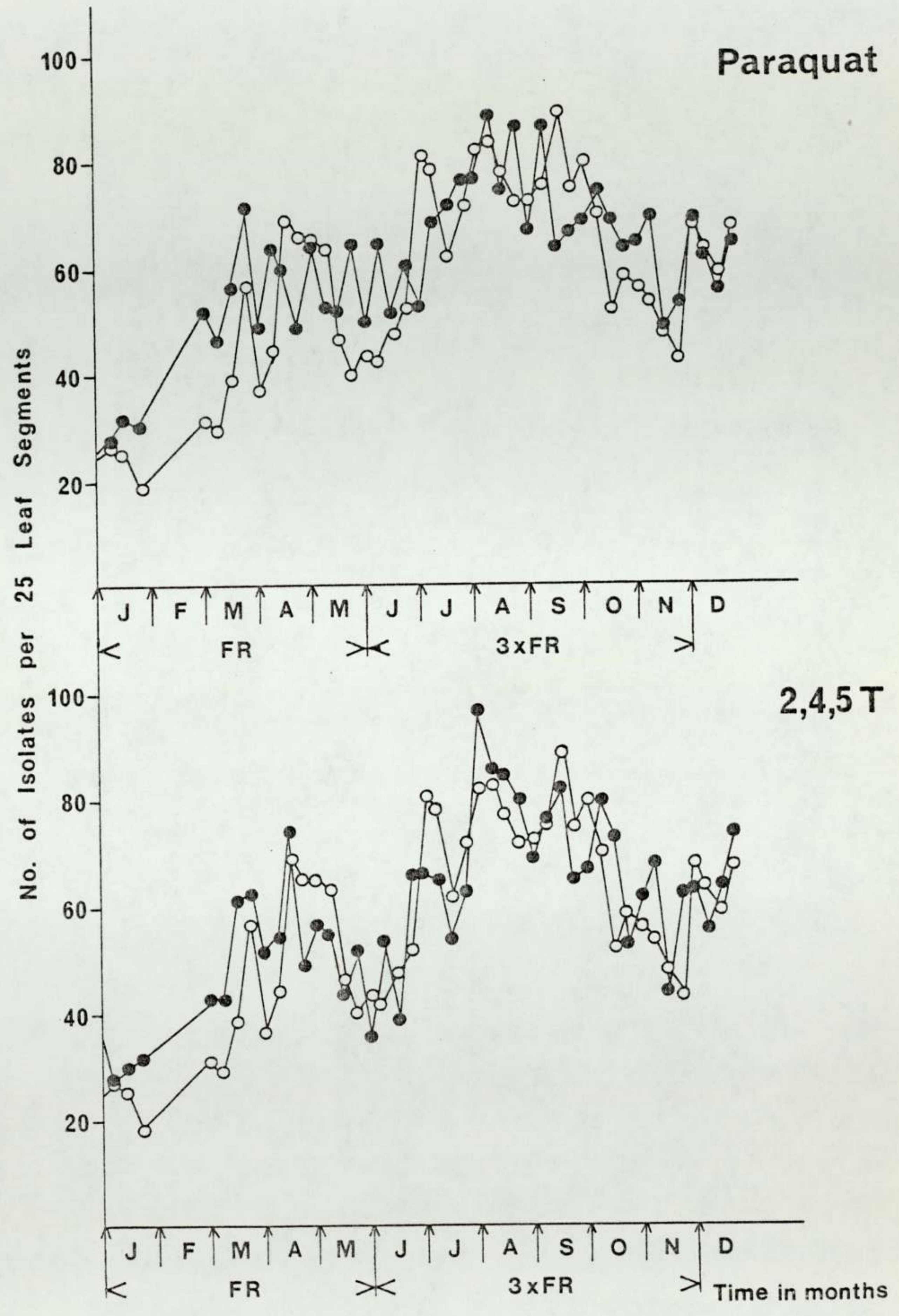
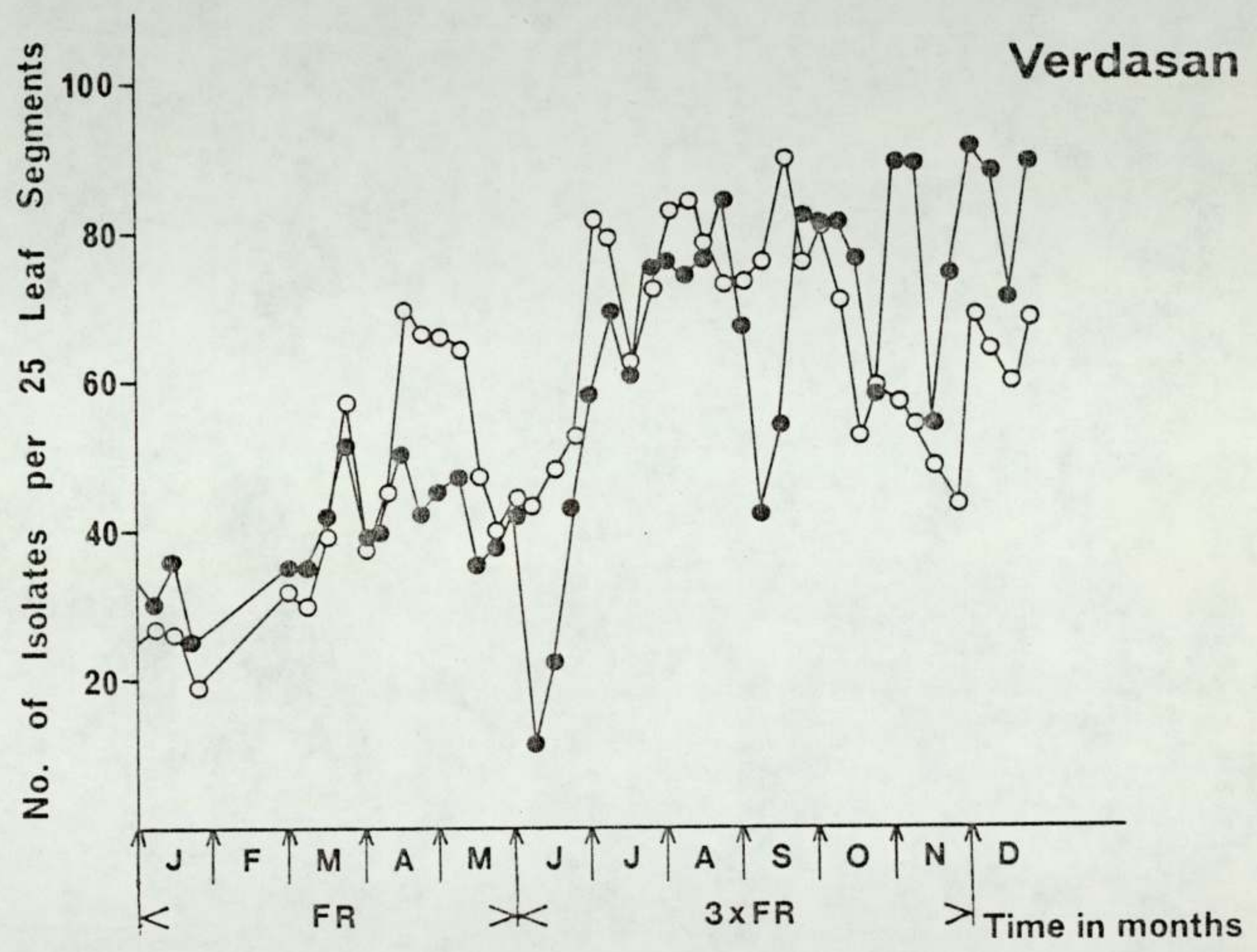

Paraquat
2,4,5 T
No. of Isolates per 25 Leaf Segments
100
80
60
40
20
J F M A M J J A S O N D
FR
3xFR
Time in months

Fig.5. The effect of VERDASAN on leaf litter fungal numbers.

O—O CONTROL

●—● AGROCHEMICAL

↑ Agrochemical application



Verdasan
No. of Isolates per 25 Leaf Segments
100
80
60
40
20
J
F
M
A
M
J
J
A
S
O
N
D
FR
3xFR
Time in months

Table 2 . Number of Fungal Isolations of Leaf Litter Fungi on CONTROL and Treated Leaves

The % reduction or increase from CONTROL numbers is given in parentheses.

		CONTROL	CAPTAN	VERDASAN	PARAQUAT	MAZIDE	2,4,5T
Overall number of isolates FR		774	659 (-14.9)	679 (-12.3)	864 (+11.6)	754 (-2.6)	825 (+6.6)
Number of isolates FR	Day 0	194	184 (-5.2)	174 (-10.3)	211 (+8.8)	187 (-3.6)	205 (+5.7)
	Day 7	185	130 (-29.7)	163 (-11.9)	201 (+8.6)	178 (-3.8)	210 (+13.5)
	Day 14	186	160 (-14.0)	156 (-16.1)	217 (+16.7)	180 (-3.2)	196 (+5.4)
	Day 28	209	185 (-11.5)	186 (-11.0)	234 (+12.0)	209 (0.0)	214 (+2.4)
Overall number of isolates 3 x FR *		1888	*1382 (-26.8)	1926 (+2.0)	1908 (+1.1)	1808 (-4.3)	1894 (+0.3)
Number of isolates 3 x FR	* Day 0	477	* 304 (-36.3)	454 (-4.8)	517 (+8.4)	476 (-0.2)	486 (+1.9)
	Day 7	443	* 361 (-18.5)	413 (-6.8)	444 (+0.2)	416 (-6.1)	441 (-0.5)
	* Day 14	449	* 347 (-22.7)	505 (+12.5)	478 (+6.5)	452 (+0.7)	464 (+3.3)
	* Day 28	519	* 370 (-28.7)	554 (+6.7)	469 (-9.6)	464 (-10.6)	503 (-3.1)

- decrease + increase

* significant at 5% level from CONTROL

Table 3. Mean Number of Species of Leaf Litter Fungi on CONTROL and Treated Leaves

The % reduction or increase from CONTROL numbers is given in parentheses.

		CONTROL	CAPTAN	VERDASAN	PARAQUAT	MAZIDE	2,4,5T
Overall mean number of species FR		13.1	13.1 (-0.4)	12.7 (-3.4)	12.9 (-1.3)	13.4(+2.1)	14.1 (+7.2)
Mean number of species FR	Day 0	12.2	12.8 (+4.9)	11.6 (-4.9)	11.8 (-3.3)	13.0 (+6.6)	13.2 (+8.2)
	Day 7	13.0	12.3 (-5.8)	14.0 (+7.7)	14.5 (+11.5)	15.5 (+19.2)	15.5 (+19.2)
	Day 14	14.5	15.0 (-3.4)	13.0 (-10.3)	13.8 (-5.2)	13.0 (-10.3)	15.0 (+3.4)
	Day 28	13.0	12.4 (-5.2)	12.4 (-5.2)	12.2 (-6.9)	12.4 (-5.2)	13.0 (0.0)
Overall mean number of species 3 x FR		15.8	* 12.1 (-23.0)	* 10.0 (-36.3)	15.1 (-4.1)	14.9 (-5.2)	14.5 (-7.9)
Mean number of species 3 x FR	Day 0	16.3	11.1 (-31.6)	10.3 (-36.8)	16.6 (+1.8)	15.9 (-2.6)	15.6 (-4.4)
	Day 7	16.0	11.3 (-29.5)	* 9.6 (-40.2)	15.3 (-4.5)	* 14.0 (-12.5)	* 13.3 (-17.0)
	Day 14	13.9	13.1 (-5.2)	10.3 (-25.8)	14.7 (+6.2)	15.4 (+11.3)	14.9 (+7.2)
	Day 28	16.9	* 12.9 (-23.7)	* 10.0 (-40.7)	* 13.9 (-17.8)	14.4 (-14.4)	14.3 (-15.3)

\- decrease + increase

* significant at 5% level from CONTROL

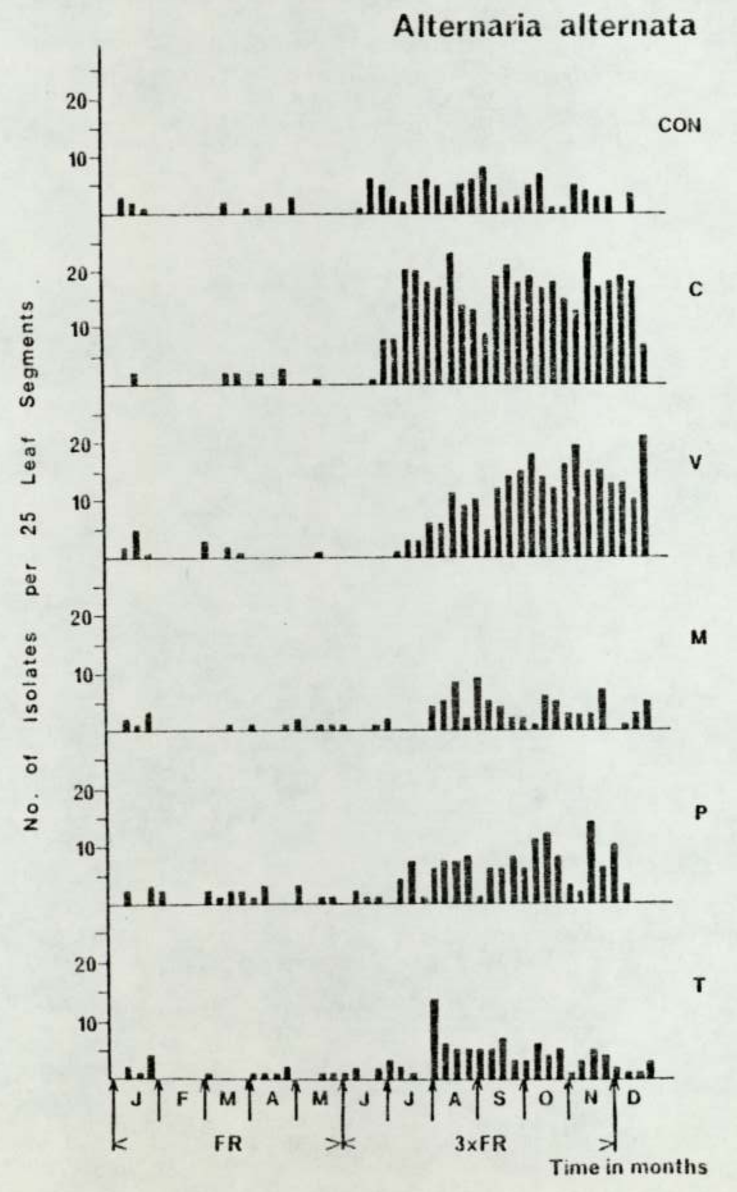
isolated from leaf litter are given in Table 3 . At normal field rate they did not significantly alter the number of species overall, and neither was the variation in numbers significantly different on any of the individual sampling days. At 3 x FR the number of species between the treatments and CONTROL differed significantly over the whole period ($F = 27.6$; $P < 0.01$). Numbers on CAPTAN and VERDASAN-treated leaves were significantly lower ($P = 0.001$) than on CONTROL and herbicide-treated leaves. In addition, numbers of species on VERDASAN-treated leaves were significantly lower ($P < 0.01$) than on those treated with CAPTAN. The variation in number of species during this period was significant on all sampling days ($P < 0.01$). Numbers on VERDASAN-treated leaves were significantly lower than CONTROL (on Days 0, 7, 14 and 28) and CAPTAN-treated leaves (on Day 7). Also, numbers on CAPTAN-treated leaves were significantly lower than CONTROL on Days 7 and 28, and than with the treatments of MAZIDE, PARAQUAT and 2,4,5T on Day 7.

The species isolated from the washed leaf litter segments are shown in Appendix 4 . The occurrence and relative abundance of the principal species are shown in Figures 6 to 8 , and Appendix 5 . Alternaria alternata, Cladosporium cladosporioides, Epicoccum purpurascens, Fusarium spp., Gliocladium roseum, Mucor hiemalis and Trichoderma spp. were most commonly isolated and in considerable numbers.

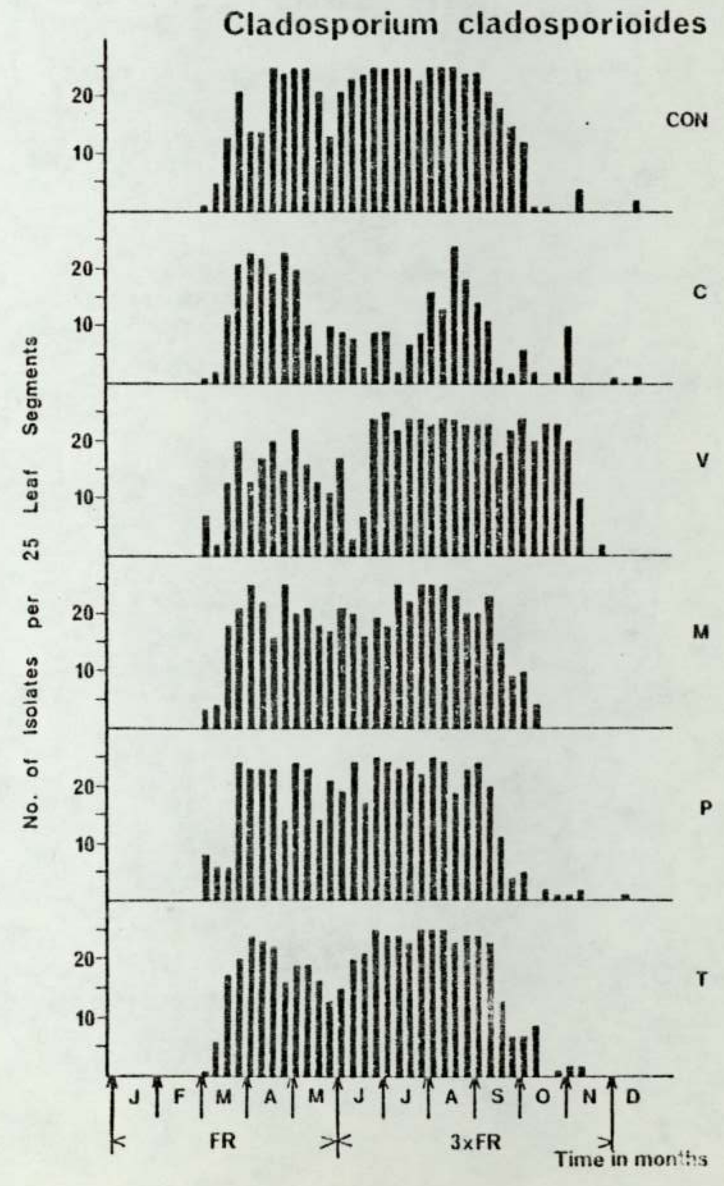
Alternaria alternata (Fig. 6) was most often isolated from the CONTROL and treated plots in the summer and autumn months. Both fungicide treatments at 3 x FR significantly ($P < 0.01$)

Fig.6. The effect of agrochemicals on the incidence of *Alternaria alternata* and *Cladosporium cladosporioides* on leaf litter.

CON	CONTROL
C	CAPTAN
V	VERDASAN
M	MAZIDE
P	PARAQUAT
T	2,4,5T



Alternaria alternata
CON
C
V
M
P
T
No. of Isolates per 25 Leaf Segments
20
10
J F M A M J J A S O N D
FR
3xFR
Time in months



Cladosporium cladosporioides
CON
C
V
M
P
T
No. of Isolates per 25 Leaf Segments
20
10
J F M A M J J A S O N D
FR
3xFR
Time in months

Fig.7. The effect of agrochemicals on the incidence of *Epicoccum purpurascens* and *Fusarium* spp. on leaf litter.

CON CONTROL

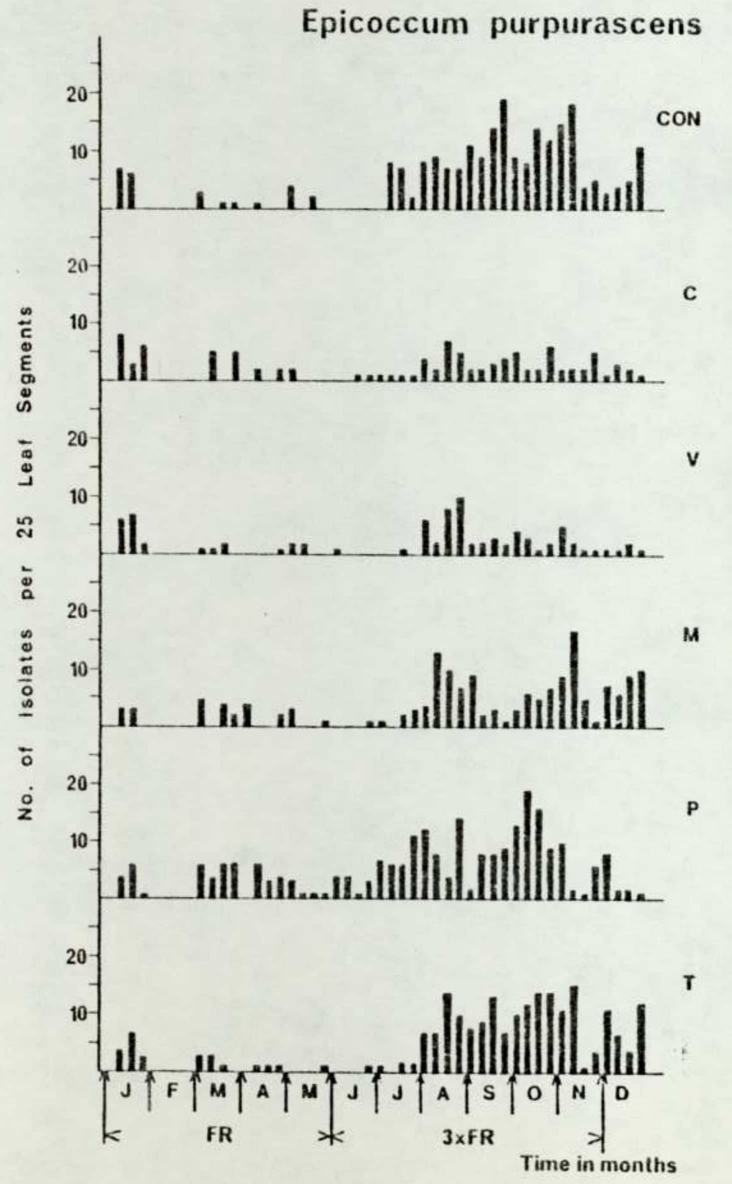
C CAPTAN

V VERDASAN

M MAZIDE

P PARAQUAT

T 2,4,5T


Epicoccum purpurascens
CON
C
V
M
P
T
No. of Isolates per 25 Leaf Segments
20
10
J F M A M J J A S O N D
FR
3xFR
Time in months

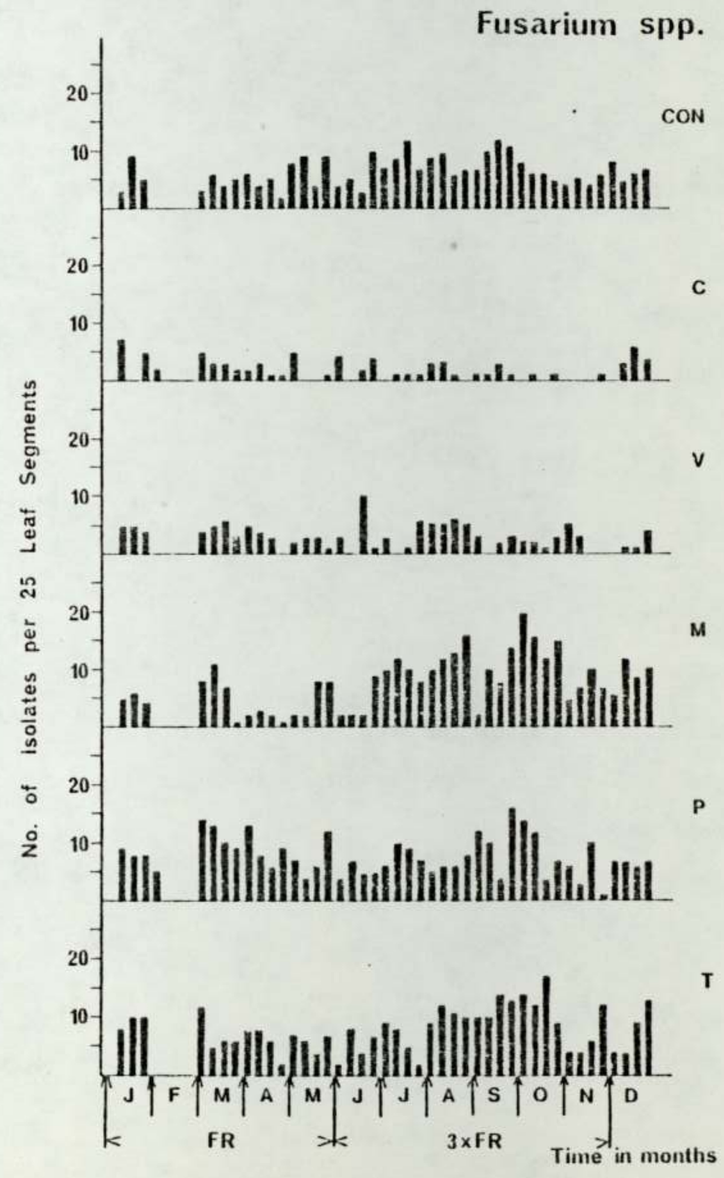

Fusarium spp.
CON
C
V
M
P
T
No. of Isolates per 25 Leaf Segments
20
10
J F M A M J J A S O N D
FR
3xFR
Time in months

Fig.8. The effect of agrochemicals on the incidence of *Mucor hiemalis* and *Ulocladium botrytis* on leaf litter.

CON	CONTROL
C	CAPTAN
V	VERDASAN
M	MAZIDE
P	PARAQUAT
T	2,4,5T

Ulocladium botrytis

CON C V M P T

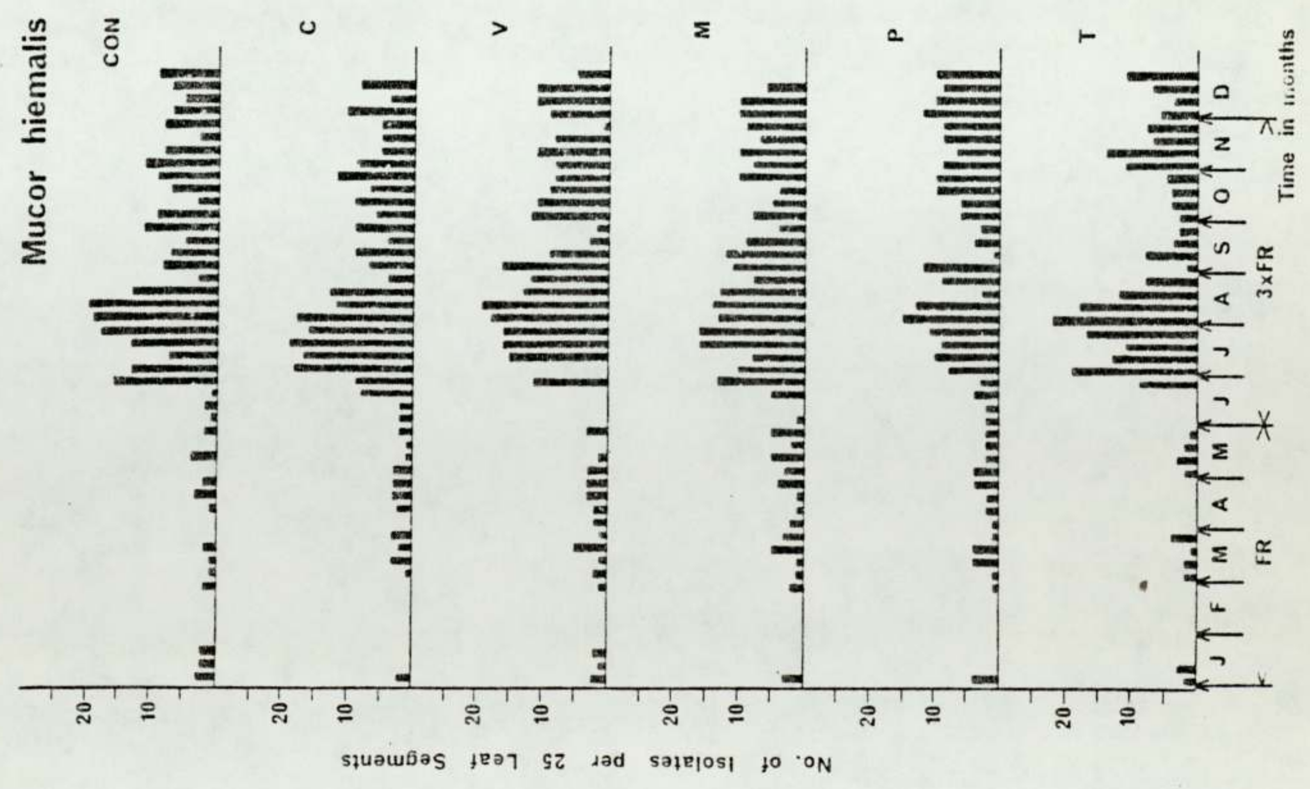
No. of Isolates per 25 Leaf Segments

20 10

J F M A M J J A S O N D

FR 3xFR

Time in months



increased the occurrence of this species. However, none of the herbicides significantly changed the rate of isolation compared with the CONTROL.

Cladosporium cladosporioides (Fig. 6) was the most frequently isolated species throughout the year in CONTROL and in treated plots. In the CAPTAN-treated plots there was a slight reduction in its occurrence in April, May and June, and this could also be seen in the VERDASAN-treated plots in May.

Epicoccum purpurascens (Fig. 7) occurred mainly in the autumn. The fungicides both caused a significant reduction ($P < 0.01$), while the herbicides did not significantly affect the rates of isolation.

Fusarium spp. (Fig. 7) were commonly isolated throughout the year. Both fungicides reduced the rate of isolation, and all three herbicides increased the rate of isolation, although not significantly.

Mucor hiemalis (Fig. 8) was most commonly isolated from the CONTROL and treated plots in the summer and autumn months. None of the agrochemicals significantly altered the occurrence of this species compared with CONTROL.

Ulocladium botrytis (Fig. 8) was infrequently isolated throughout the year from CONTROL plots and from those treated with herbicides. However, while it was similar to CONTROL levels at FR, at 3 x FR it was more frequently isolated after treatment with CAPTAN, and was abundant in the VERDASAN-treated plots.

The changes in occurrence of these species (expressed as a percentage of the CONTROL plots) after treatment at FR and 3 x FR

are summarised in Table 4. These data represent the number of times that each species was isolated over each separate sampling period and treatment, expressed as a percentage of CONTROL. There is less emphasis in the figures for Ulocladium botrytis than appears in Appendix 5 because this fungus was isolated in greater abundance on any one sampling occasion than in CONTROL plots, rather than on more occasions.

Table 4. Frequencies of Occurrence of Leaf Litter Fungi expressed as a Percentage of the CONTROL

	NORMAL FIELD RATE					3 x NORMAL FIELD RATE				
	C	V	M	P	T	C	V	M	P	T
Alternaria alternata	87	114	142	157	157	104	95	87	108	104
Cladosporium cladosporioides	93	93	93	93	93	78	81	63	70	74
Epicoccum purpurascens	100	125	112	187	125	108	87	104	112	100
Fusarium spp.	87	94	100	100	100	67	78	100	100	100
Ulocladium botrytis	110	100	89	56	89	183	217	158	150	75

C CAPTAN

V VERDASAN

M MAZIDE

P PARAQUAT

T 2,4,5T

Discussion

Maximum numbers of fungi were isolated from leaf litter during the summer and autumn months from CONTROL leaves. The peak occurrences of the leaf litter fungi coincided with those of the phylloplane reported by other workers (di Menna, 1971; Kuthubutheen, 1977), and also with those of air-borne spora shown by Gregory and Hirst (1957) and Harvey (1967).

The most important actively growing fungal components of the leaf litter of *Agrostis tenuis* were found to be *Alternaria alternata*, *Cladosporium cladosporioides*, *Epicoccum purpurascens*, *Fusarium* spp., and *Ulocladium botrytis*. In addition, *Gliocladium roseum*, *Mucor hiemalis*, *Penicillium* spp., *Phoma* and *Trichoderma* spp. were also isolated frequently. These represent the forms which were isolated from serially washed and plated leaf segments. Generally, the isolation of these species is in agreement with earlier studies on green, senescing and decaying plant material (Webster, 1956, 1957; Hudson and Webster, 1958; Pugh, 1958; Hudson, 1962; Hering, 1965; Yadav, 1966; Preece and Dickinson, 1971; Pugh and Mulder, 1971; Dickinson and Preece, 1976).

Alternaria alternata has been found to be a constant component of the phylloplane mycoflora (Kerling, 1958; Webster, 1956, 1957; Friend, 1965; Hogg and Hudson, 1966; Bainbridge and Dickinson, 1972; Kuthubutheen, 1977). In addition, it has been commonly isolated from yellow and senescing leaves (Hudson, 1962; Kerling, 1965; Pugh and Mulder, 1971) and leaf litter (Hering, 1965). Kuthubutheen (1977) recorded a somewhat higher incidence of *A. alternata* on *Agrostis tenuis* in Nottingham than was found in the

present study, but in both studies it was commonly isolated with maximum occurrence between June and December. It may, therefore, be regarded as predominantly a leaf surface coloniser which is able to persist in the senescing leaves and fresh litter.

The absence of Aureobasidium pullulans in this study is surprising, since in a similar study of Agrostis tenuis, Kuthubutheen (1977) isolated it from green leaves throughout the year with a maximum frequency of 75% from August to October. However, Dickinson (1965) did not record it on green or yellow leaves of Halimione. The role of Aureobasidium pullulans in the decomposition of leaf litter has been the matter of some speculation and it is not completely understood if it is a true litter inhabiting species or not. Smit and Weiringa (1953) regarded it as a major component and chief pectinolytic organism of freshly fallen leaf litter although it tended to disappear in older litter. However, Hering (1967) showed that the rate at which A. pullulans decomposed oak leaf litter, in vitro, was very low and he could find no evidence of its high pectinolytic activity as claimed by Smit and Weiringa (1953). A. pullulans, therefore, may be primarily a very early leaf coloniser having the ability to persist as microsclerotia or chlamydospores for a short while in the litter layer (Pugh and Buckley, 1971).

Cladosporium cladosporioides and Cladosporium herbarum are known to be consistent components of the air spora, phylloplane and leaf litter. In the present study, C. cladosporioides was isolated at a frequency of 76% from CONTROL leaves, and the increase in C. herbarum during the summer and autumn months coincided with its

seasonal maximum in the air spora (Hyde and Williams, 1953; Harvey, 1967) which Gregory and Hirst (1957) attributed to the accumulation of dead plant material. The association of C. herbarum as a primary coloniser of decaying plant tissue has been noted previously by White (1945), Westerdijk (1949), Webster (1956, 1957), Hudson and Webster (1958), Hudson (1962) and Pugh and Mulder (1971).

Hogg and Hudson (1966) found C. herbarum associated with necrotic areas of living beech leaves, and Hudson (1962) described it as having "at least a limited parasitic phase with a more marked phase of saprophytic spread and survival". However, in vitro studies have shown that C. herbarum does not markedly affect senescence of barley leaves (Skidmore and Dickinson, 1973) and carries out little decomposition of fresh litter (Hering, 1967). Kuthubutheen (1977) found that numbers of C. cladosporioides on green leaves were reduced by VERDASAN and that this fungus showed some degree of growth in media containing CAPTAN.

Epicoccum purpurascens is generally regarded as primarily a litter inhabiting fungus which is able to initiate colonisation of green leaves (e.g. Friend, 1965 on lime; Bainbridge and Dickinson, 1972 on potatoes; Kuthubutheen, 1977 on Agrostis tenuis). In the present study, E. purpurascens was commonly isolated from washed leaf litter with maximum occurrence between July and December. It was regarded as one of the first dominant members of decaying material by Webster (1956) on cocksfoot, and Hudson and Webster (1958) on Agropyron. Hering (1956) recorded it from leaf litter of oak, birch and hazel.

In the present study Mucor hiemalis was isolated throughout

the year from CONTROL leaves with maximum occurrence between June and September. Treatment with the agrochemicals did not markedly affect its occurrence. Members of the Mucorales were very infrequently found by Hudson and Webster (1958) on decaying stems of *Agropyron repens*, and Webster (1957) concluded that they play little part in the above ground phase of decomposition. However, *Mucor hiemalis* was constantly isolated from decaying stems of *Heracleum sphondylium* by Yadav (1966), and Pugh (1958) recorded it from leaf litter of *Carex paniculata*. These observations, and those from the present study suggest that *M. hiemalis* is an important member of the mycoflora of decaying plant material.

Ulocladium botrytis occurs saprophytically on a variety of plant substrates including litter and has been shown to inhabit wood, paper, textiles and excrement (Joly, 1964). Its role as a cellulose decomposer has also been shown (White *et al.*, 1948). In the present study, *U. botrytis* was isolated throughout the year in low frequencies on CONTROL leaves and leaves treated with MAZIDE, PARAQUAT and 2,4,5T. However, it was isolated in high frequencies on VERDASAN-treated leaves at 3 x FR. The high frequency of occurrence of *U. botrytis* may be attributed to a suppression or elimination of its competitors due to VERDASAN treatment allowing it to proliferate. This may imply that the fungus has some tolerance as a consequence of repeated exposures to the fungicide, or a natural tolerance. It is possible that it had adapted to VERDASAN during the two years that the plots had been sprayed as Parry and Wood (1958) and Partridge and Rich (1962) demonstrated the ability of various fungi to adapt to high concentrations of copper and mercury

as a result of repeated treatment with these elements.

Kuthubutheen (1977) noted the high incidence of *Chrysosporium pannorum* on green leaves treated with Verdasan following a report by Williams and Pugh (1975) that this fungus was found to be more resistant to the organo-mercurial fungicide *in vitro* than a number of other saprophytic fungi. The same workers also showed that agar medium permitted growth of *C. pannorum* at higher concentrations of Verdasan than did liquid medium. They ascribed this to the presence, in the agar medium, of mercury chelating radicals such as thiol groups noted by Greenaway (1973).

When applied at their normal field rates, the two fungicides CAPTAN and VERDASAN, and the herbicide MAZIDE, reduced the total number of fungal isolations from leaf litter, although the differences were not significant from CONTROL numbers. At three times the normal field rates, numbers of isolations were reduced significantly by CAPTAN although the other chemicals had negligible effects. The reductions caused by CAPTAN were significant on all sampling days following its application, and numbers of isolations were lowest immediately following application. Fungal numbers were initially drastically reduced by VERDASAN applied at 3 x FR, although towards the end of the year numbers increased to more than that of CONTROL. These results at first seem surprising since previous studies have shown that VERDASAN is more toxic in its effects on fungal populations than is CAPTAN (Wainwright and Pugh, 1973; Kuthubutheen and Pugh, 1979).

Wainwright and Pugh (1973) showed that VERDASAN was more effective than CAPTAN, at lower concentrations, at increasing

levels of ammonification and decreasing levels of nitrification in the soil, CAPTAN has also been shown to be less inhibitory than VERDASAN to the growth, germination, cellulolytic and amylolytic capabilities of mesophylic fungi. However, Kuthubutheen and Pugh (1977) showed that although numbers of mesophiles on VERDASAN-treated leaves were nearly half those on CAPTAN-treated leaves, numbers of thermophiles on leaves treated with these two fungicides differed only slightly.

The effects of herbicides on the mycoflora of plant remains have been little studied. Wilkinson and Lucas (1969a) showed that fungi differ in their ability to tolerate PARAQUAT and showed that it can alter, and sometimes reverse, the outcome between fungi competing for plant material pre-treated with the herbicide. It was subsequently shown (Wilkinson and Lucas, 1969b) that a qualitative change in such fungi might affect the persistence of pathogenic species capable of saprophytic survival in these tissues, either through direct antagonism or through changes in the rate of decomposition of the infected material. Mitzkouski (1959) showed that 2,4-dichlorophenoxyacetic acid (2,4 D) and 4-chloro-2-methyl-phenoxyacetic acid (MCPA) have no harmful effects on the soil microflora but do adversely affect the epiphytic microflora of sprayed plants. Indeed, most studies on herbicide/microflora interaction have been concentrated on the soil flora. It is generally understood that most herbicides, when used at normal field rates, do not significantly alter soil microbial populations (Fletcher, 1960; Bollen, 1961). However, several reports (Klyuchnikov and Petrova, 1951; Mashtakov _et al._, 1962) have shown that the quality of the

microflora may change following herbicide application. Mashtakov *et al*.(1962) showed in field tests that although total fungal numbers remained unchanged on the whole, members of *Aspergillus*, *Fusarium*, *Mucor* and *Phycomyces* increased, while *Alternaria*, *Cladosporium* and *Rhizopus* were present in treated samples but not in the control. The nature and extent of the herbicidal effect has also been shown to vary with soil type, some genera being affected in one soil type but not in another (Mashtakov *et al*., 1962; Tu and Bollen, 1968). Houseworth and Tweedy (1973) attributed the increase in fungal numbers in crop soils treated with atrazine to the decomposition of bean and wheat plant material killed by this herbicide.

In this present study the herbicides MAZIDE, PARAQUAT and 2,4,5T did not significantly alter the species composition or total fungal numbers over the whole sampling period on leaf litter even when applied at abnormally high concentrations.

However, when numbers of isolations from treated leaves were compared with CONTROL on each sampling day, then numbers were reduced significantly on Day 7 with MAZIDE and 2,4,5T-treated leaves, and on Day 28 with PARAQUAT-treated leaves. Frequencies of individual species were also affected: occurrence of *Fusarium* spp. was 144, 110, and 133 % higher than CONTROL on leaves treated with 3 x FR of MAZIDE, PARAQUAT and 2,4,5T respectively. *Epicoccum purpurascens* was reduced most by MAZIDE.

The results presented in this study, showing little alteration of the fungal flora in herbicide-treated plots and little change over each sampling month, suggest that the short and long term effects of herbicides are minimal.

Further work is needed, particularly on combinations of treatments of herbicides and fungicides, as little work has been done in this area. Nash and Harris (1969) have worked on the effects of combinations of s-triazine and the fungicide Dexon on soil micro-organisms. Also, Atrazine is often applied to the same crop with either Captan or Thiram (Houseworth and Tweedy, 1973). The microbial breakdown of one class of compounds may be affected by the other, leading to elevated levels in the soil, which may have a deleterious effect on those organisms responsible for organic matter decomposition.

CHAPTER II

GROWTH OF BASIDIOMYCETES

GROWTH OF BASIDIOMYCETES

Introduction

During the course of studies on soil fungi, members of the Basidiomycetes have often been overlooked because of difficulties in isolation and identification. Warcup (1957), with his hyphal isolation technique, showed that Basidiomycetes can be recovered from the soil, and Warcup and Talbot (1962) were able to identify several species.

However, the ability of soil inhabiting Basidiomycetes to decompose litter and its main constituents, cellulose and lignin, has been shown (Lindeberg, 1944, 1948). Fries (1955) and Hering (1967) indicated that the ability to decompose these constituents is very common amongst the Basidiomycetes. More recently, Frankland (1975) showed that *Mycena galopus* can decompose about 20% of freshly fallen litter within 6 months. Other active litter decomposers have been found amongst the genera of *Clavaria*, *Clitocybe*, *Marasmius*, and *Stropharia*, and many wood destroying fungi found on decaying trees have decomposed litter effectively *in vitro*: *Armillaria mellea*, and species of *Flammulina*, *Hypholoma* and *Pholiota* (Lindeberg, 1946; Mikola, 1954).

Basidiomycetes also form an important group of fungi forming mycorrhizal associations with plants. The benefits of introducing mycorrhizal infection at the time of transplanting plant stock, in order to encourage the development of a sturdy root system and healthy plant, are well known in agricultural (Gerdemann, 1968; Hayman and Mosse, 1971; Mosse and Hayman, 1971) and forestry

practices (Bjorkman, 1961; Marx, 1970, 1973; Iyer *et al*., 1971).

It was decided, therefore, to investigate the presence of Basidiomycetes in litter and other substrates, and to work on the aspects of the physiology of the species isolated, along with species obtained from other sources. The need for such studies was emphasised by Pugh (1980) in his examination of the relationships between life strategies and ecology of fungi.

Materials and Methods

Collection, Isolation, Maintenance and Inoculation of Basidiomycetes

i. Collection

The sources of the Basidiomycetes used in this study are shown in Table 5 .

ii. Isolation

Various methods were employed to isolate Basidiomycete colonies from sporophore tissue, leaf litter, and soil particles:

a. Sporophore tissue - Palmer (1969) described a method whereby Basidiomycete colonies may be isolated from sporophore tissue. In this study, young sporophores of *Coprinus comatus* were collected and transported immediately to the laboratory. 1 mm^3 blocks of stipe tissue were aseptically cut and transferred onto Oxoid malt extract agar (MA) incorporating 6 mg/1 of aureomycin to inhibit bacterial growth. Within 48 hours, tufts of hyphae could be seen growing from the blocks of sporophore tissue and subsequently across the agar, This process was repeated with *Phallus impudicus*, this time the tissue from the 'egg' stage being used.

b. Leaf litter - Hunt and Cobb (1971) described a selective medium (HC) for the isolation of Basidiomycetes. Its composition is given in Appendix 3 . Two Basidiomycetes, designated 109B and 235M, both bearing clamp connections, were isolated from leaf litter of *Agrostis tenuis*. Initially many sterile forms appeared growing from the litter.

Table 5. Sources of the Basidiomycetes used in this study

Species	Origin	Ecological type / natural substrate
Agaricus arvensis Schaeff. ex Secr.	University of Aston culture collection	
Agaricus bisporus (Lange) Pilat	University of Aston culture collection	Coprophilic
Amanita muscaria (Link ex Fr.)Hooker	U.S.D.A. Beltsville U.S.A.	Mycorrhizal (Trappe, 1962)
Basidiomycete 109B	Leaf litter of Agrostis tenuis	Litter inhabiting
Basidiomycete 235M	Leaf litter of Agrostis tenuis	Litter inhabiting
Boletus variegatus Sow. ex Fr.	Institute of Terrestrial Ecology, Midlothian	Mycorrhizal (Trappe, 1962)
Coprinus comatus (Mull. ex Fr.)Gray	From sporophore tissue on grassland,Birmingham	Wood-destroying (Webster, 1970)
Cyathus stercoreus Haller ex Pers.	University of Aberdeen	Wheat straw and soil (Warcup and Talbot,1962)
Lepista nuda (Bull. ex Fr.)Cooke	University of Aston culture collection	Mycorrhizal (Trappe, 1962)
Lepista saeva (Fr.) Orton	University of Aston culture collection	
Paxillus involutus (Batsch ex Fr.)Fr.	Institute of Terrestrial Ecology, Midlothian	Mycorrhizal (Trappe, 1962)
Phallus impudicus (Linn.) Pers.	From sporophore tissue among beech litter in deciduous woodland	Decayed wood (Grainger, 1962) and possibly mycorrhizal (Trappe, 1962)
Pleurotus sajor-caju (Fr.) Singer	University of Aston culture collection	
Russula emetica (Schaeff. ex Fr)Gray	U.S.D.A. Beltsville U.S.A.	Mycorrhizal (Trappe, 1962)

Subsequent transfer of the hyphae onto MA revealed <u>Alternaria alternata</u>, <u>Mucor</u> spp., <u>Trichoderma</u> spp., and <u>Ulocladium botrytis</u> to be present. However, some forms remained sterile on HC and MA, and may possibly have been Basidiomycetes lacking clamp connections and which were therefore not used for further study.

c. Soil - Warcup's (1955) hyphal isolation technique was employed to see if Basidiomycetes could be isolated from soil immediately below the litter region of <u>Agrostis tenuis</u>. On the assumption that Basidiomycetes may sink enmeshed with the heavier soil particles, 5 g of the soil were repeatedly washed with tap water in a 250 ml beaker, the suspension allowed to settle, and the supernatant decanted off. This procedure was repeated five times and finally the sediment was examined microscopically for the presence of fragments of fungal hyphae. These were picked out and plated onto HC medium. No Basidiomycetes were successfully isolated by this method, although many colonies of <u>Mucor</u> spp. and <u>Mortierella</u> spp. were obtained.

iii. <u>Maintenance of Cultures</u>

The mycorrhizal species were maintained on slopes of a modified Hagem's medium (HMA) described by Modess (1941) and listed in Appendix 3 ; all other Basidiomycetes were kept on MA. All stock cultures were kept at $5^{\circ}C$ and were subcultured every three months.

iv. <u>Inoculation</u>

Inocula for Petri plate and flask cultures were cut

from the growing margin of colonies growing on either HMA (for mycorrhizal species) or MA (for non-mycorrhizal species) with a sterile 5 mm cork borer. These inocula were transferred to Petri plates containing 20 ml of appropriate agar medium, where they were placed centrally with the surface of the mycelial mat downwards, or into flasks and floated on the surface of the culture medium. Flotation of the mycelial discs is the usual inoculation technique employed in investigations of Basidiomycetes, having first been used by Melin and Lindeberg (1939). In this way, it is possible to obtain good aerobic conditions which greatly promote the growth rate of higher fungi as shown by Modess (1941) in his growth rate comparisons between submerged and floating cultures. The weight of the mycelial plug, calculated as the mean weight of 25 such plugs taken from the inoculum plate and dried at 90°C to constant weight, was subtracted from the final dry weight of the mycelium from flask cultures.

Effect of Temperature on Growth Rate

9 cm Petri plates containing approximately 20 ml of cool HMA or MA, which had been autoclaved for 15 minutes at 120°C and 15 psi were inoculated with mycelial discs of the test fungus growing on either HMA or MA. The plates were incubated at 5°, 10°, 20°, 25°, 30° and 35°C. Five replicate plates for each temperature for every fungus were used. The colony diameters were taken as the mean of two diameters at right angles to each other. Plates were measured at intervals depending on their growth rate, and linear extension rates were calculated during the log phase of growth.

Dry Weight Increase

100 ml Erlenmeyer flasks, each containing 20 ml of the basic liquid medium (BLM) shown in Appendix 3 , were autoclaved for 15 minutes at 120°C and 15 psi. Each flask was inoculated with a disc of the test fungus growing on either HMA or MA, and incubated at their optimum temperature for growth as standing cultures. Three replicate flasks for each consecutive sample for each fungus were used. For dry weight analysis, flasks were removed and the mycelial mats harvested by suction filtration onto pre-weighed, dried, and cooled Whatman No. 1 filter papers. After drying to constant weight at 90°C, the mycelial mats were allowed to cool in a dessicator and were reweighed. Growth rates were calculated during the log phase of growth.

Results

Effect of Temperature on Growth Rate

The results are summarised in Figure 9 and are tabulated in Appendix 6, and indicate the optimum growth temperatures of the six species of fungi under investigation. All six species grew at 5°C, the lowest temperature used. Four of the species investigated grew optimally at between 20°C and 25°C (Basidiomycete 235M, Coprinus comatus, Boletus variegatus and Phallus impudicus). Paxillus involutus showed a lower optimum temperature, around 20°C, while Cyathus stercoreus had its optimum near 30°C.

In general, the two mycorrhizal species (P. involutus and B. variegatus) and Phallus impudicus did not grow above 30°C, whereas the other three non-mycorrhizal species grew above this temperature.

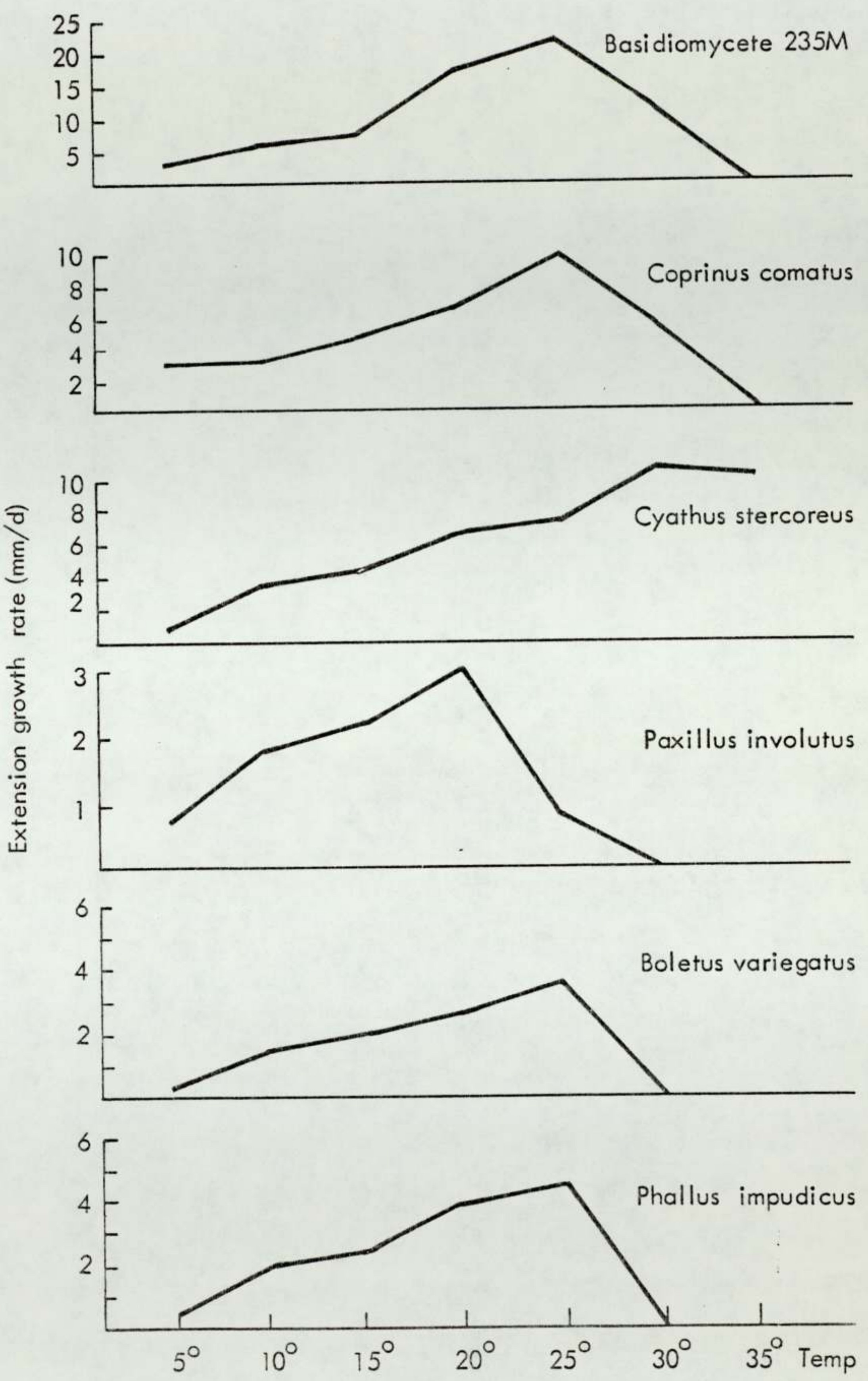
Dry Weight Increase

The three non-mycorrhizal species, 235M, Coprinus comatus and Cyathus stercoreus, showed relatively higher growth rates than did Paxillus involutus, Boletus variegatus and Phallus impudicus (Table 6). The growth data are shown in Appendix 6.

Table 6. Growth rate (mg dry weight/day) of Basidiomycetes at their optimum temperature for growth.

Basidiomycete 235M	33.5	Boletus variegatus	6.3
Cyathus stercoreus	9.4	Paxillus involutus	4.5
Coprinus comatus	7.7	Phallus impudicus	3.3

Fig.9. Effect of temperature on linear extension rate of six Basidiomycetes on agar medium.



Basidiomycete 235M
Coprinus comatus
Cyathus stercoreus
Paxillus involutus
Boletus variegatus
Phallus impudicus
Extension growth rate (mm/d)
25
20
15
10
5
10
8
6
4
2
10
8
6
4
2
3
2
1
6
4
2
6
4
2
5°
10°
15°
20°
25°
30°
35°
Temp

Fig.10. Growth of six species of Basidiomycetes in liquid medium at their optimum temperature for growth.

● Basidiomycete 235M

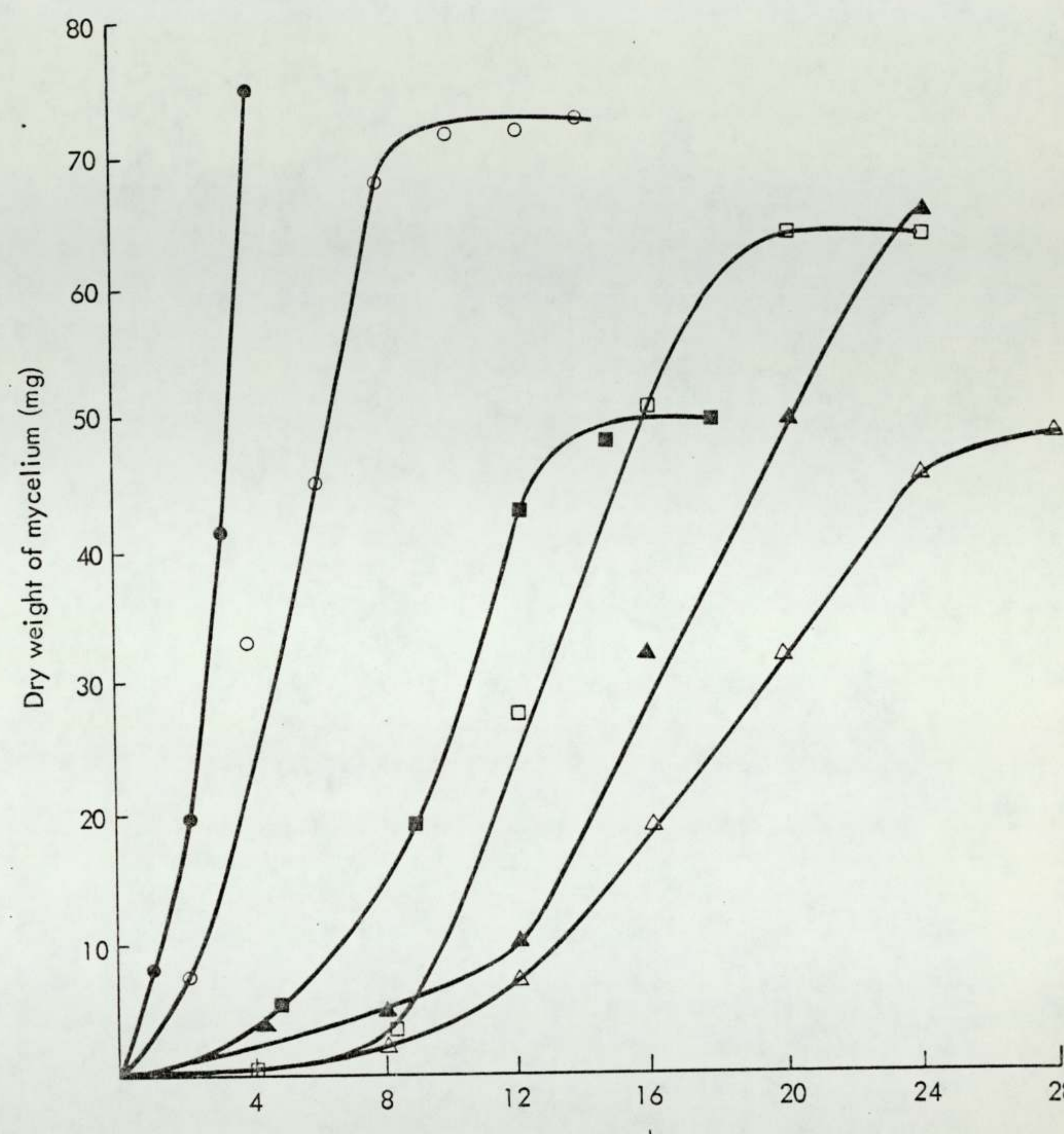
□ *Boletus variegatus*

■ *Coprinus comatus*

○ *Cyathus stercoreus*

▲ *Paxillus involutus*

△ *Phallus impudicus*


Dry weight of mycelium (mg)
80
70
60
50
40
30
20
10
4
8
12
16
20
24
days

In addition, it is evident from Figure 10 that not only were growth rates slower in the mycorrhizal species and <u>Phallus impudicus</u>, but the lag phase was, in general, considerably longer. Conversely, those species with a shorter lag phase also showed a faster growth rate.

Discussion

In earlier studies, Basidiomycetes have mainly been found to be mesophilic in their temperature requirements. Three mycorrhizal species of Boletus studied by Melin (1925) showed optimum growth at 25°C, while Mikola (1948) found a slightly lower optimum for species of Amanita and Lactarius. Norkrans (1950) found that mycorrhizal Tricholoma species had optima within the range 18°to 30°C, while Marx (1969) showed that Pisolithus tinctorius grew best at 30°to 35°C. Laiho (1970) reported the temperature maximum for eight strains of Paxillus involutus to be about 30°C, with all strains being killed at 32°C.

Of the various wood-destroying Hymenomycetes studied by Humphrey and Siggers (1933) and Björkman (1946) belonging to the genera Polyporus, Sterium and Poria, the great majority have maximum growth at temperatures of 28°C or higher. On the other hand, several Mycena species grew best at 20°C (Fries, 1949).

Treschow (1944) found that Agaricus bisporus grew best at a temperature range between 20°C and 27°C with an optimum at 24°C. Coprophilous species tend to have higher temperature optima: species of Coprinus investigated by Rege (1927) showed a high optimum of 30°to 35°C. The ability to grow at higher temperatures was confirmed by Fries (1956), who found various Coprinus species to grow best at 30°to 35°C, with good growth at 44°C in C. fimentarius. Cooney and Emerson (1964) singled out Coprinus as the only genus in the Basidiomycetes known to contain thermotolerant species, and Kuthubutheen and Pugh (1977) reported Coprinus delicatulus, which they isolated from green leaves of Agrostis tenuis, as a

thermophilic species, noting growth and sporophore production at 45°C. A temperature of 30°C was shown to favour the growth of <u>C. cinereus</u> by Seal and Kelley (1980).

In the present study the five species used were mesophilic in their temperature requirements, while the coprophile <u>Cyathus stercoreus</u> showed maximum growth at 30°C and 35°C. All six species exhibited growth at 5°C and there were indications that <u>Coprinus comatus</u> could probably grow quite well at lower temperatures as there was little change in growth rates at 10°C and 5°C. Melin (1925) found that three species of <u>Boletus</u> grew well at 10°C and two of them continued to grow at 6°C, while Lobanow (1960) reported that the temperature minimum of mycorrhizal fungi was 1° to 5°C. However, temperature requirements can depend on the origin of the strains used: Moser (1958) showed that the minimum for a strain of <u>Paxillus involutus</u> isolated from a valley was 2°to 8°C, whereas a mountain strain grew at -2° to 4°C.

Soil inhabiting Basidiomycetes have long been regarded as slow growing organisms. However, Basidiomycete <u>235M</u> is exceptional in showing a rate of growth comparable with many non-Basidiomycetes. <u>Phallus impudicus</u> and the two mycorrhizal species tested were slower growing than the other non-mycorrhizal fungi. Pugh and MacDonald (1980) suggested that growth rate may be used as a criterion for distinguishing between mycorrhizal and non-mycorrhizal fungi. However, further studies are needed since few attempts have been made at explaining the apparent physiological differences between these two groups of fungi.

CHAPTER III

EFFECT OF AGROCHEMICALS ON GROWTH OF BASIDIOMYCETES

EFFECT OF AGROCHEMICALS ON GROWTH OF BASIDIOMYCETES

Introduction

Romell (1938) pointed to the fundamental physiological difference between litter decomposing fungi and those forming mycorrhizae with trees. While the litter decomposers are able to decompose cellulose and lignin, as shown by Falck (1930) and later by Lindeberg (1944) in pure culture experiments, according to Romell: "the obligate tree mycorrhizal fungi seem to be practically unable to break down dead organic residues, under conditions prevailing in nature, so they must derive their energy food from living and functioning roots of trees".

Later, however, Lindeberg (1948) was able to show that a strain of the mycorrhizal *Boletus subtomentosus* decomposed litter *in vitro*, after Romell (1939) had found that this species formed fruit bodies in areas which had been isolated by trenching from all roots which were attached to living trees. This led Norkrans (1950) to suggest that the difference in the cellulase forming ability between litter decomposing and mycorrhiza forming types is probably a quantitative rather than a qualitative one.

Other physiological differences between mycorrhizal and non-mycorrhizal fungi have pointed to differences between the two groups: Lindeberg (1948) found that extracellular polyphenol oxidases are produced only by the litter decomposers. More recently, it was shown by Olsen *et al.* (1971) that extracts of aspen leaves, which contained benzoic acid and catechol, had a greater inhibitory effect on the growth of mycorrhizal *Boletus* species than on litter

decomposing Marasmius species. They drew attention to Lindeberg's (1948) findings, suggesting that the ability of the litter decomposers to produce polyphenol oxidases may explain this difference.

In the light of these findings and those of the previous chapter, it was decided to investigate the ability of mycorrhizal and non-mycorrhizal fungi to grow in the presence of increasing concentrations of agrochemicals.

In the past, the effects of these compounds on the formation of the mycorrhizal association with plants have received more attention than their effect on growth of either litter decomposing or mycorrhiza forming species. Reduction in forest productivity has been blamed on the eradication of mycorrhizal fungi in the rhizosphere (Iyer and Wilde, 1965).

There have been several reports on the effects of fungicides (Jalali and Domsch, 1975; Paget et al., 1976; Sutton and Sheppard, 1976; de Bertoldi et al., 1977; Boatman et al., 1978) and other pesticides (Ocampo and Hayman, 1980) on the physiology of vesicular-arbuscular mycorrhizae, and it has been shown that agrochemicals can adversely affect ectomycorrhizal formation (Wilde and Persidsky, 1956; Hacskaylo and Palmer, 1957; Persidsky and Wilde, 1960; Smith, 1973).

Materials and Methods

Species

The same six species used in the growth studies (Chapter 2) were again used, together with: *Agaricus arvensis*, *A. bisporus*, Basidiomycete 109B, *Boletus greveillei*, *Coprinus atromentarius*, *Lepista nuda*, *L. saeva* and *Pleurotus sajor-caju* (Table 5, Chapter 2).

Effect of Agrochemicals on Linear Extension Rate

Known volumes of Hagem's agar (Appendix 3) or Oxoid malt extract agar were autoclaved at 15 psi for 15 minutes. The fungicides CAPTAN and VERDASAN were suspended in sterile distilled water to give stock solutions containing 100 ppm. Appropriate volumes of these were added to the cooled sterile medium to give final concentrations of 0.1, 0.5, 0.75, 1.0, 2.5, and 5.0 ppm for VERDASAN, and 10, 25, 50, 75, and 100 ppm of CAPTAN.

Appropriate amounts of the herbicides MAZIDE and PARAQUAT were dissolved in known volumes of agar medium to give final concentrations of 100, 250, 500, 1000, 2000, and 3000 ppm for MAZIDE, and 10, 25, 50, 100, 250, and 500 ppm for PARAQUAT. 20 ml aliquots of agrochemical-incorporated medium were dispensed into each of 9 cm Petri dishes. Growth medium without agrochemical addition was used as CONTROL. Each Petri dish was inoculated with a disc of fungal mycelium cut from colonies growing on either HMA or MA. Five replicate plates for each concentration of agrochemical for every test fungus were used. The plates were incubated at the optimum temperature for growth of each fungus. The colony diameters were taken as the mean of two diameters at right angles to each other. Linear extension rates were calculated during the log phase of

growth.

Effect of Agrochemicals on Dry Weight Production

Appropriate amounts of herbicide stocks were added to basic liquid medium (BLM) to give final concentrations of 500, 1000, 2000, 3000, 4000, and 8000 ppm for MAZIDE, and 5, 10, 25, 50, 100, and 250 ppm for PARAQUAT. Flasks were autoclaved for 15 minutes at 15 psi. Appropriate amounts of VERDASAN stock solution were added to cool sterile BLM to give final concentrations of 0.1, 0.25, 0.5, 0.75, 1.0, and 2.5 ppm. Flasks containing 20 ml of agrochemical-incorporated BLM were inoculated with the test fungi and incubated at their optimum temperature for growth in liquid medium. BLM without agrochemical addition was used as CONTROL. Five replicate flasks for each concentration of agrochemical for every fungus were used. For dry weight analysis, flasks were removed, the mycelia harvested by suction filtration onto preweighed, oven dried Whatman No. 1 filter papers, and dried to constant weight at 90°C.

Tolerance of VERDASAN

Several species of both mycorrhizal and non-mycorrhizal fungi were grown in the presence of increasing concentrations of VERDASAN from 0.05 ppm to 5.0 ppm for liquid culture growth.

Results

Linear Extension

The effects of agrochemicals on the linear extension rate of the six species are shown in Figures 11 and 12 , Table 7 and Appendix 7 . All species showed growth in the absence of agrochemicals on CONTROL plates (Appendix 7). 235M, Coprinus comatus and Cyathus stercoreus had the highest growth rates on MA, while the mycorrhizal forms, Boletus variegatus, Paxillus involutus and Russula emetica had slowest growth rates on HMA. Growth of these three species was even further retarded on MA.

In general, the fungi were tolerant of MAZIDE at the concentrations used: 235M was the least tolerant of this herbicide. CAPTAN, VERDASAN and PARAQUAT showed some similarities in their effects at the concentrations used: while 235M and Coprinus comatus were more tolerant of VERDASAN and PARAQUAT than the other species, only Cyathus stercoreus showed a greater tolerance of CAPTAN than the other species. Boletus variegatus, Paxillus involutus and Russula emetica were least tolerant of these three agrochemicals. Cyathus stercoreus showed a growth response similar to the mycorrhizal species in the presence of PARAQUAT but was more tolerant of VERDASAN than these species.

Concentrations of CAPTAN from 10 ppm to 100 ppm allowed the growth of the three non-mycorrhizal species, while the mycorrhizal forms showed less tolerance of the fungicide (Fig. 1). No growth of B. variegatus and P. involutus occurred at 75 ppm of CAPTAN, nor of R. emetica at 25 ppm of CAPTAN.

While none of the species tested grew at 2.5 ppm of

Fig.11. The effects of CAPTAN and VERDASAN on the linear extension rate of Basidiomycetes.

○—○ Basidiomycete 235M

●—● *Boletus variegatus*

△—△ *Coprinus comatus*

▲—▲ *Cyathus stercoreus*

□—□ *Paxillus involutus*

■—■ *Russula emetica*

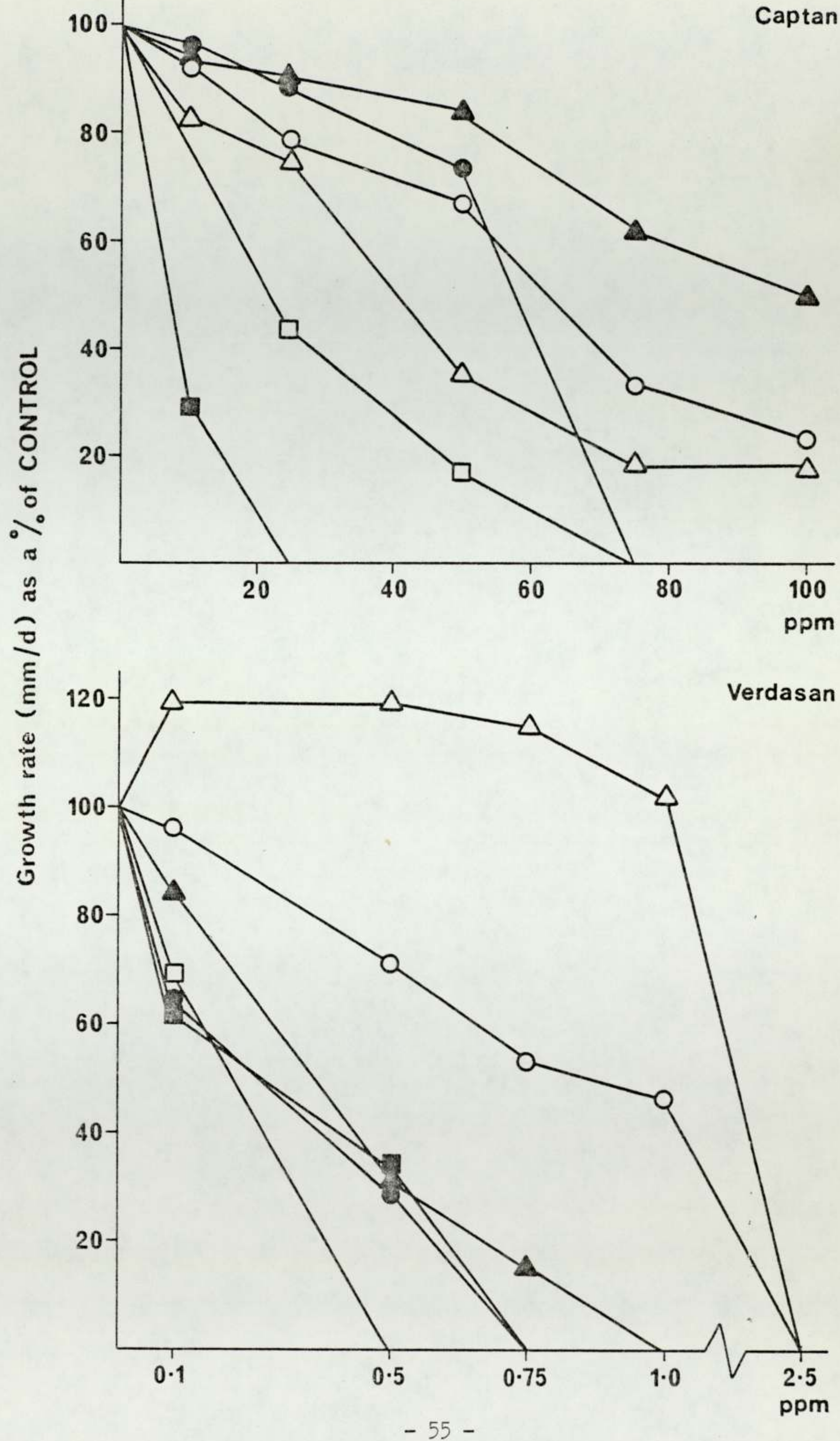

Captan
100
80
60
40
20
20
40
60
80
100
ppm
Growth rate (mm/d) as a %of CONTROL
Verdasan
120
100
80
60
40
20
0·1
0·5
0·75
1·0
2·5
ppm

Fig.12. The effects of MAZIDE and PARAQUAT on the linear extension rate of Basidiomycetes.

○—○ Basidiomycete <u>235M</u>

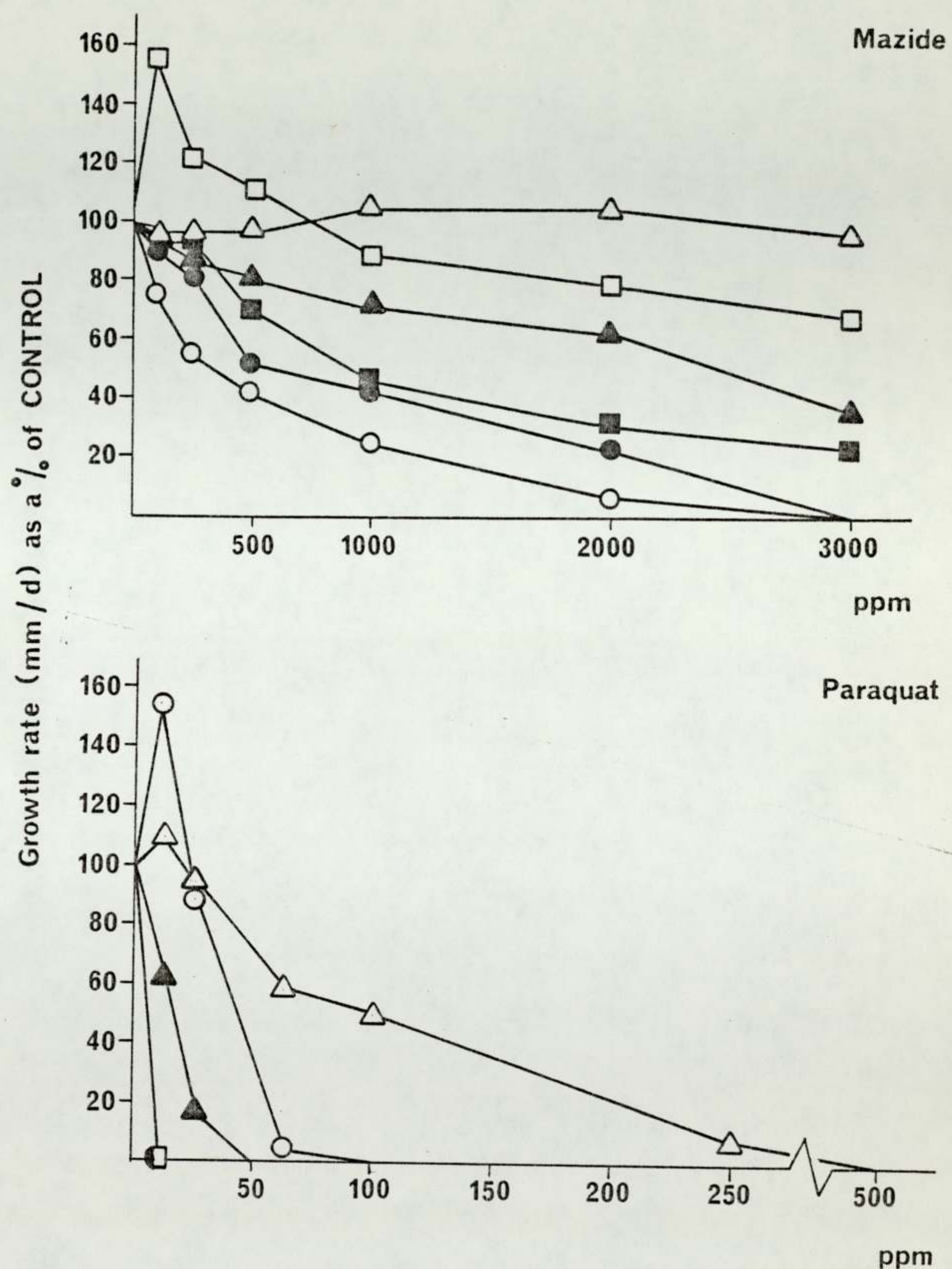
●—● <u>Boletus variegatus</u>

△—△ <u>Coprinus comatus</u>

▲—▲ <u>Cyathus stercoreus</u>

□—□ <u>Paxillus involutus</u>

■—■ <u>Russula emetica</u>


Mazide
160
140
120
100
80
60
40
20
500
1000
2000
3000
ppm
Growth rate (mm/d) as a % of CONTROL
Paraquat
160
140
120
100
80
60
40
20
50
100
150
200
250
500
ppm

VERDASAN, only 235M and Coprinus comatus were able to grow at 1.0 ppm of the fungicide (Fig. 11). VERDASAN allowed the growth of B. variegatus and R.emetica at 0.5 ppm, and of P. involutus only at 0.1 ppm. Cyathus stercoreus showed no growth at 1.0 and 2.5 ppm of VERDASAN.

MAZIDE at 3000 ppm prevented the growth of 235M and Boletus variegatus although all the other species tested continued to grow at this concentration, (Fig 12).

Varying degrees of tolerance of PARAQUAT were shown: B. variegatus and P. involutus showed no growth at 10 ppm to 500 ppm of the herbicide, although Coprinus comatus showed no growth only at 500 ppm of PARAQUAT (Fig 12). Growth of 235M and Cyathus stercoreus was inhibited at 100 ppm and 50 ppm of PARAQUAT respectively.

When the number of "no growth" occasions was considered (Table 7), Coprinus comatus, with 2, emerged as the most tolerant species. The two generally more sensitive mycorrhizal species, Boletus variegatus and Paxillus involutus, both with 12, had noticeably more than the other non-mycorrhizal species: 235M with 5, and Cyathus stercoreus with 6 "no growth" occasions.

The linear extension rates of the six species tested with different concentrations of agrochemicals are given in Appendix 7 and are expressed as a percentage of the CONTROL in Table 7 .

The linear extension rate of 235M was reduced appreciably by CAPTAN at 50 ppm to 100 ppm and its rate decreased slowly with increasing concentrations of VERDASAN until at 1.0 ppm it was only half that of CONTROL.

Table 7 . Effect of Increasing Agrochemical Concentration on Linear Extension Rate of Fungi at their Optimum Temperatures for Growth expressed as a percentage of CONTROL.

AGROCHEMICAL	Concentration (ppm)	235M	Boletus variegatus	Coprinus comatus	Cyathus stercoreus	Paxillus involutus	Russula emetica
CAPTAN	10	92	96	83	93	NT	29
	25	78	89	75	90	43	-
	50	67	74	35	84	17	-
	75	33	-	18	62	-	-
	100	23	-	18	50	-	-
VERDASAN	0.1	96	64	119	84	69	62
	0.5	71	29	119	31	-	38
	0.75	53	-	115	15	-	-
	1.0	46	-	102	-	-	-
	2.5	-	-	-	-	-	-
MAZIDE	100	76	90	95	98	156	92
	250	56	81	97	86	122	93
	500	42	52	97	80	111	69
	1000	25	42	104	71	89	46
	2000	7	23	104	62	78	31
	3000	-	-	95	35	67	23
PARAQUAT	10	155	-	108	62	-	NT
	25	88	-	92	16	-	NT
	50	2	-	57	-	-	NT
	100	-	-	51	-	-	NT
	250	-	-	7	-	-	NT
	500	-	-	-	-	-	NT
Number of "no growth" occasions		5	12	2	6	12	

- No growth NT Not tested

The linear extension rate of _Boletus variegatus_ was most drastically reduced by CAPTAN at 75 ppm where growth was inhibited completely, and in VERDASAN at 0.5 ppm the rate was less than a third that of CONTROL. At a concentration of only 500 ppm of MAZIDE the linear extension rate was nearly halved, and no growth occurred in any of the concentrations of PARAQUAT that were tested.

The linear extension rate of _Coprinus comatus_ showed a marked reduction in CAPTAN at 50 ppm although in concentrations of VERDASAN from 0.1 ppm to 1.0 ppm this was the only fungus tested that showed a rate which was comparable with that of CONTROL. The rate was little affected by any of the concentrations of MAZIDE tested; PARAQUAT at a concentration of 50 ppm markedly reduced the rate.

The linear extension rate of _Cyathus stercoreus_ in the presence of CAPTAN at 75 ppm to 100 ppm, VERDASAN at 0.5 ppm, and PARAQUAT at 10 ppm to 25 ppm, was markedly less than on CONTROL plates. With MAZIDE, the rate decreased noticeably at 2000 ppm to 3000 ppm.

The linear extension rate of _Paxillus involutus_ was markedly less than CONTROL in CAPTAN at 25 ppm tp 50 ppm, in VERDASAN at 0.5 ppm to 1.0 ppm, and in PARAQUAT where growth was completely inhibited even at 10 ppm. However, extension rates in all concentrations of MAZIDE were comparable with CONTROL, and on three occasions (at 100, 250 and 500 ppm of MAZIDE), were higher.

The linear extension rate of _Paxillus involutus_ was drastically reduced by CAPTAN at 10 ppm to 100 ppm, and also by VERDASAN at 0.1 ppm to 2.5 ppm, as compared with the CONTROL. With CAPTAN, all species behaved similarly with decreased

growth at increasing concentrations. Coprinus comatus was the only species which showed a tolerance of VERDASAN up to 1.0 ppm; all other species showed decreased growth at concentrations greater than CONTROL.

With MAZIDE, Coprinus comatus and Cyathus stercoreus were little affected, and Paxillus involutus showed increased growth at lower concentrations. 235M and Boletus variegatus, however, showed decreased growth at increasing concentrations of MAZIDE.

The growth of 235M and Coprinus comatus was increased slightly at low concentrations of PARAQUAT: growth of all the other species was reduced at increasing concentrations of the herbicide.

Colony morphology was often changed with increasing concentrations of agrochemicals, and with the higher concentrations of VERDASAN brown pigments diffused into the agar medium from Boletus variegatus. The lag period increased with increasing concentration and these were generally longer with the mycorrhizal species.

Effect of Agrochemicals on Mycelial Dry Weight Production

The effects of agrochemicals on the growth of the six Basidiomycetes are shown in Figures 13, 14, 15, Table 8 and Appendix 8. All six species tested showed growth in CONTROL flasks without agrochemical addition, and increasing agrochemical concentration significantly affected mycelial dry weight production.

In general, the fungi were tolerant of MAZIDE (Fig. 13) at the concentrations used: only Phallus impudicus showed a marked intolerance. PARAQUAT and VERDASAN (Figs. 14 and 15) showed some similarities in their effects at the concentrations used: 235M and Coprinus comatus were more tolerant than the other species. Boletus

Fig.13. The effect of MAZIDE on mycelial dry weight production by Basidiomycetes.

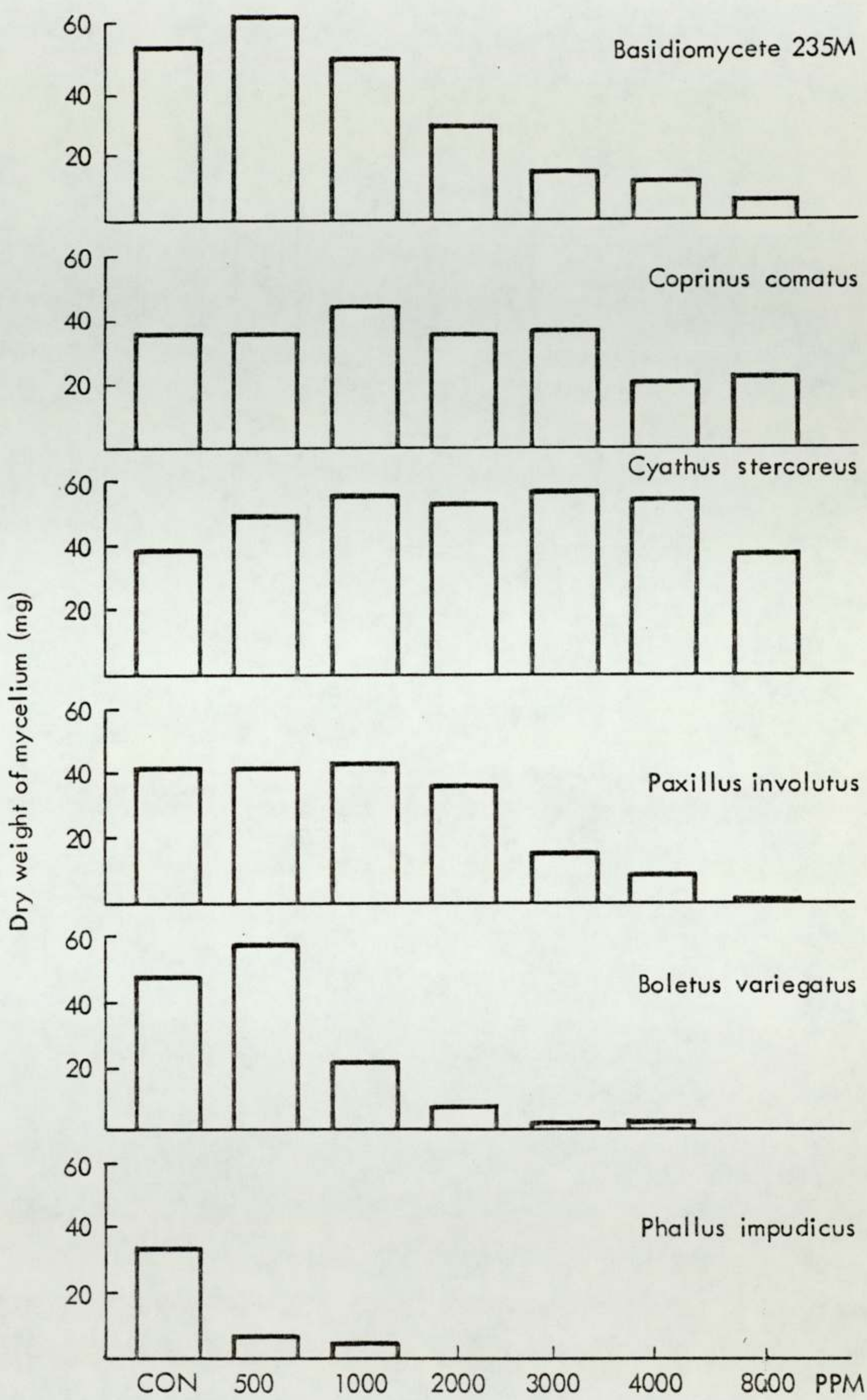

60
40
20
Basidiomycete 235M
60
40
20
Coprinus comatus
Cyathus stercoreus
60
40
20
Dry weight of mycelium (mg)
60
40
20
Paxillus involutus
60
40
20
Boletus variegatus
60
40
20
Phallus impudicus
CON
500
1000
2000
3000
4000
8000
PPM

Fig.14. The effect of PARAQUAT on mycelial dry weight production by Basidiomycetes.

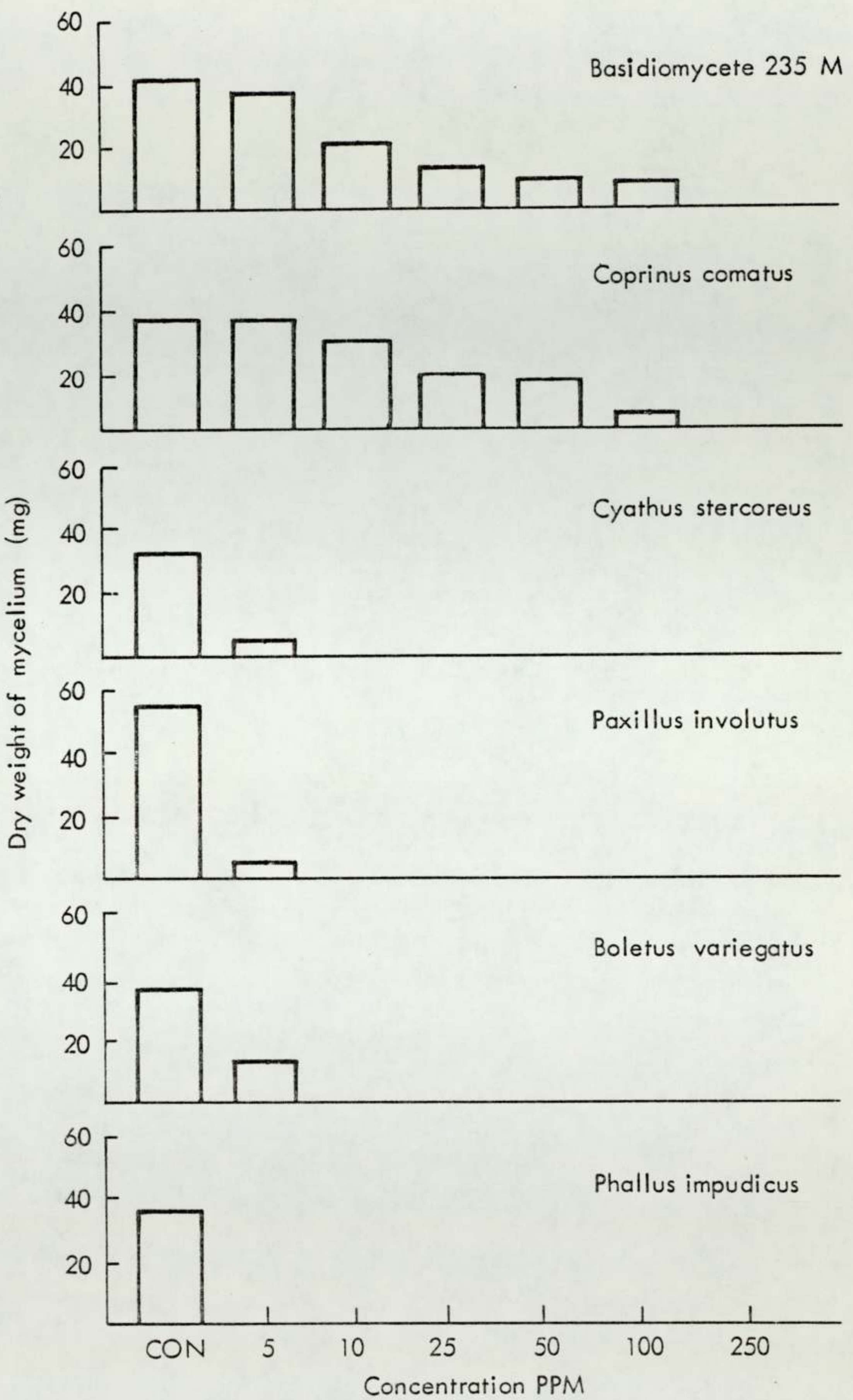
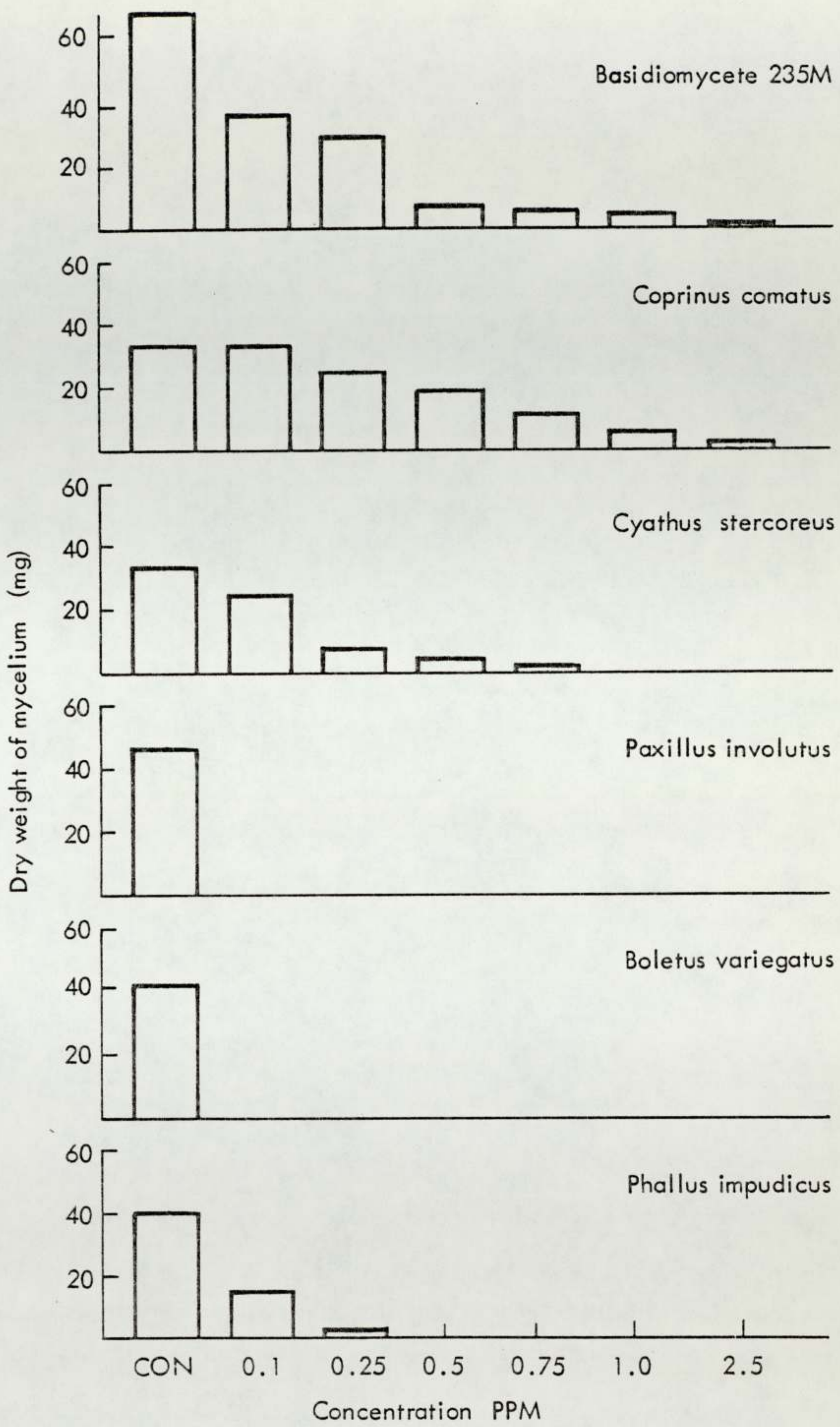

Basidiomycete 235 M
Coprinus comatus
Cyathus stercoreus
Paxillus involutus
Boletus variegatus
Phallus impudicus
Dry weight of mycelium (mg)
60
40
20
CON
5
10
25
50
100
250
Concentration PPM

Fig.15. The effect of VERDASAN on mycelial dry weight production by Basidiomycetes.


Basidiomycete 235M
Coprinus comatus
Cyathus stercoreus
Paxillus involutus
Boletus variegatus
Phallus impudicus
Dry weight of mycelium (mg)
60
40
20
CON
0.1
0.25
0.5
0.75
1.0
2.5
Concentration PPM

variegatus and Paxillus involutus were the least tolerant, showing no growth at 10 ppm of PARAQUAT and 0.1 ppm of VERDASAN. Cyathus stercoreus and Phallus impudicus showed some growth patterns similar to the mycorrhizal species in the presence of PARAQUAT, but both were more tolerant of VERDASAN than these species.

The growth of 235M in MAZIDE at 2000 ppm to 8000 ppm, in PARAQUAT at 10 ppm to 250 ppm, and in VERDASAN at 0.1 ppm to 2.5 ppm was significantly less than CONTROL.

Coprinus comatus, however, showed slightly greater tolerance, where growth in MAZIDE at 4000 ppm to 8000 ppm, and in VERDASAN at 0.25 ppm to 2.5 ppm, was significantly less than CONTROL. C. comatus and 235M were the only species capable of growth in 1.0 ppm to 2.5 ppm of VERDASAN.

Although growth of Cyathus stercoreus in the lowest concentration of PARAQUAT was significantly less than CONTROL, MAZIDE at a concentration of 500 ppm to 4000 ppm had a significant stimulatory effect on growth as compared with CONTROL.

Paxillus involutus and Boletus variegatus behaved similarly in the presence of PARAQUAT and VERDASAN, growth being significantly reduced at the lowest concentrations tested as compared with CONTROL. However, while growth of P. involutus in MAZIDE at 3000 ppm to 8000 ppm was significantly less than CONTROL, MAZIDE at 500 ppm had a significant stimulatory effect on the growth of B. variegatus as compared with CONTROL.

The growth of Phallus impudicus in all concentrations of each agrochemical tested was significantly less than that in CONTROL.

When the number of "no growth" occasions was considered

Table 8 . Effect of Increasing Agrochemical Concentration on Mean Dry Weight Production of Fungi at their Optimum Temperatures for Growth expressed as a percentage of CONTROL.

AGROCHEMICAL	Concentration (ppm)	235M	Boletus variegatus	Coprinus comatus	Cyathus stercoreus	Paxillus involutus	Phallus impudicus
VERDASAN	0.5	54	-	101	74	-	40
	0.25	44	-	75	25	-	6
	0.5	11	-	58	16	-	-
	0.75	10	-	32	7	-	-
	1.0	7	-	18	-	-	-
	2.5	4	-	12	-	-	-
MAZIDE	500	117	121	99	130	102	21
	1000	94	48	129	143	103	18
	2000	58	13	99	138	87	-
	3000	31	4	101	150	39	-
	4000	25	8	61	143	27	-
	8000	16	-	68	99	5	-
PARAQUAT	5	92	38	98	15	11	-
	10	55	-	80	-	-	-
	25	36	-	48	-	-	-
	50	27	-	45	-	-	-
	100	30	-	-	-	-	-
	250	-	-	-	-	-	-
Number of "no growth" occasions		1	12	2	7	11	14

- No growth

(Table 8), 235M with 1, Coprinus comatus with 2, and Cyathus stercoreus with 7, emerged as the most tolerant species. The mycorrhizal species, Paxillus involutus and Boletus variegatus, with 11 and 12 respectively, and Phallus impudicus with 14 "no growth" occasions, were much more sensitive.

Tolerance of VERDASAN

The ability of mycorrhizal and non-mycorrhizal Basidiomycetes to grow in increasing concentrations of the fungicide VERDASAN is shown in Table 9.

The mycorrhizal species and Phallus impudicus generally showed less tolerance of VERDASAN than the other species. However, although Coprinus comatus could continue to grow at 2.5 ppm of VERDASAN, Coprinus atromentarius could not grow at a fifth of this concentration.

Table 9 . Ability of Basidiomycetes to grow in VERDASAN

Species	Concentration ppm							
	0.05	0.1	0.25	0.5	0.75	1.0	2.5	5.0
Basidiomycete 235M	+	+	+	+	+	+	+	–
Basidiomycete 109B	+	+	+	+	+	+	+	–
Coprinus comatus	+	+	+	+	+	+	+	–
Pleurotus sajor-caju	+	+	+	+	+	+	+	–
*Lepista nuda	+	+	+	+	+	+	+	–
Agaricus arvensis	+	+	+	+	+	+	–	–
Lepista saeva	+	+	+	+	+	+	–	–
Cyathus stercoreus	+	+	+	+	+	–	–	–
Agaricus bisporus	+	+	+	+	–	–	–	–
*Boletus greveillei	+	+	+	+	–	–	–	–
Coprinus atromentarius	+	+	+	–	–	–	–	–
Phallus impudicus	+	+	+	–	–	–	–	–
*Paxillus involutus	+	–	–	–	–	–	–	–
*Boletus variegatus	+	–	–	–	–	–	–	–

\+ growth
– no growth
* known mycorrhizal species

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Discussion

The agrochemicals used were generally found to reduce fungal growth, although in some cases growth was increased at lower concentrations: MAZIDE increased mycelial dry weight production of both Basidiomycete 235M and Coprinus comatus, although these increases were not significantly greater than those of the CONTROL. Significant increases in dry weight production did however occur with Boletus variegatus and Cyathus stercoreus in the presence of lower concentrations of MAZIDE, although the linear extension rate decreased with increasing MAZIDE concentration with these two species. Factors present in the herbicide responsible for growth stimulation in liquid cultures may have been adsorbed onto the agar in a similar way that fungicides have been shown to be inactivated in agar medium (Greenaway, 1973; Williams and Pugh, 1975). Da Silva et al. (1977) reported a stimulation of dry weight production of Boletus luteus and B. variegatus by low concentrations of 2,4-dichlorophenoxyacetic acid (2,4D) and 2,4,5T.

Linear extension rates were also appreciably increased by the agrochemicals in some cases: with Basidiomycete 235M by PARAQUAT at 10 ppm to 1000 ppm; with Coprinus comatus by VERDASAN at 0.1 ppm to 0.75 ppm; and with Paxillus involutus by MAZIDE at 100 ppm to 500 ppm. Only in the case of P. involutus was there a corresponding increase of mycelial dry weight at lower concentrations of the agrochemical.

In all other cases, dry weight production and linear extension rates were reduced by increasing the agrochemical concentration and these fungi showed similar patterns of growth in the presence of agrochemicals, both on agar and in liquid media. One exception was

Basidiomycete 235M: MAZIDE had a more deleterious effect on linear extension rate than on dry weight production.

The results of Chapter 2 suggested that there were some physiological differences between mycorrhizal and non-mycorrhizal species. In the presence of agrochemicals there were again some differences in the tolerance shown by these different groups. The mycorrhizal fungi, Boletus variegatus, Paxillus involutus and Russula emetica showed generally lower linear extension rates in the presence of the agrochemicals than did the non-mycorrhizal species. One exception was P. involutus in the presence of MAZIDE, which showed rates comparable with the tolerant Coprinus comatus.

Dry weight production was also differently affected by the agrochemicals in the two groups of fungi: with the exception of Phallus impudicus the mycorrhizal species were less tolerant than the non-mycorrhizal species to the three agrochemicals used. At concentrations likely to exist in field situations all species, except Phallus impudicus, were tolerant of MAZIDE. With PARAQUAT and VERDASAN, however, all of the test fungi were inhibited below the field application rate.

The behaviour of Phallus impudicus indicates many similarities with the known mycorrhizal Boletus variegatus and Paxillus involutus species. However, Grainger (1962) described Phallus impudicus as growing saprophytically on leaf mould and decayed wood, while Trappe (1962) reported it as a possible mycorrhizal species. Further laboratory and field experiments are needed to see whether P. impudicus is in fact truly mycorrhizal, and if the reaction of Basidiomycetes to agrochemicals in general can be used as a criterion

to distinguish between the mycorrhizal and non-mycorrhizal groups.

Thus, the four agrochemicals tested could be expected, on the basis of the recorded data,to have more deleterious effects on mycorrhizal activity than on decomposition in general. If any of these chemicals interfere with the establishment of symbiosis between the ectotroph and its plant host, the former may soon be eliminated from the ecosystem. Since higher plant growth is frequently dependent on the fungal partner, the agrochemicals would also have an adverse effect on their growth. This could be particularly important in marginal lands, where the use of these compounds is likely to be great as they are brought into cultivation (Pugh and MacDonald, 1980).

CHAPTER IV

THE EFFECTS OF AGROCHEMICALS ON MEMBRANES OF BASIDIOMYCETES

THE EFFECTS OF AGROCHEMICALS ON MEMBRANES OF BASIDIOMYCETES

Introduction

The measurement of metabolite leakage as an indicator of membrane disruption in fungi has been used by Harris and Dodge (1972b) and by Smith, Lyon and Pugh (unpublished results). The leakage of metabolites experienced can be correlated with equivalent cell damage or lysis, and observed by electron microscope study (Smith and Lyon, 1977).

In the past, the extent of metabolite loss from membranes has been used to determine the viability of plant and fungal propagules. Matthews and Bradcock (1967) demonstrated that excessive leakage of metabolites from seeds of Pisum sativum indicated not only that they were in poor physical condition, but also were more prone to colonisation by pathogenic organisms. Blakeman (1973) demonstrated that spores of Botrytis cinerea which were exposed to leaching for long periods, lost their viability through the leakage, with a subsequent loss of endogenous substances required for germination.

Leakage of amino acids and nucleic acids was detected above the maximum growth temperature of the psychrophilic bacterium Vibrio marinus by Haight and Morita (1966), who postulated that leakage of cellular components was one of the major reasons for loss of viability by this organism. Ward (1971) demonstrated that a similar process occurred in Basidiomycetes, where amino acids and carbohydrate leakage occurred at temperatures above the optimum for growth. It has also been shown that the thermophilic Mucor pusillus, in comparison with the mesophilic species Mucor hiemalis and Rhizopus stolonifer, demonstrated little efflux of metabolites at high temperatures, indicating the more thermostable nature of its

membrane structure (Smith, Lyon and Pugh, unpublished results).

The leakage of metabolites from organisms is normally a passive diffusion process, which can either be related to an alteration in membrane structure (Simon, 1974), or to a combination of both an alteration in membrane structure and interference with the uptake mechanisms of membranes (Ismail B. Sahid, Lyon and Smith, in press).

The aim of the present study is to investigate whether two non-mycorrhizal Basidiomycetes, 235M and *Cyathus stercoreus*, which have shown some tolerance of agrochemicals in growth studies, have membranes of greater stability than the more susceptible mycorrhizal *Boletus variegatus*, in the presence of agrochemicals.

The herbicide PARAQUAT, and the organo-mercurial fungicide VERDASAN were used, since they have both been shown to interfere with membrane structure and uptake mechanisms of membranes (Rothstein, 1959; Stokes and Turner, 1971; Harris and Dodge, 1972b). In addition, SODIUM AZIDE was used: this compound has been used in the past as a biodegradable herbicide, and has been shown to inhibit active uptake of metabolites across cellular membranes by inhibiting nuclear phosphorylation (Mettler, 1972).

Materials and Methods

Effect of Increasing Concentrations of Agrochemical on Leakage

The effects of PARAQUAT, SODIUM AZIDE, and VERDASAN on the permeability of the membranes of the three fungi, Basidiomycete 235M, *Boletus variegatus*, and *Cyathus stercoreus* were investigated. Individual 100 ml Erlenmeyer flasks, each containing 20 ml of the basic liquid medium, were sterilised for 15 minutes at 120°C and 15 psi, and were each inoculated with a plug of mycelium taken from the margin of actively growing colonies on either HMA or MA. The flasks were incubated in a Gallenkamp orbital shaker rotating at 100 rpm, at the optimum growth temperature of the fungus. The resulting colonies were removed from their growth medium by suction filtration and washed with an excess of glass distilled water which was kept at the optimum temperature for growth for the particular species. Colonies were then quickly transferred to fresh 100 ml Erlenmeyer flasks containing a range of PARAQUAT concentrations between 50 ppm and 1000 ppm, a range of SODIUM AZIDE concentrations between 0.33 ppm and 65.1 ppm, and a range of VERDASAN concentrations between 0.1 ppm and 50 ppm, in glass distilled water and kept at the optimum temperature for the respective species. Flasks containing glass distilled water alone were used as CONTROLS, and five replicate flasks for each concentration of agrochemical were used. During the incubation period the flasks were gently shaken at 90 rpm in an orbital shaker. Care was taken not to damage the fungal mycelium in any of the preparation stages.

After the incubation period the colonies were again harvested by suction filtration onto dried and preweighed No. 1 Whatman filter

papers, and dried to constant weight at 90°C to determine their dry weight. The filtrates from each colony were centrifuged at 2000 x g for 15 minutes to remove any residual mycelial debris prior to assay for metabolites.

Time course of Metabolite Leakage in the Presence of Agrochemicals

In order to determine the time course of metabolite leakage from the fungi, only one concentration of PARAQUAT (1000 ppm), SODIUM AZIDE (65.1 ppm), and VERDASAN (100 ppm) was used. The colonies were prepared in the manner described above, transferred to flasks containing the agrochemicals, and gently shaken at 90 rpm in an orbital shaker. Five replicate flasks for every fungus and for each consecutive sample over a 24 hour incubation period were used. The flasks were sampled at 0, 1, 2, 4, 8, and 24 hours after the start of the incubation period, the filtrates centrifuged at 2000 x g for 15 minutes, and the dry weight of each colony determined as previously described.

Potassium Assay

The levels of potassium in the filtrates were assayed with an EEL flame photometer and compared with a previously constructed standard curve (Appendix 9).

Phosphate Assay

Aliquots of 1 ml of filtrate were mixed with 1 ml of 0.5N trichloroacetic acid (TCA) and 1 ml of colour reagent. The colour reagent was prepared by mixing together 4 ml of 16% ammonium molybdate in 10N sulphuric acid with 35 ml of distilled water and 2 g of ferrous sulphate. The optical density (O.D.) of the resulting blue colour was measured at a wavelength of 660 nm against a reagent

blank in a Beckman DB spectrophotometer, and the concentration of phosphate in each aliquot determined by comparison with a previously constructed standard curve (Appendix 10). Since PARAQUAT interferes with this assay, aliquots of filtrate containing the herbicide were treated by a modification of the method used by Calderbank and Yuen (1964) in order to remove PARAQUAT (Appendix 11).

Amino Acids Assay

Total amino acids were evaluated by a technique similar to that of Yemm and Cocking (1955). To determine the total amounts of amino acids, 0.5 ml of 0.2M pH 5 citrate buffer was mixed with 1.0 ml aliquots of diluted filtrate. Subsequently, 1.0 ml of ninhydrin working solution, which was composed of 120 ml of 9.58 mg/l ninhydrin in methoxyethanol, mixed with 40 mg of ascorbic acid in 4.0 ml distilled water, was added to each aliquot. The samples were heated in a boiling water bath for 15 minutes and subsequently cooled for 15 minutes in cold running water. The O.D. of the resulting blue colour was measured against a reagent blank in a Beckman DB spectrophotometer at a wavelength of 520 nm, and the concentration of amino acids in each aliquot determined by comparison with a previously constructed standard curve (Appendix 12).

Results

The amounts of potassium, phosphate and total amino acids recorded from flasks containing the three species of fungi, Basidiomycete 235M, *Boletus variegatus* and *Cyathus stercoreus* in the presence of the chemicals are shown in Figures 16, 17 and 18 and are summarised in Appendix 13. All three species showed some leakage in CONTROL flasks, without chemical addition. In general, 235M released greater amounts of potassium, phosphate and amino acids in CONTROL flasks than did *B. variegatus* and *C. stercoreus*.

The Effect of Increasing Concentrations of Agrochemicals on Potassium Leakage

The results (Fig. 16) indicate that the net loss of potassium from the three fungi in the presence of PARAQUAT and VERDASAN was significant and the amounts leaked increased with increasing concentrations of chemicals. However, in the presence of SODIUM AZIDE, while the net loss of potassium from *Cyathus stercoreus* and *Boletus variegatus* was significant and increased significantly with increasing concentration, there was no such effect with Basidiomycete 235M.

At the concentrations used, VERDASAN caused the greater net loss of potassium from the three species of fungi, and SODIUM AZIDE the least.

The net loss from *B. variegatus* in the presence of only 50 ppm of PARAQUAT was nearly four times that of CONTROL, while the amounts recorded from 235M and *C. stercoreus* were only slightly but still significantly increased.

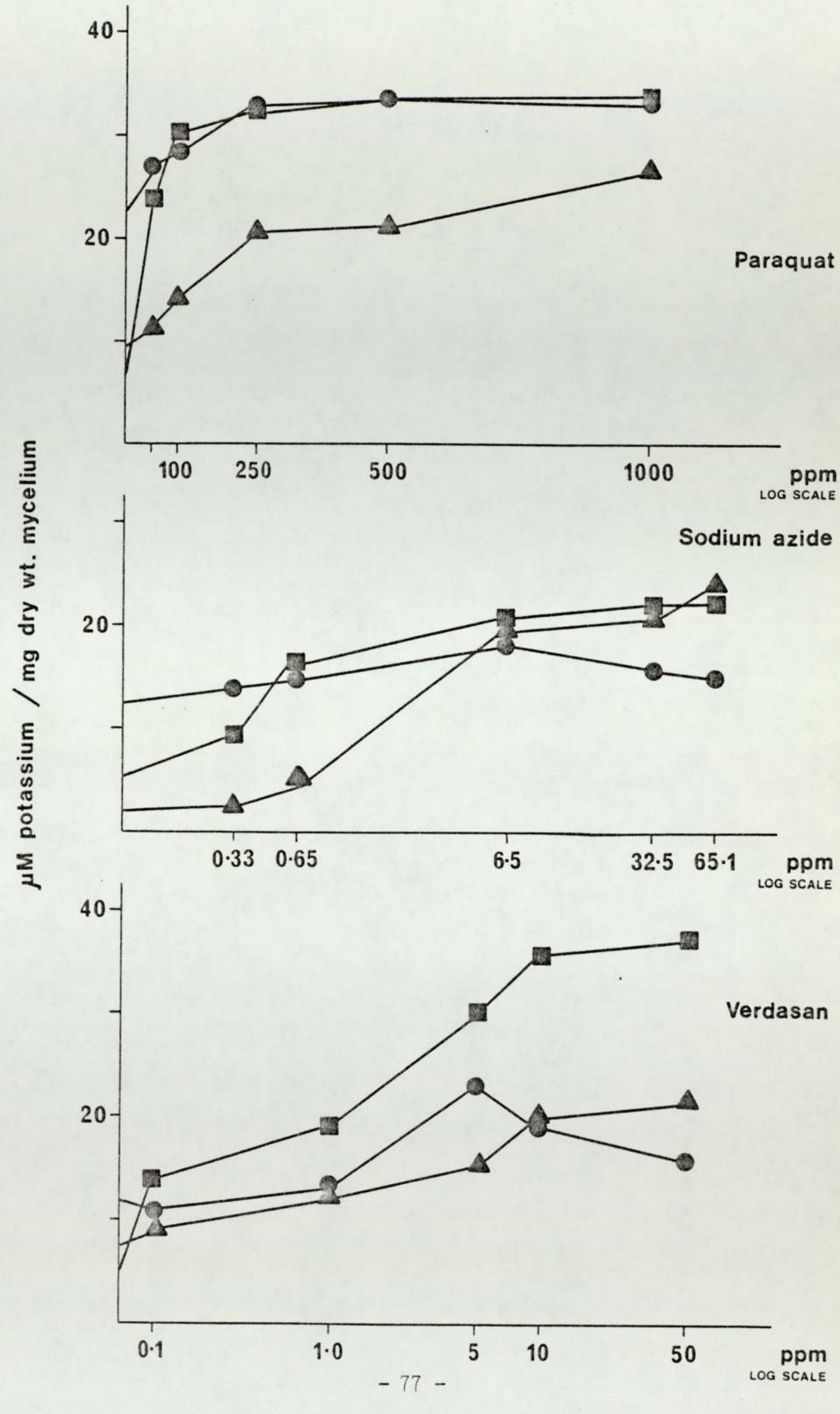
In the presence of SODIUM AZIDE, *B. variegatus* and *C. stercoreus* showed similar patterns of leakage, with the amounts

Fig.16. The effects of PARAQUAT, SODIUM AZIDE and VERDASAN on the leakage of potassium from Basidiomycetes.

●—● Basidiomycete 235M

■—■ *Boletus variegatus*

▲—▲ *Cyathus stercoreus*


40
20
Paraquat
100
250
500
1000
ppm
LOG SCALE
μM potassium / mg dry wt. mycelium
Sodium azide
20
0·33
0·65
6·5
32·5
65·1
ppm
LOG SCALE
40
Verdasan
20
0·1
1·0
5
10
50
ppm
LOG SCALE

reaching a maximum at around 6.51 ppm , and tending to remain constant at the higher concentrations of this chemical.

The Effect of Increasing Concentration of Agrochemicals on Phosphate Leakage

The results (Fig. 17) indicate that the net loss of phosphate from the three fungi in the presence of PARAQUAT and VERDASAN was significant, and the amounts leaked increased with increasing chemical concentration. However, while increasing SODIUM AZIDE concentration significantly increased the amount of phosphate lost from *Boletus variegatus* and *Cyathus stercoreus*, there was no such effect with Basidiomycete *235M* .

In the presence of PARAQUAT and VERDASAN, *235M* and *C. stercoreus* showed similar patterns of leakage, with only small increases over each concentration range: *B. variegatus*, however, exhibited much greater increases in the amounts of phosphate leaked.

Effect of Increasing Concentrations of Agrochemicals on Amino Acids Leakage

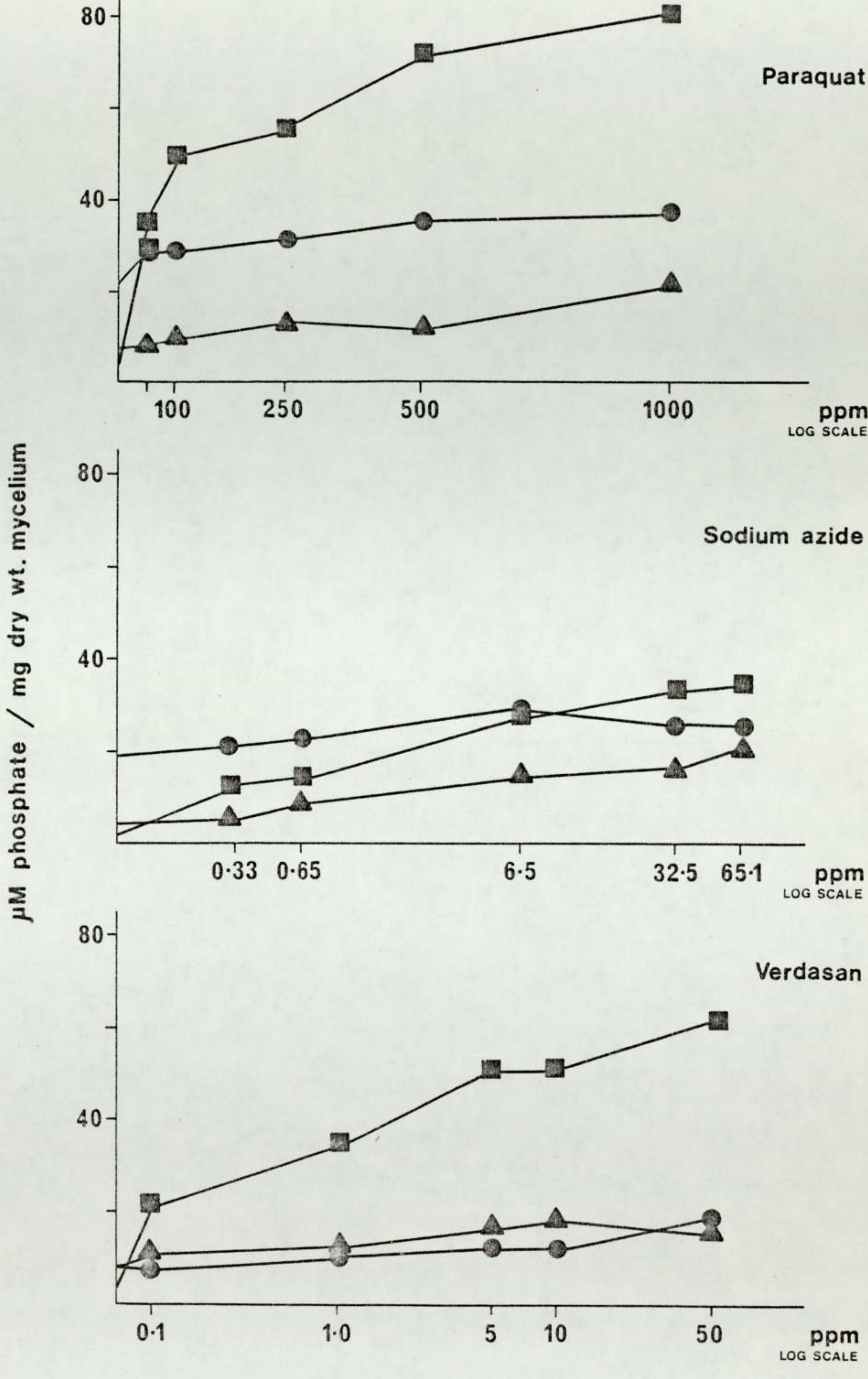
The results indicate (Fig. 18) that the net loss of amino acids from the three fungi in the presence of PARAQUAT, SODIUM AZIDE and VERDASAN was significant, and in all cases the amounts leaked increased with increasing concentration. Compared to the CONTROL, Basidiomycete *235M* generally lost the least amount of amino acids in the presence of the three chemicals, and *Boletus variegatus* the greatest.

Fig.17. The effects of PARAQUAT, SODIUM AZIDE and VERDASAN on the leakage of phosphate from Basidiomycetes.

●—● Basidiomycete 235M

■—■ *Boletus variegatus*

▲—▲ *Cyathus stercoreus*



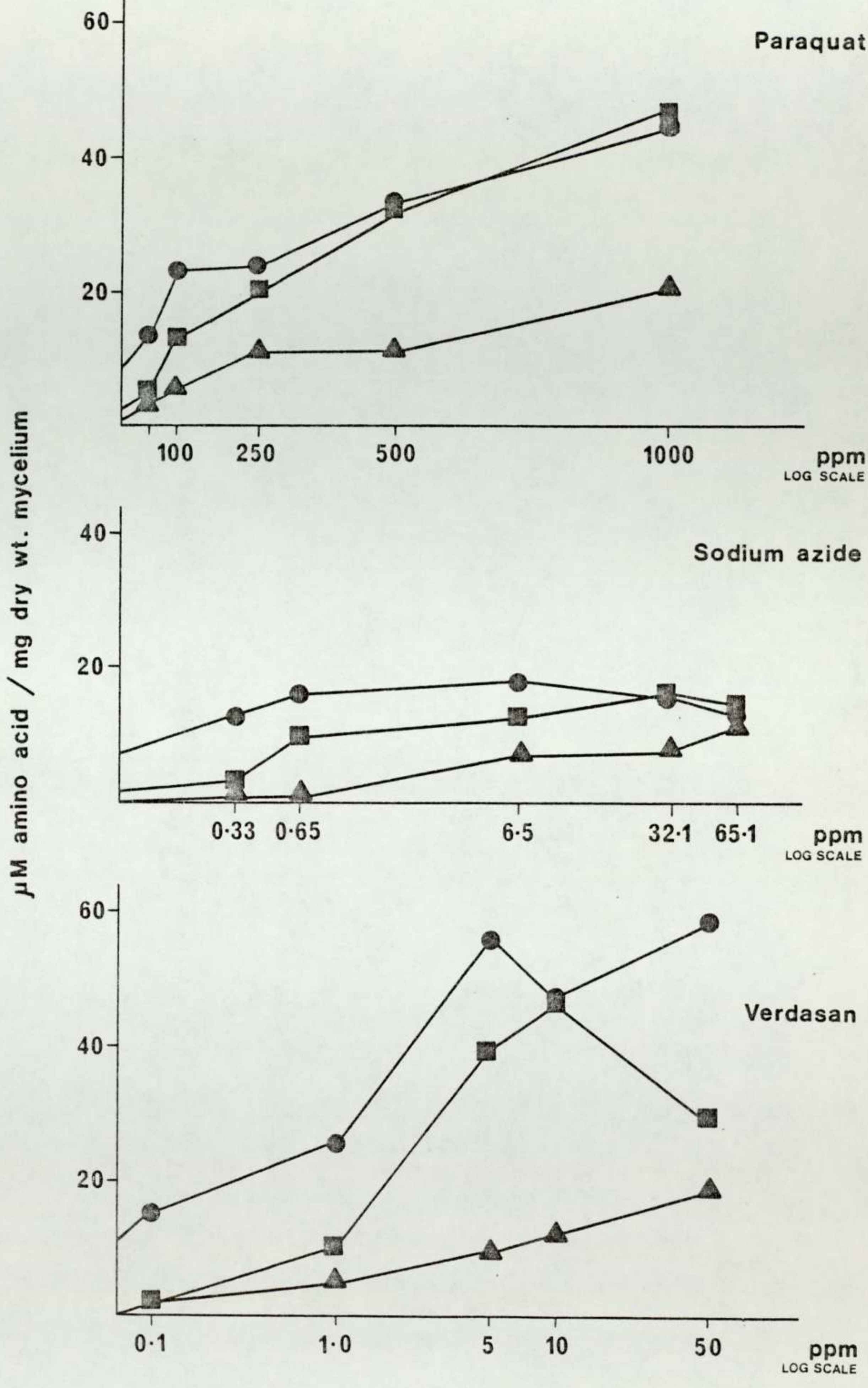
80
40
Paraquat
100
250
500
1000
ppm
LOG SCALE
µM phosphate / mg dry wt. mycelium
80
40
Sodium azide
0·33
0·65
6·5
32·5
65·1
ppm
LOG SCALE
80
40
Verdasan
0·1
1·0
5
10
50
ppm
LOG SCALE

Fig.18. The effects of PARAQUAT, SODIUM AZIDE and VERDASAN on the leakage of amino acids from Basidiomycetes.

●—● Basidiomycete 235M

■—■ *Boletus variegatus*

▲—▲ *Cyathus stercoreus*



60
40
20
Paraquat
100
250
500
1000
ppm
LOG SCALE
μM amino acid / mg dry wt. mycelium
40
20
Sodium azide
0·33
0·65
6·5
32·1
65·1
ppm
LOG SCALE
60
40
20
Verdasan
0·1
1·0
5
10
50
ppm
LOG SCALE

Results

Time Course of Potassium Leakage

The effects of PARAQUAT, SODIUM AZIDE and VERDASAN on the net loss of potassium over the 24 hour incubation period from Basidiomycete 235M, Boletus variegatus and Cyathus stercoreus are shown in Figs. 19, 20 and 21, and Appendix 14.

In CONTROL flasks all three species leaked some potassium, and with B. variegatus the amounts released increased steadily for 8 hours, after which no further significant leakage occurred. This pattern was also shown by C. stercoreus. With 235M, potassium was lost at a steady rate, a maximum being reached after 4 hours. Significantly less potassium was leaked from 235M after 24 hours than at the maximum point.

The three chemicals caused a significant increase in the net loss of potassium from B. variegatus and C. stercoreus ($p < 0.001$). However, while the amounts lost from 235M in the presence of VERDASAN were also significantly increased when compared with CONTROL ($p < 0.001$), no such effect was seen with either PARAQUAT or SODIUM AZIDE (Figs. 19, 20 and 21).

Significant amounts of potassium ($p < 0.05$) were lost from B. variegatus and C. stercoreus after only 1 hour in the presence of the three chemicals, as compared with CONTROL. However, the amounts lost from 235M only became significant after 8 hours with VERDASAN, and continued to be significant to the end of the incubation period.

The amounts of potassium leaked from B. variegatus in the presence of PARAQUAT and VERDASAN (Figs 19 and 21) increased rapidly for 2 hours, but thereafter slowed down. Maximum potassium loss

was recorded after 18 hours in the presence of these two agrochemicals. With SODIUM AZIDE (Fig 20) potassium was lost rapidly for 4 hours, at which time a maximum was reached. There was no further change in the amounts of potassium leaked for the next 14 hours, although after 24 hours significantly less ($p < 0.05$) was recorded than at the maximum point.

The loss of potassium from C. stercoreus in the presence of the three chemicals showed similar patterns to those of B. variegatus. Maximum loss occurred after 18 hours in the presence of both PARAQUAT and VERDASAN. However, with SODIUM AZIDE, the amounts lost increased steadily, with maximum amounts being recorded at the end of the incubation period.

With VERDASAN, the amounts of potassium lost from 235M for the first 2 hours were comparable with the CONTROL. In the treated flasks, maximum potassium loss occurred after 8 hours, and thereafter decreased up until the end of the experiment, where amounts recorded were significantly less than the maximum.

The amounts of potassium leaked from 235M in the presence of PARAQUAT and VERDASAN were not significantly different from the CONTROL over the 24 hour incubation period, and in VERDASAN the amounts lost for the first 2 hours were also comparable with the CONTROL. However, in VERDASAN-treated flasks a significant amount of potassium was lost after 8 hours when compared with the CONTROL, but thereafter the amounts recorded decreased up until the end of the experiment, where significantly less amounts were recorded than after 8 hours.

Fig.19. Time course of potassium leakage from Basidiomycetes in the presence of PARAQUAT.

●—● Basidiomycete 235M (treatment)
○—○ Basidiomycete 235M (CONTROL)
■—■ *Boletus variegatus* (treatment)
□—□ *Boletus variegatus* (CONTROL)
▲—▲ *Cyathus stercoreus* (treatment)
△—△ *Cyathus stercoreus* (CONTROL)

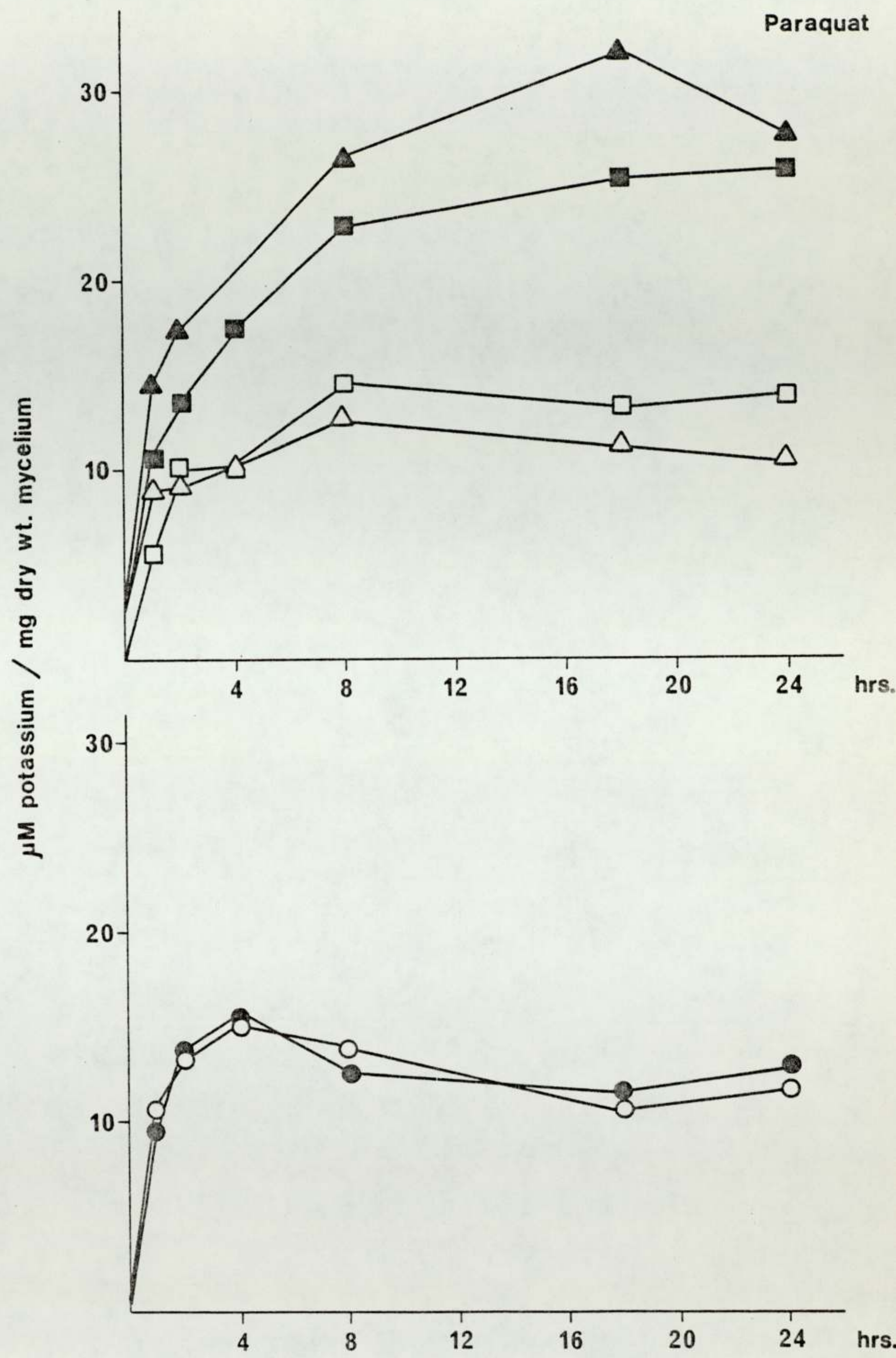
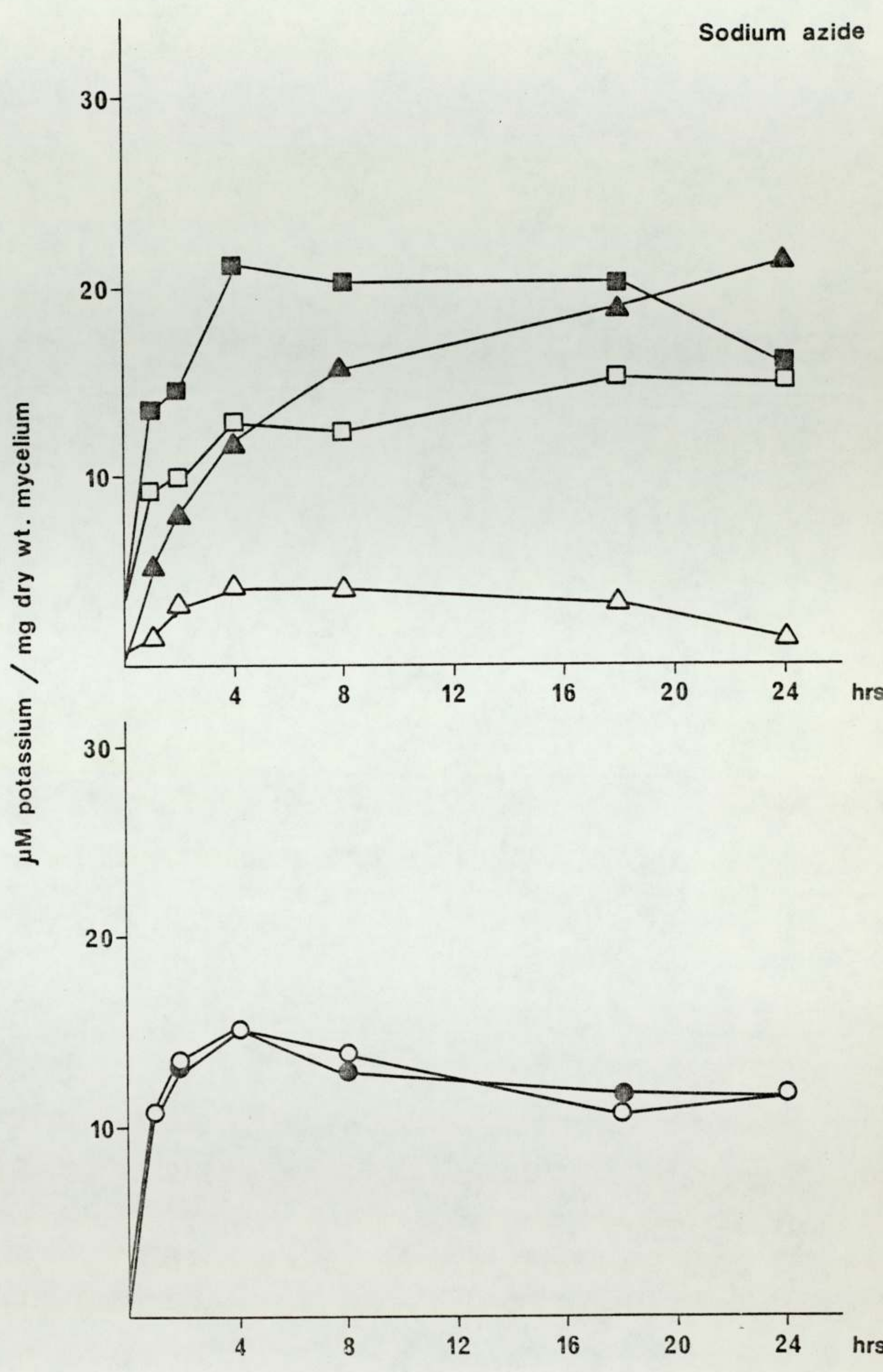

Paraquat
30
20
10
μM potassium / mg dry wt. mycelium
4
8
12
16
20
24
hrs.
30
20
10
4
8
12
16
20
24
hrs.

Fig.20. Time course of potassium leakage from Basidiomycetes in the presence of SODIUM AZIDE.

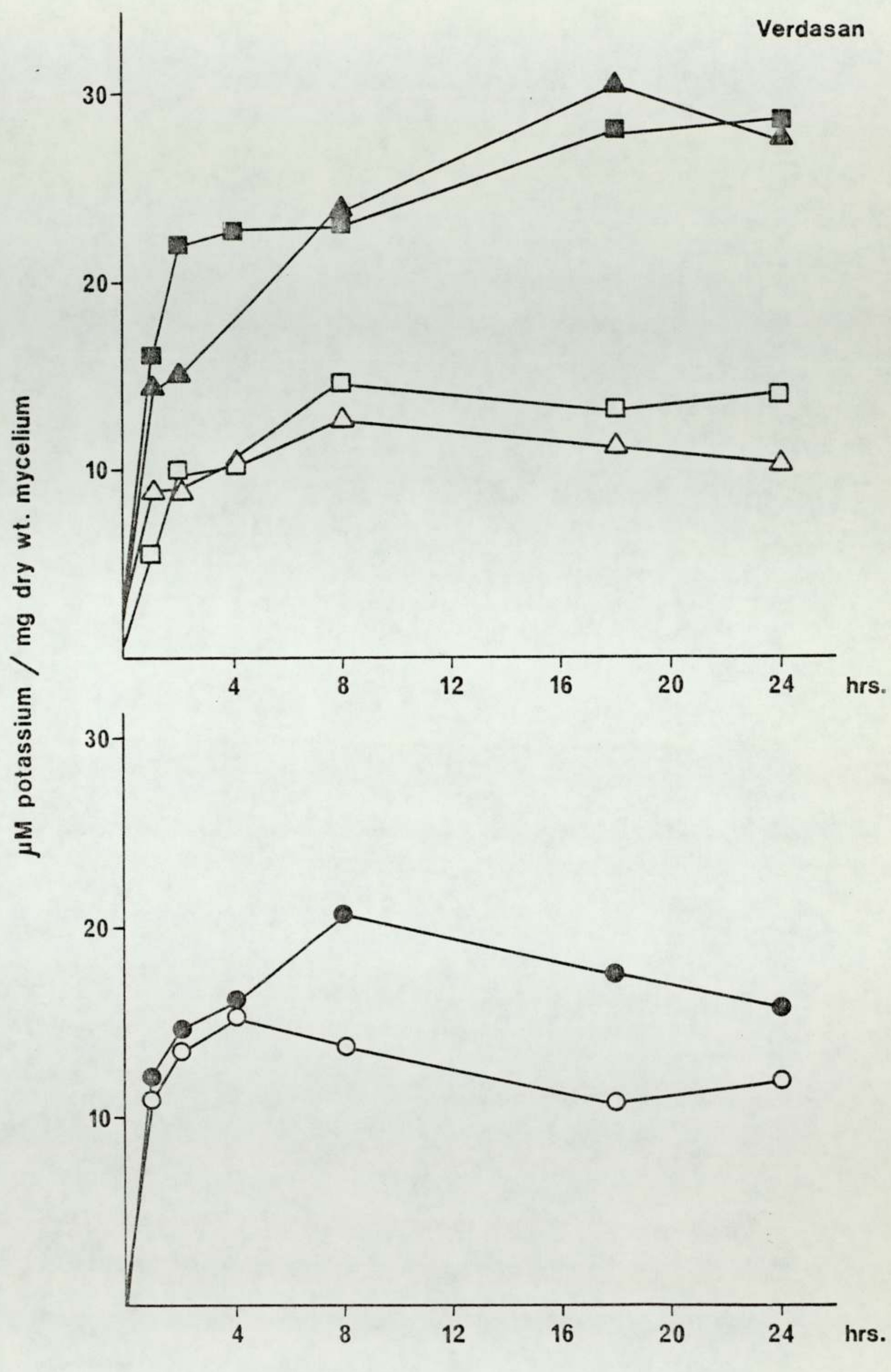
●—● Basidiomycete 235M (treatment)
○—○ Basidiomycete 235M (CONTROL)
■—■ *Boletus variegatus* (treatment)
□—□ *Boletus variegatus* (CONTROL)
▲—▲ *Cyathus stercoreus* (treatment)
△—△ *Cyathus stercoreus* (CONTROL)



Sodium azide
μM potassium / mg dry wt. mycelium
30
20
10
4
8
12
16
20
24
hrs
30
20
10
4
8
12
16
20
24
hrs

Fig.21. Time course of potassium leakage from Basidiomycetes in the presence of VERDASAN.

●—● Basidiomycete 235M (treatment)
○—○ Basidiomycete 235M (CONTROL)
■—■ *Boletus variegatus* (treatment)
□—□ *Boletus variegatus* (CONTROL)
▲—▲ *Cyathus stercoreus* (treatment)
△—△ *Cyathus stercoreus* (CONTROL)


Verdasan
30
20
10
μM potassium / mg dry wt. mycelium
4
8
12
16
20
24
hrs.
30
20
10
4
8
12
16
20
24
hrs.

Time Course of Phosphate Leakage

The effects of PARAQUAT, SODIUM AZIDE and VERDASAN on the net loss of phosphate over the 24 hour incubation period from Basidiomycete 235M, Boletus variegatus and Cyathus stercoreus are shown in Figs. 22, 23 and 24, and Appendix 14.

In CONTROL flasks, all three species leaked some phosphate, and the amount leaked from B. variegatus was generally greater than from either 235M or C. stercoreus. The amounts of phosphate leaked increased with time, and in all three species a maximum was reached, after which a decreasing amount of phosphate was recorded.

The three chemicals caused a significant increase ($p < 0.001$) in the net loss of phosphate from the three species compared with CONTROL (Figs. 22, 23, 24). With PARAQUAT, significant amounts ($p < 0.05$) of phosphate were leaked after 2 hours with B. variegatus and 235M, and after 4 hours with C. stercoreus. In the presence of SODIUM AZIDE, significant amounts were leaked from 235M after 2 hours, and after 4 hours from B. variegatus and C. stercoreus. However, VERDASAN had more immediate effects, since significant amounts were leaked from B. variegatus after 1 hour, and from 235M and C.stercoreus after 2 hours.

The amounts of phosphate leaked from B. variegatus in the presence of PARAQUAT and VERDASAN increased rapidly for about 4 hours, and although the rate of release slowed thereafter, the amounts released were increasing significantly ($p < 0.05$) up until the end of the incubation period. With SODIUM AZIDE, phosphate was released linearly with time for 8 hours, after which little or no further loss occurred.

The amounts of phosphate released from _Cyathus stercoreus_ in the presence of PARAQUAT and VERDASAN increased rapidly for 4 hours but thereafter slowed down. Maximum phosphate loss was recorded after 18 hours in VERDASAN and there was no significant change thereafter. With PARAQUAT, however, the amounts released increased until the end of the incubation period. In the presence of SODIUM AZIDE, phosphate loss progressed steadily for 18 hours, after which no significant further loss occurred. The amounts of phosphate released by _235M_ showed similar patterns in the presence of PARAQUAT and SODIUM AZIDE : the amounts released reached a maximum after 4 hours, and after 18 hours significantly less phosphate was recorded in treated flasks.

Fig.22. Time course of phosphate leakage from Basidiomycetes in the presence of PARAQUAT.

●—● Basidiomycete 235M (treatment)
○—○ Basidiomycete 235M (CONTROL)
■—■ *Boletus variegatus* (treatment)
□—□ *Boletus variegatus* (CONTROL)
▲—▲ *Cyathus stercoreus* (treatment)
△—△ *Cyathus stercoreus* (CONTROL)

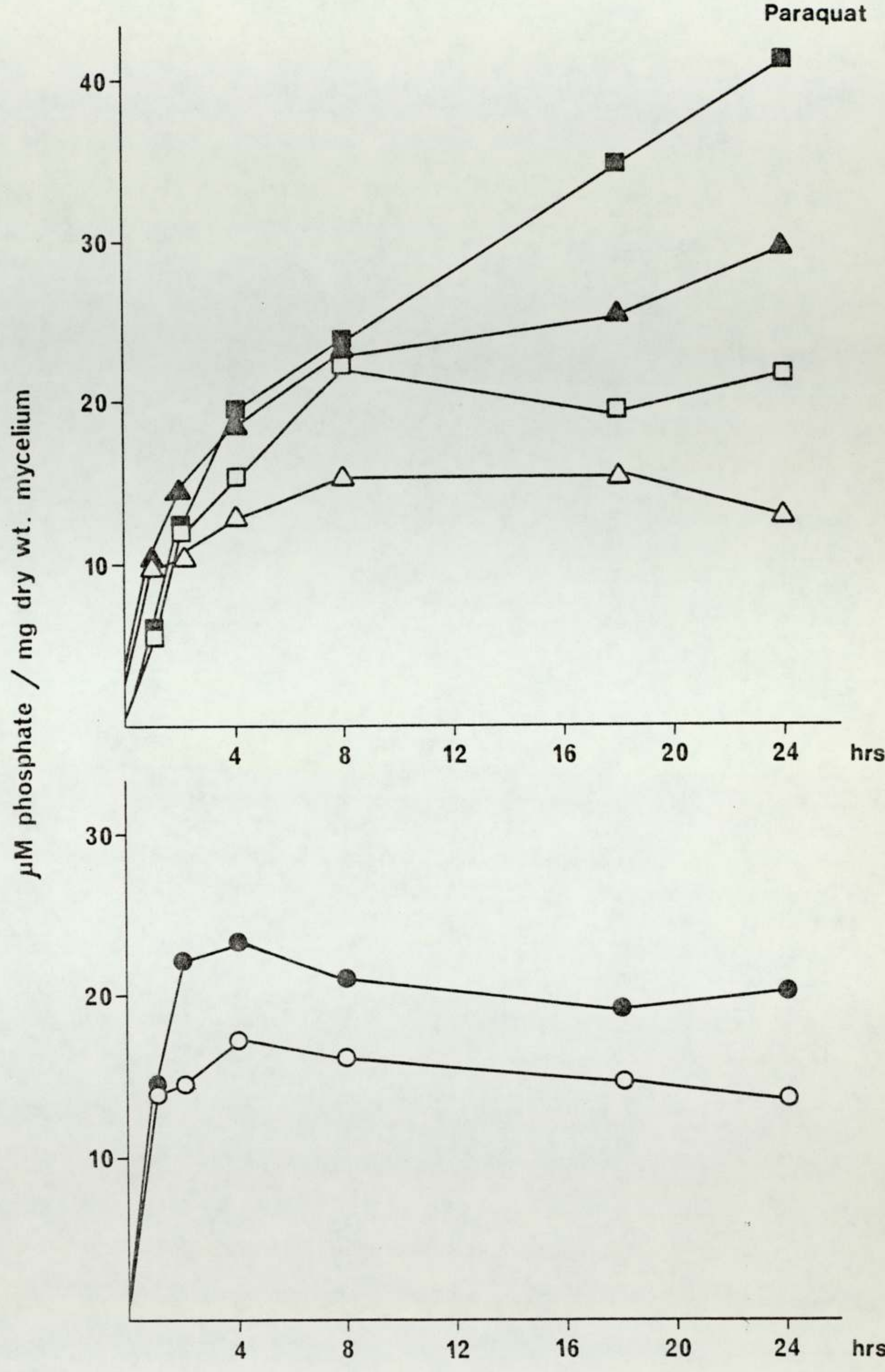
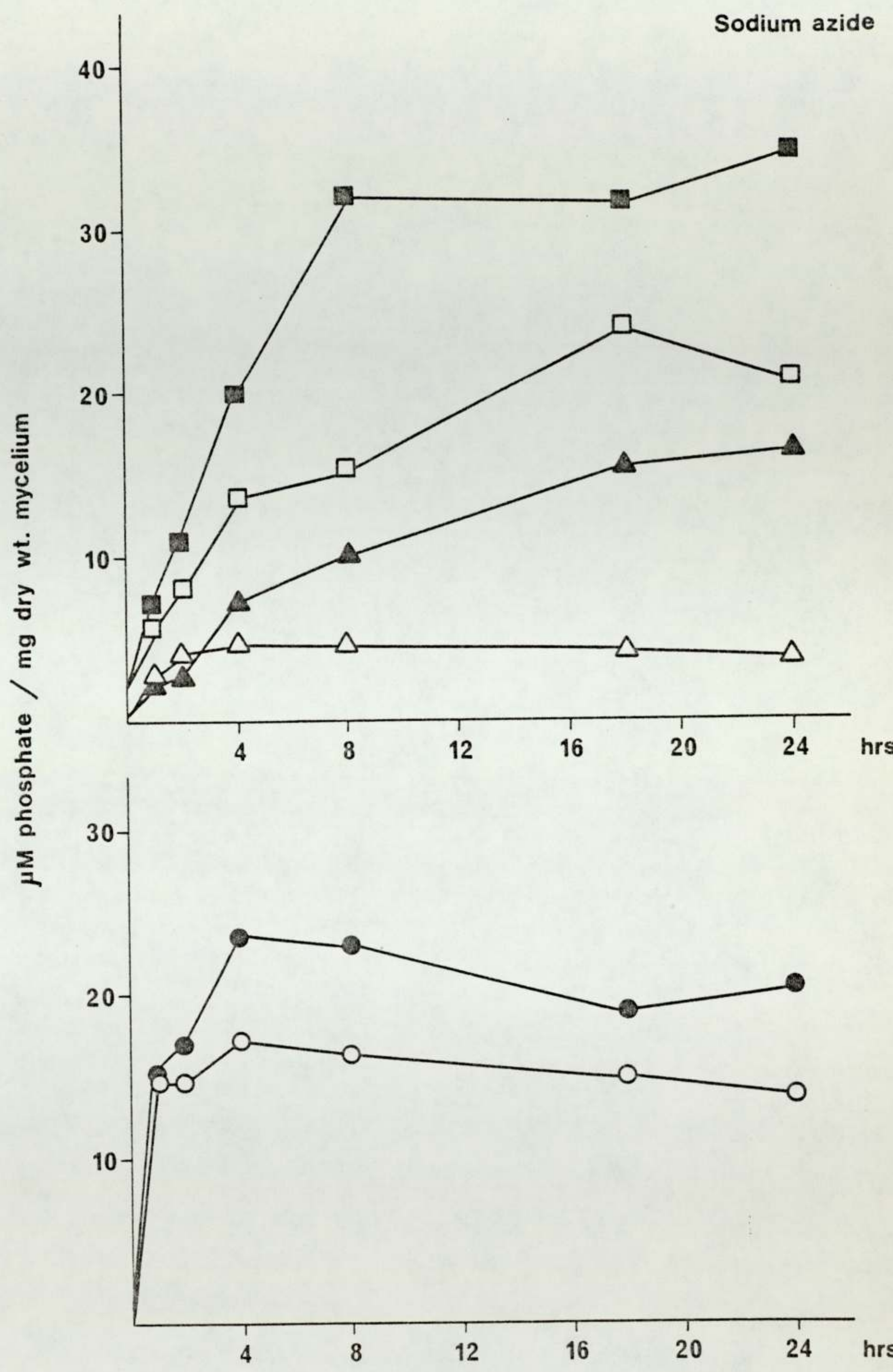

Paraquat
40
30
20
10
4
8
12
16
20
24
hrs
μM phosphate / mg dry wt. mycelium
30
20
10
4
8
12
16
20
24
hrs

Fig.23. Time course of phosphate leakage from Basidiomycetes in the presence of SODIUM AZIDE.

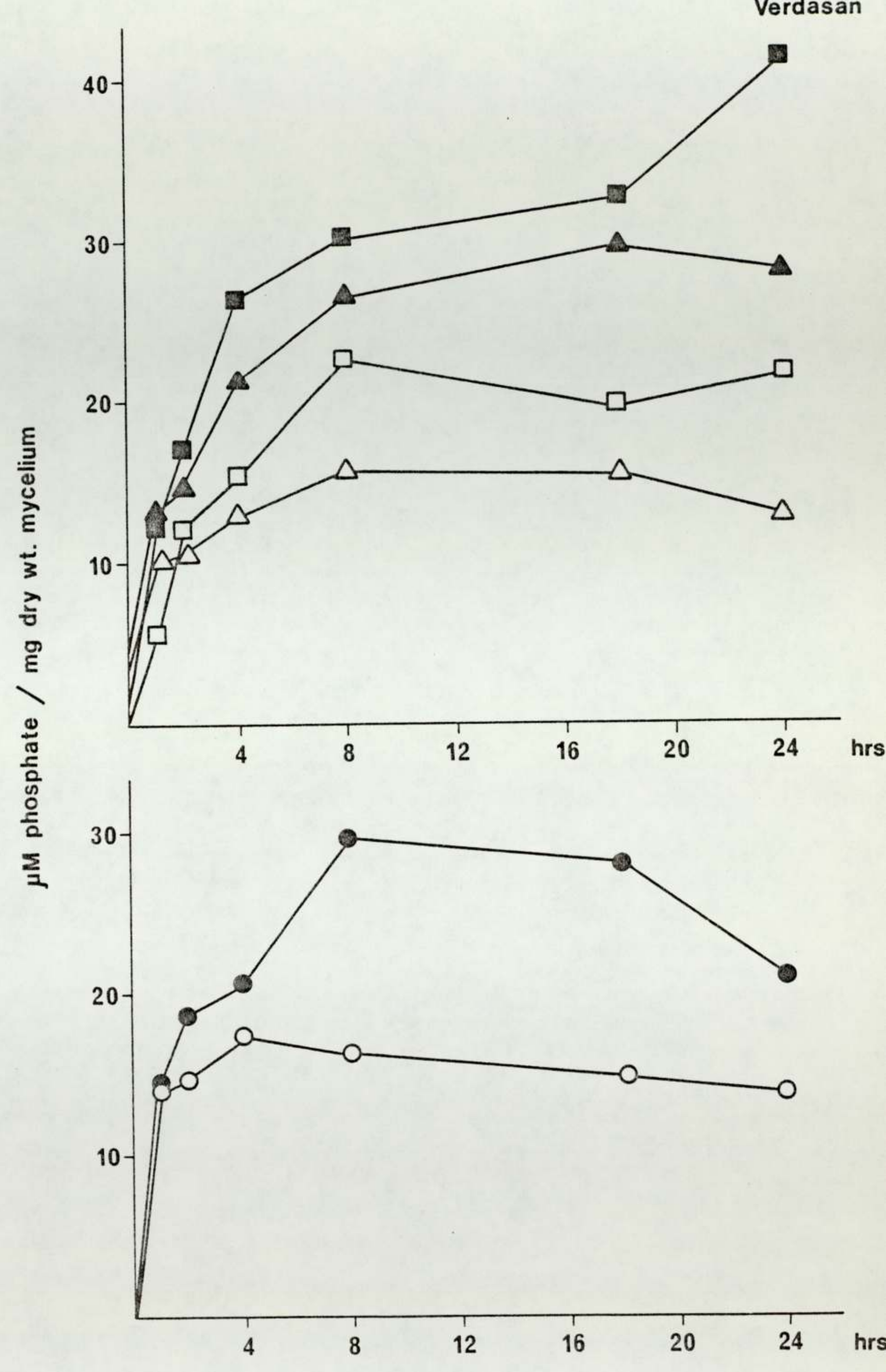
●—● Basidiomycete 235M (treatment)
○—○ Basidiomycete 235M (CONTROL)
■—■ *Boletus variegatus* (treatment)
□—□ *Boletus variegatus* (CONTROL)
▲—▲ *Cyathus stercoreus* (treatment)
△—△ *Cyathus stercoreus* (CONTROL)



Sodium azide
40
30
20
10
4
8
12
16
20
24
hrs
μM phosphate / mg dry wt. mycelium
30
20
10
4
8
12
16
20
24
hrs

Fig.24. Time course of phosphate leakage from Basidiomycetes in the presence of VERDASAN.

●—● Basidiomycete 235M (treatment)
○—○ Basidiomycete 235M (CONTROL)
■—■ *Boletus variegatus* (treatment)
□—□ *Boletus variegatus* (CONTROL)
▲—▲ *Cyathus stercoreus* (treatment)
△—△ *Cyathus stercoreus* (CONTROL)


Verdasan
40
30
20
10
4
8
12
16
20
24
hrs
μM phosphate / mg dry wt. mycelium
30
20
10
4
8
12
16
20
24
hrs

Time Course of Amino Acid Leakage

The effects of PARAQUAT, SODIUM AZIDE and VERDASAN on the net loss of amino acids over the 24 hour incubation period from Basidiomycete 235M, Boletus variegatus and Cyathus stercoreus are shown in Figs. 25, 26 and 27, and Appendix 14.

In CONTROL flasks, all three species leaked some amino acids, and generally similar amounts were leaked from B. variegatus and C. stercoreus. With both these species, the amounts of amino acids lost increased slowly with time, and a maximum was reached after which no further loss occurred. There was a tendency for less amino acids to be recorded after 24 hours than at the maximum period, although only in the case of B. variegatus was this reduction significant ($p < 0.05$).

The three chemicals caused a significant increase ($p < 0.001$) in the net loss of amino acids from the three species of fungi, compared with CONTROL (Figs. 25, 26 and 27).

A measure of leakage rate of the three fungi in the presence of the chemicals may be made by comparing the times at which the amounts of amino acids leaked became significantly different from CONTROL. With PARAQUAT, significant amounts were lost from all these fungi after 4 hours, and these amounts were significant at subsequent sampling times. With SODIUM AZIDE, significant amounts were lost from Cyathus stercoreus after 2 hours and from 235M and Boletus variegatus after 4 hours. Significant amounts of amino acids leaked fastest from B. variegatus in the presence of VERDASAN after only 1 hour, although these were recorded after 4 hours from C. stercoreus, and after 8 hours from 235M. With Boletus variegatus in the presence

of SODIUM AZIDE, the loss of amino acids progressed at a slower rate than with PARAQUAT for 18 hours, after which little loss occurred. However, in the presence of VERDASAN, amino acid loss progressed rapidly for about 4 hours and thereafter slowed down. After 8 hours no change in the amount lost occurred.

The amounts of amino acids leaked from Cyathus stercoreus in the presence of PARAQUAT increased linearly over the whole time course without a maximum being reached. With SODIUM AZIDE and VERDASAN the pattern of leakage was similar to that with PARAQUAT, except that after 18 hours a maximum was reached.

The pattern of amino acid leakage from 235M was similar in the presence of the three agrochemicals, although the amounts lost in the presence of VERDASAN tended to be higher than in the other two chemicals. Maximum amounts of amino acids were lost after 4 hours with PARAQUAT, and after 8 hours with SODIUM AZIDE and VERDASAN.

Fig.25. Time course of amino acids leakage from Basidiomycetes in the presence of PARAQUAT.

●—● Basidiomycete 235M (treatment)
○—○ Basidiomycete 235M (CONTROL)
■—■ *Boletus variegatus* (treatment)
□—□ *Boletus variegatus* (CONTROL)
▲—▲ *Cyathus stercoreus* (treatment)
△—△ *Cyathus stercoreus* (CONTROL)

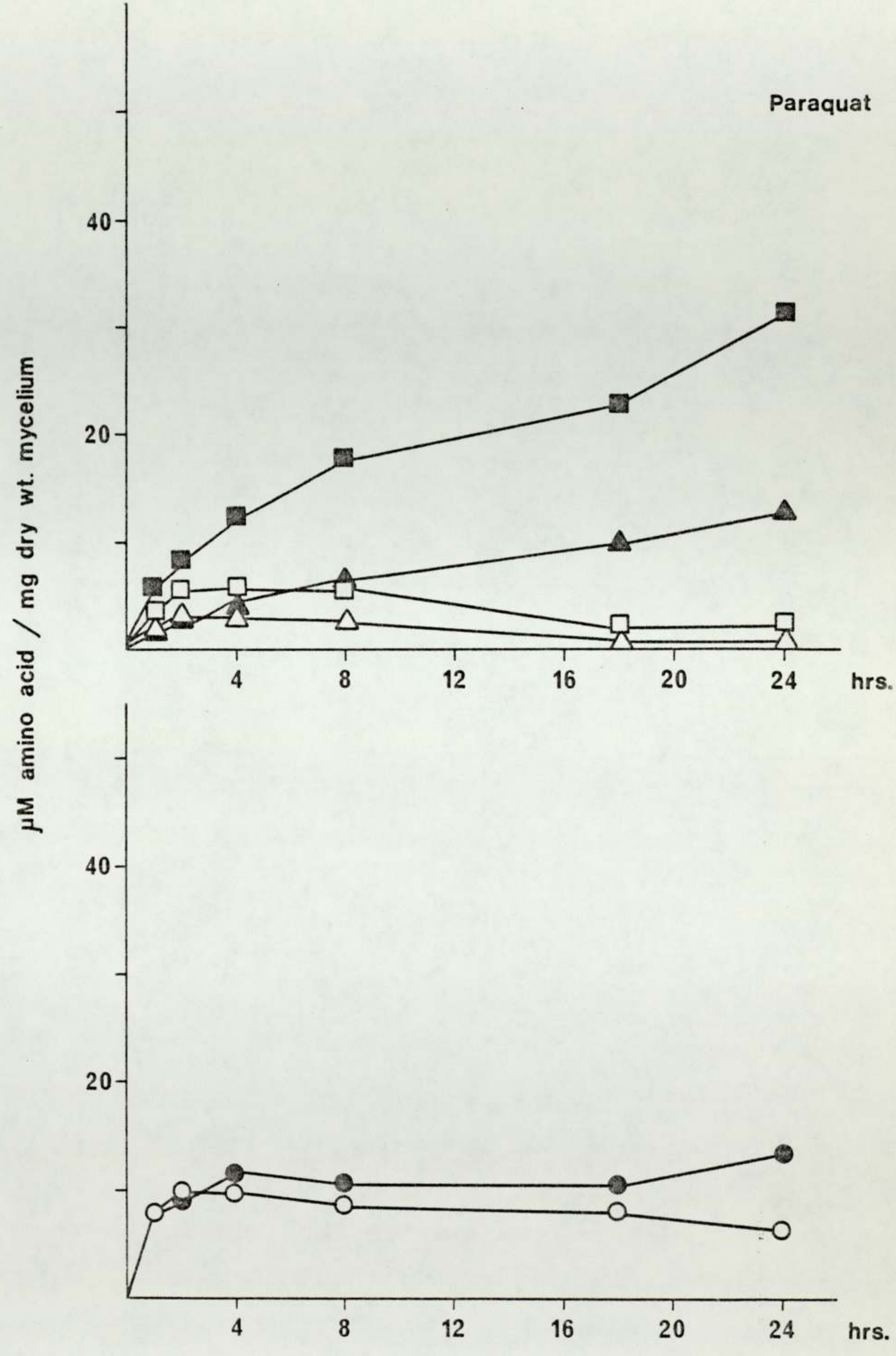

Paraquat
40
20
4
8
12
16
20
24
hrs.
μM amino acid / mg dry wt. mycelium
40
20
4
8
12
16
20
24
hrs.

Fig.26. Time course of amino acids leakage from Basidiomycetes in the presence of SODIUM AZIDE.

●—● Basidiomycete 235M (treatment)
○—○ Basidiomycete 235M (CONTROL)

■—■ *Boletus variegatus* (treatment)
□—□ *Boletus variegatus* (CONTROL)

▲—▲ *Cyathus stercoreus* (treatment)
△—△ *Cyathus stercoreus* (CONTROL)

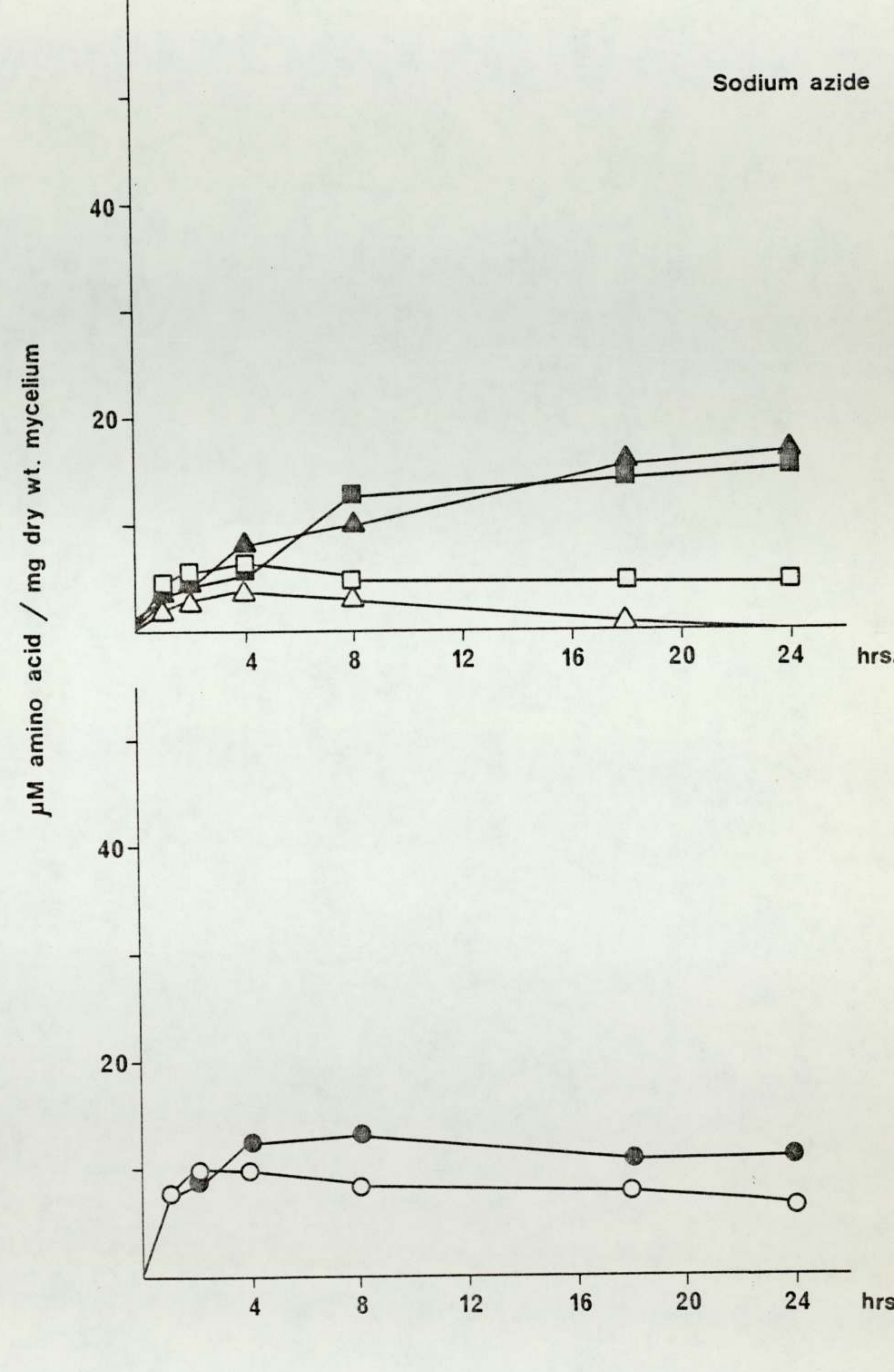
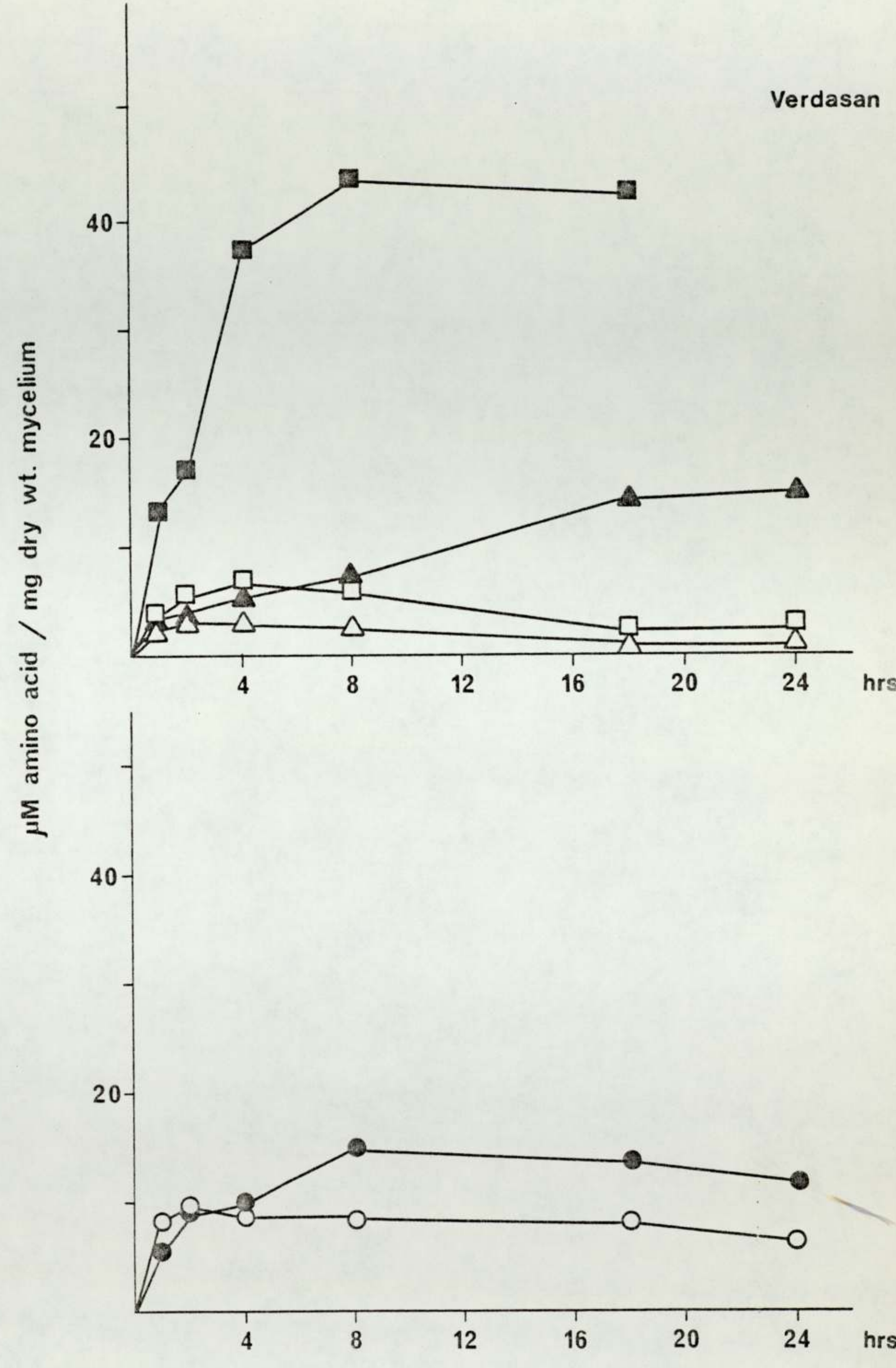

Sodium azide
µM amino acid / mg dry wt. mycelium
40
20
4
8
12
16
20
24
hrs.
40
20
4
8
12
16
20
24
hrs

Fig.27. Time course of amino acids leakage from Basidiomycetes in the presence of VERDASAN.

●—● Basidiomycete <u>235M</u> (treatment)
○—○ Basidiomycete <u>235M</u> (CONTROL)
■—■ <u>Boletus variegatus</u> (treatment)
□—□ <u>Boletus variegatus</u> (CONTROL)
▲—▲ <u>Cyathus stercoreus</u> (treatment)
△—△ <u>Cyathus stercoreus</u> (CONTROL)


Verdasan
40
20
4
8
12
16
20
24
hrs.
μM amino acid / mg dry wt. mycelium
40
20
4
8
12
16
20
24
hrs.

Discussion

The fungi isolated in the presence of the three agrochemicals demonstrated leakage of metabolites. Basidiomycete 235M, which showed greater tolerance of both PARAQUAT and VERDASAN in growth studies (Chapter 3) than either Boletus variegatus or Cyathus stercoreus, also displayed lower rates of leakage.

Compared with the CONTROL, the net losses of potassium, phosphate, and amino acids from 235M in the presence of PARAQUAT and VERDASAN were less than from the other two species. In addition, the susceptible B. variegatus showed the greatest leakage of phosphate and amino acids in the presence of the three chemicals over the concentration range tested, although the amounts of potassium released were not so drastically altered (Figs. 16, 17, and 18).

The rate of release of significant amounts of the individual metabolites also showed some indication of the tolerance of the fungi to the agrochemicals: the amounts of potassium which leaked from C. stercoreus and B. variegatus in the presence of PARAQUAT and VERDASAN became significant after only 1 hour of incubation (Figs.19 and 20). However, with 235M in the presence of PARAQUAT, there was no change in the net loss of potassium with time, and in VERDASAN a significant loss of potassium was recorded only after 8 hours and from then until the end of the incubation period.

A similar pattern was observed with the loss of amino acids by the three fungi in the presence of VERDASAN (Fig.27): the most susceptible species, B. variegatus, showed a significant net loss after only 1 hour of incubation and at subsequent times, while the more tolerant C. stercoreus and 235M showed a significant loss after

4 hours and 8 hours, respectively.

As an indication of tolerance to the agrochemicals, the rate of phosphate release exhibited no clear patterns within the three species of fungi, although significant amounts of phosphate were lost twice as fast from Boletus variegatus than from both 235M and Cyathus stercoreus in the presence of PARAQUAT and SODIUM AZIDE (Figs.22 and 23).

The primary mode of action of PARAQUAT (a bipyridylium herbicide) in green plants is a result of its success in competing with nicotinamide adenine dinucleotide phosphate (NADP) for electrons emanating from photosystem I of the photosynthetic electron transport pathway within the chloroplasts (Akhavein and Linscott, 1968). A number of rapid physiological and structural changes were shown to occur after plants were treated with PARAQUAT: an increase in membrane permeability occurred, resulting in the loss of potassium ions (Harris and Dodge, 1972a), and the production of malondialdehyde, a breakdown product of unsaturated fatty acids associated with the lipid element of membranes (Dodge, 1971). Later, Harris and Dodge (1972b) noted that these changes were correlated with ultrastructural changes in flax cotyledon cells which included rupture of the tonoplast, rapid disintegration of chloroplasts and the swelling and rupturing of mitochondria. They postulated that due to the rapidity of the structural breakdown, other disruptive processes besides the disruption of the photosynthetic chain were operating. The rapid breakdown of structure was more likely to be due to the production of free radicals or hydrogen peroxide, which subsequently reacted with the membranes causing loss of cell compartmentalisation (Harris and

Dodge, 1972b).

Another mechanism of PARAQUAT action was suggested by Stokes and Turner (1971) who observed a rapid rise in oxygen uptake by Chlorella incubated in the dark in the presence of another bipyridyl herbicide, diquat. They postulated that diquat uncoupled oxidative phosphorylation with the subsequent release of hydrogen peroxide. They also noted that a build up of citric acid occurred, due to a block in the tricarboxylic acid cycle and α-ketoglutarate which was probably caused by hydrogen peroxide production.

If free radical and hydrogen peroxide production occurs in fungi it would probably follow this mechanism (Smith, 1976). In the present study, the release of cellular metabolites by the three fungi in the presence of PARAQUAT may have been due to this mechanism. However, Smith (1976) found that out of four Mucoraceous fungi tested, the leakage of potassium was correlated with malondialdehyde production and therefore free radical and hydrogen peroxide production in only one species.

As well as inhibiting metabolic phosphorylation, mercury based compounds are also known to affect the fungal membrane. The membrane defect produced by mercury results from a direct interaction of the metal with membrane ligands (Rothstein, 1959). Mercury reacts with any sulphydryl group in the membrane structure, probably by the formation of -S-Hg-S- bridges. The formation of such bridges constitutes a molecular stress, and when this stress reaches a threshold level for a given cell a generalised breakdown of the membrane results, with the release of cellular constituents. The first sites of action will be at the surface membranes of the cell, followed, as

the metal diffuses inwards, by reactions within the interior of the cell (Rothstein, 1959).

The mechanisms of metabolite transport across living membranes have also been studied. The uptake of potassium in fungi was recognised by Slayman (1970) to be effected by a potassium-sodium-hydrogen ion pump. The size of the potential and the rapidity with which it is reduced by metabolic inhibitors suggests that the pump is driven by a metabolic process (Slayman and Tatum, 1965). A close relationship between the size of the potential and the level of adenosine triphosphate (ATP) in Neurospora was noted by Slayman (1970).

SODIUM AZIDE has long been recognised as a metabolic inhibitor. Its activity can be ascribed to the inhibition of nuclear phosphorylation (Mettler, 1972). Phosphate transport across membranes appears to require the provision of metabolite energy by the fungus. Harley et al. (1956) showed that phosphate uptake in beech mycorrhizas was greatly diminished by sodium azide.

Respiratory energy is also required for the active transport of amino acids across cell membranes. It was shown that the accumulation of amino acids is greatly reduced by the two metabolic inhibitors dinitrophenol (DNP) and sodium azide in Botrytis (Jones and Watson, 1962; Jones, 1963) and in Neurospora (DeBusk and DeBusk, 1965). In the later case considerable loss of amino acids was seen to occur.

Thus, the patterns of metabolite loss from the three fungi under investigation can be seen to be correlated with the modes of action of the different agrochemicals: the leakage of potassium,

phosphate, and amino acids from the fungi in the presence of SODIUM AZIDE may be due to a reduction in ATP production. The pattern of leakage over an increasing concentration range suggests that no gross lysis of cells occurs, since the net amounts released remain constant after a maximum concentration effect has been reached. As the levels of ATP drop, the metabolites will diffuse down a concentration gradient into the medium and, when all available ATP has been utilised, will not be taken up by the transport system.

This may also be the mechanism operating with PARAQUAT. Stokes and Turner (1971) have shown that the bipyridyl herbicides uncouple oxidative phosphorylation, depriving the treated organisms of ATP. However, the far greater effects observed with this herbicide compared with SODIUM AZIDE suggests that the membrane is either directly or indirectly disrupted, which may lead to gross cell lysis at the higher concentrations.

The direct action of VERDASAN on the cell membranes resulted in a very rapid loss of metabolites which was apparent in the two species, Cyathus stercoreus and Boletus variegatus, which showed little tolerance of this fungicide in growth studies, compared with 235M. The rate of release of potassium and amino acids from 235M in the presence of VERDASAN was much slower than with the other two species.

Thus, the loss of viability of these fungi in the presence of high concentrations of agrochemicals may be correlated with gross leakage of cellular metabolites. Even at lower concentrations, at which the fungi initially remain viable, slight membrane damage could lead to a loss of important growth products with a concomitant

change in intracellular pH which may have deleterious effects on the cell.

Further work is needed in this area, particularly to explain the differences in the reaction of the susceptible and tolerant species to the agrochemicals. Smith and Lyon (1976) showed that susceptibility of two Mucoraceous species of fungi, *Mucor hiemalis* and *Zygorhynchus moelleri*, to Paraquat was greater than that of two Imperfect forms, *Aspergillus niger* and *Penicillium frequentans*, and noted that this susceptibility was strongly correlated with the accumulation of the herbicide in the mycelium. Susceptibility to Paraquat may therefore be connected with permeability of the membranes. Smith, Lyon and Ismail B. Sahid (1976) also showed that these two groups of fungi differed in the rate at which Paraquat was broken down within the mycelium, the Imperfect species breaking down Paraquat faster than the Mucoraceous species.

Similar work is needed with mycorrhizal and non-mycorrhizal Basidiomycetes, along with studies on the rates at which toxic compounds are taken up into their mycelium, in order to explain the differences in the susceptibility of these two groups to agrochemicals.

CHAPTER V

EFFECT OF VERDASAN ON BASIDIOMYCETE 235M C_x CELLULASE ACTIVITY

Effect of VERDASAN on Basidiomycete 235M

C_x Cellulase Activity

Introduction

Previous research (Reese *et al.* 1950; Selby and Maitland, 1967; Boretti *et al.*, 1973) indicates that cellulases are a complex of enzymes with various activities: the C_1 component, whose action is still unclear, is unspecified and is required for the hydrolysis of native cellulose; the C_x components, also necessary to initiate attack on native cellulose, are required for short chain degradation; and the β-glucosidases act on cellobiose and short chains to form glucose (Reese, 1975). The results of Wood (1975) suggest that crystalline cellulose is effectively rendered soluble by the synergistic action of the C_1 and C_x enzymes, and not by C_1 alone as was originally reported by Reese *et al.* (1950).

A wide variety of techniques have been described to assay the cellulolytic ability of micro-organisms. Halliwell (1963) remarked that confusion has arisen over the mechanisms of cellulase production because of the use of numerous cellulosic substrates, ranging from highly complex cotton fibres to simpler soluble compounds such as carboxymethylcellulose (CMC).

Assays for cellulase activity have included: gravimetric determinations on residual cellulose (Lembeck and Colmer, 1967); measurement of clearing zones on cellulose agar (Rautela and Cowling, 1966; Savory *et al.*, 1967); microscopic examination of cellulose particles in agar after growth of a test organism (Walsh and Stewart, 1969); determination of reducing sugars after incubation of

cultures with cellulosic substrates (Kelman and Cowling, 1967; Reese and Mandels, 1963); change in viscosity of CMC (Talboys, 1958); and loss in tensile strength of cotton fibres (Marsh, 1949; Reese and Downing, 1951).

The C_x enzyme may be assayed by reducing sugar production from CMC by a method shown by Gascoigne and Gascoigne (1960). The presence of C_1 and other components of the cellulase system do not affect this assay: it is satisfactory for the quantitative measurement of C_x in pure, mixed and crude preparations of cellulase (Mandels, 1975).

Although the effects of metallic ions (Jermyn, 1952; Reese and Mandels, 1957), oxidising and reducing agents (Basu and Whitaker, 1953; Sison _et al_., 1958), and sugars (Norkrans, 1950; Mandels and Reese, 1957) on cellulase activity in micro-organisms are well documented, the interaction of commonly used agrochemicals with this process has received little attention.

In addition, characterisation of Basidiomycete cellulase has been neglected in the past, the most recent reports being from van Sumère _et al_. (1957) and Mandels and Reese (1960). The importance of these fungi in the degradation of cellulosic substrates makes it desirable to obtain more information on their cellulolytic activity in the presence of agrochemicals, at a time when they are becoming increasingly used in many land practices.

Hence this study was undertaken using Basidiomycete _235M_, using Whatman cellulose powder as an insoluble form of cellulose. This species was isolated from leaf litter of _Agrostis tenuis_, where it may have come into contact with VERDASAN. It was appropriate,

therefore to examine its ability to degrade cellulose in the presence of this organo-mercurial fungicide.

Materials and Methods

Medium for Cellulase Production

The medium for cellulase production was identical to that used by Reese and Mandels (1963) and is shown in Appendix 15. The carbohydrate used as a substrate to induce cellulase production was Whatman cellulose powder (CF 11). 5 mm discs of mycelium of 235M grown on MEA at 25°C and taken from the growing edge of colonies, were transferred into 100 ml Erlenmeyer flasks containing 20 ml of cool medium which had been autoclaved for 15 minutes at 120°C and 15 psi. The flasks were incubated at 25°C for 6 days in a Gallenkamp orbital shaker rotating at 100 rpm. At the end of this period the colonies were removed from their growth medium by suction filtration onto pre-weighed filter papers, dried to constant weight at 90°C, and their dry weight determined.

Effect of Temperature on Cellulase Activity

The method described by Gascoigne and Gascoigne (1960) was used to assay the cellulase activity of the filtrates from 235M. A 1 ml aliquot of enzyme sample was added to test tubes containing 9 ml of a solution containing 1% CMC in 0.055M sodium-citrate buffer of pH 5.4. The tubes were incubated at a range of temperatures from 10°C to 65°C for 24 hours, after which the amount of reducing sugar produced was determined as shown below. Five replicate tubes for each incubation temperature were used.

Effect of pH on Cellulase Activity

The cellulase assay was performed using a range of sodium-citrate buffers of pH 3.5 to 7.5, and at the optimum temperature for

cellulase activity. The tubes were incubated for 24 hours, after which the amount of reducing sugar produced was determined as shown below. Five replicate tubes for each pH were used.

Effect of VERDASAN on Cellulase Activity

The effect of increasing concentrations of VERDASAN on 235M C_x cellulase activity was determined at optimum conditions of temperature and pH. Appropriate amounts of stock solution of VERDASAN were added to the assay mixture to give final concentrations of VERDASAN of 0.1, 0.5, 1.0, 5.0, and 10.0 ppm (a.i.) before the enzyme solution was added. The tubes were incubated for 24 hours, after which the amount of reducing sugar produced was determined as shown below. Five replicate tubes for each concentration of fungicide were used.

Reducing Sugar Determination

Miller's (1959) modification of a technique used by Sumner and Somers (1944) was used. At the end of the incubation period, 1 ml of the enzyme-substrate mixture was immediately added to 3 ml of dinitrosalycilic acid (DNSA) reagent (Appendix 15). This solution was boiled in a water bath for 15 minutes, cooled to room temperature, and its absorbance determined in a Beckman SP500 spectrophotometer at a wavelength of 540 nm. Reducing sugar determinations were determined from a previously constructed standard curve (Appendix 16) prepared from aqueous solutions of D-glucose in the range 0.1 ppm to 1.0 ppm. A second standard curve (Appendix 16) was constructed by adding VERDASAN at a concentration of 50 ppm to the range of glucose solutions in order to determine whether the fungicide, even at greater than the highest concentration used, interfered with the

reducing sugar determination. VERDASAN was shown not to interfere with the assay and so was used in further experiments.

Results

Effect of Temperature on Cellulase Activity

The effect of temperature on Basidiomycete <u>235M</u> C_x activity is shown in Figure 28 and Appendix 17 .

The temperature optimum for activity occurs between 40°C and 50°C Maximum activity with the CMC substrate occurs at 45°C with a 1.3% decrease in total activity at 50°C, and a 37% decrease at 40°C. An incubation temperature of 45°C was used for further cellulase assays.

Effect of pH on Cellulase Activity

The effect of pH on Basidiomycete <u>235M</u> C_x cellulase activity is shown in Figure 29 and Appendix 18 .

The results indicate a pH optimum in the range of pH 5.0 to 5.8. The highest C_x cellulase activity occurred at pH 5.4. There was no activity at pH 4.6 and below. A drop in activity occurred above pH 6.2 with only 32% of the total activity occurring at pH 7.5. A sodium-citrate buffer at the pH optimum of 5.4 was used for further cellulase assays.

Effect of VERDASAN on Cellulase Activity

The effects of different concentrations of VERDASAN on the Basidiomycete <u>235M</u> C_x cellulase activity are shown in Figure 30 and Appendix 19 .

The rate of reducing sugar production in the CONTROL was approximately linear over the first two hours; the rate then decreased after 3 hours until the end of the experiment after 5 hours. VERDASAN, at all concentrations, significantly reduced ($P < 0.05$)

Fig.28. The effect of TEMPERATURE on Basidiomycete <u>235M</u> C_x cellulase activity expressed as the amount of reducing sugars released by 1 ml of fungal filtrate from a CMC substrate in 24 hr at pH 5.4.

Fig.29. The effect of pH on Basidiomycete <u>235M</u> C_x cellulase activity expressed as the amount of reducing sugars released by 1 ml of fungal filtrate from a CMC substrate in 24 hr at an optimum temperature of $45^{\circ}C$.

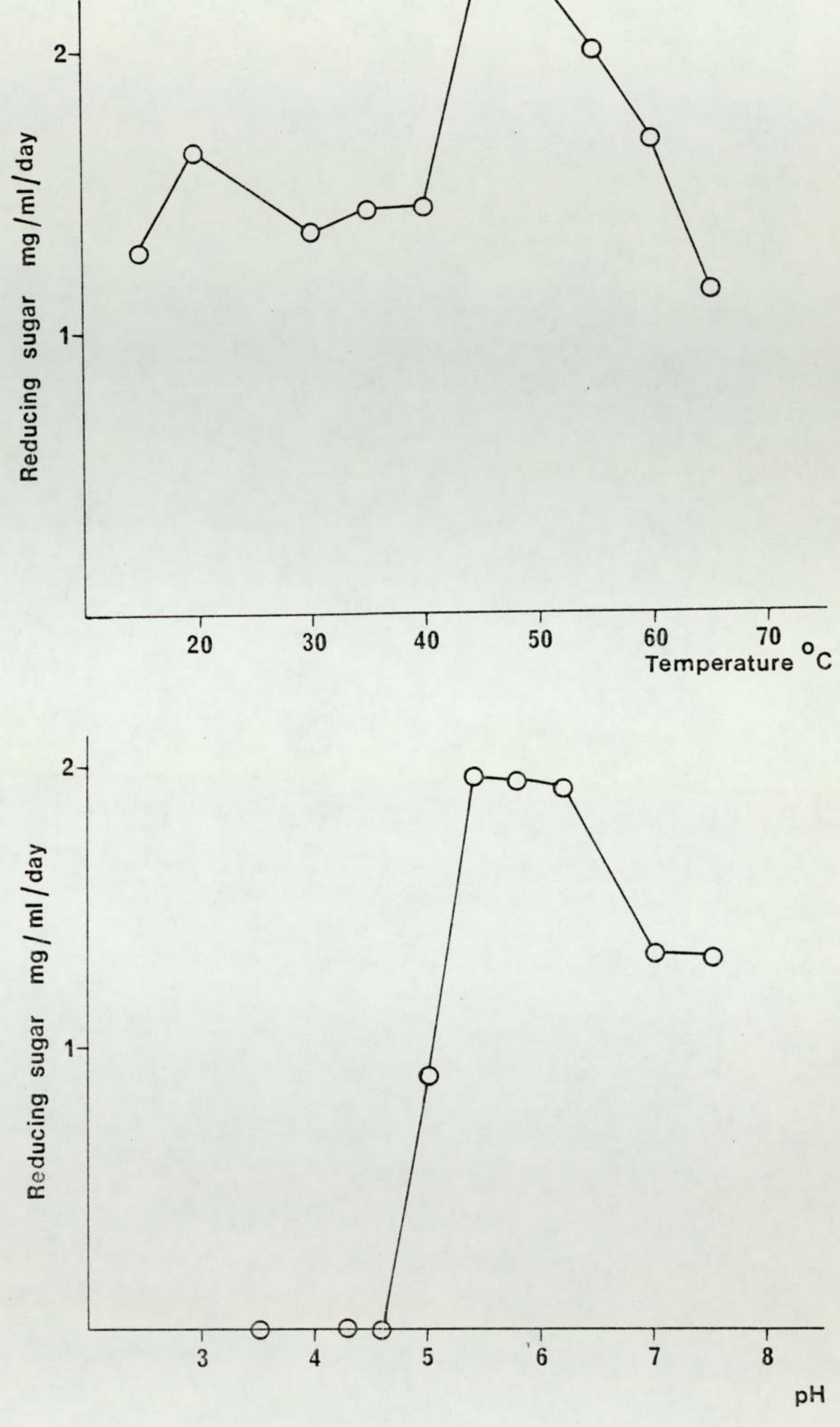

Reducing sugar mg/ml/day
2
1
20
30
40
50
60
70
Temperature °C
Reducing sugar mg/ml/day
2
1
3
4
5
6
7
8
pH

Fig.30. The effect of increasing concentrations of VERDASAN on the rate of reducing sugar production from a CMC substrate by Basidiomycete 235M C_x cellulase under conditions of optimum temperature and optimum pH.

○ CONTROL (without VERDASAN)

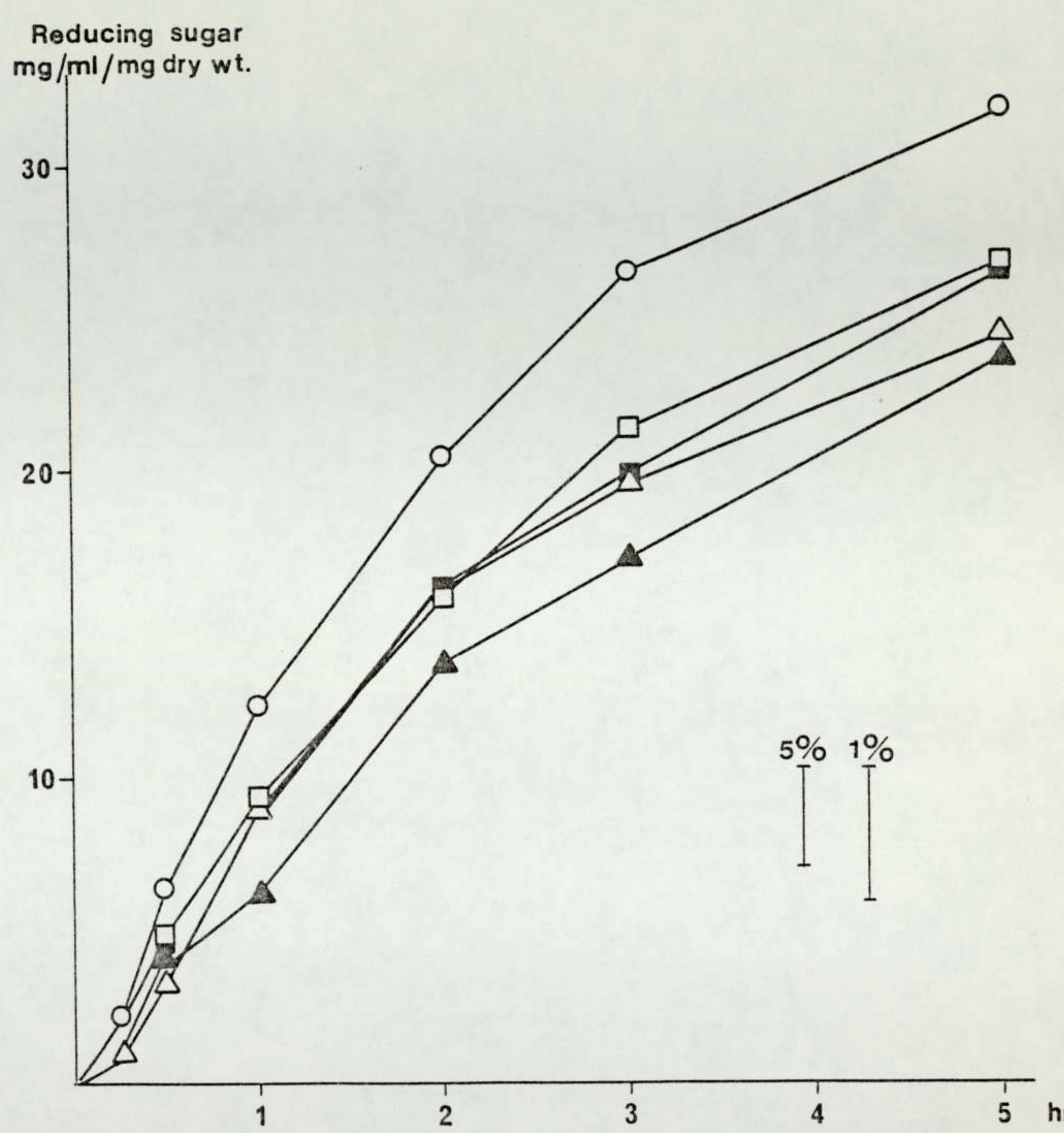
□ 0.5 ppm

■ 1.0 ppm

△ 5.0 ppm

▲ 10.0 ppm

For clarity, the data for the addition of 0.1 ppm and 2.5 ppm of VERDASAN are not shown. The complete results are tabulated in Appendix 19.



Reducing sugar
mg/ml/mg dry wt.
30
20
10
5%
1%
1
2
3
4
5
hr

the total amount of reducing sugar produced during the 2, 3, 4 and 5 hour incubation periods.

Discussion

When compared with other enzymes or enzyme systems, cellulases characteristically have high temperature optima (Ghose and Kostick, 1969). Dixon and Webb (1964) stated that optimum temperature is determined by the balance between the effect of temperature on the rate of reaction and its effect on the rate of enzyme destruction. Basidiomycete 235M showed a broad temperature range with maximum reducing sugar production at 45°C and a minor peak occurring at 20°C. The occurrence of two peaks may suggest the presence of a dual enzyme system with different temperature optima. Maximum reducing sugar production from CMC was observed at 55°C in *Poronia oedipus* (Denison and Koehn, 1977) and *P. punctata* (Robertson and Koehn, 1978) and at 50°C in the thermophile *Talaromyces dupontii* (Oso, 1978). Only one peak of activity was observed with these fungi.

In general, enzymes are only active over a limited range of pH, and in most cases a definite peak is observed (Dixon and Webb, 1964). The optimum pH range for cellulase activity typically falls within pH 4 to 6, although optimum values of pH 3.2 and pH 7 have been reported (Reese *et al.*, 1952; Sison *et al.*, 1958). The optimum pH between 5 and 5.8, with a maximum at pH 5.4 for Basidiomycete 235M C_x cellulase hydrolysis, seems consistent with the data observed for other fungi. *Poronia oedipus* was shown to have a pH optimum in the range pH 4.4 to 5 for the C_x system (Denison and Koehn, 1977), and *P. punctata* demonstrated major and minor peaks of C_x activity at pH 4.8 and 5.6 respectively, suggesting the presence of a dual enzyme system (Robertson and Koehn, 1978). Optimum C_x

activity of Talaromyces emersonii occurred between pH 5 and 7 with a maximum at pH 6 (Oso, 1978), and in Trichoderma koningii the optimum was at pH 5.0 (Toyama, 1956).

VERDASAN was shown in this study to significantly decrease the total amount of reducing sugar produced by the activity of C_x cellulase on CMC. There are contradictory findings concerning the influence of mercury ions on cellulase activity. Although Jermyn (1952) considered that cellulase activity was enhanced by mercury in Aspergillus oryzae, most other workers have found inhibition of its activity (Grassman et al., 1933, on Aspergillus oryzae; Marsh et al., 1953, on Myrothecium verrucaria; Reese and Mandels, 1957, on Trichoderma viride). Basu and Whitaker (1953) showed that cellulase activity of M. verrucaria at 10^{-5}M of mercuric chloride was less than 50% of the control and at 10^{-3}M was completely inhibited.

The C_x cellulase activity of Basidiomycete 235M was also found to be progressively reduced by increasing concentrations of VERDASAN and its behaviour, therefore, correlates well with most of the previous work outlined above.

This reduction is considered to be potentially important in nature as this species was isolated from leaf litter of Agrostis tenuis where it could be particularly vulnerable to freshly applied agrochemicals such as VERDASAN.

GENERAL DISCUSSION

The agrochemicals used in this study included the broad spectrum fungicides CAPTAN and VERDASAN, and the herbicides MAZIDE, PARAQUAT, SODIUM AZIDE and 2,4,5T. These compounds produced different effects on the fungi in field and laboratory studies when applied at their normal field rates and at elevated rates.

The concentrations at which the agrochemicals come into contact with the surface layers of soil are frequently greater than the maximum application rate quoted by their manufacturers, since these rates are calculated to a depth of soil which may exceed 30 cm. This is perhaps an oversimplification of the situation since :

a) the surface layers of soil will encounter the initial total application.

b) the accumulation of these compounds may occur in plant tissues to give locally high concentrations which may persist for a considerable time.

Bollen (1961) calculated that a dose rate of Verdasan of 22.7 Kg/ha (50 lb/acre) corresponds to 50 ppm if distribution to a depth of 17 cm (6 in) is uniform. The recommended rate of Verdasan application is 8.5 Kg/ha (7.5 lb/acre). Initially, little of the chemical is likely to penetrate the surface layers of soil to a depth of more than 1.25 cm (0.5 in) : this would result in a concentration of approximately 100 ppm in the uppermost layer if the fungicide becomes evenly distributed there (Williams, Pugh and Morris, 1972).

Perhaps of greater importance is the accumulation of herbicides by plants because, following the death of the plant, the plant tissue becomes subject to microbial decomposition. Calderbank and Tomlinson (1968) found that immediately after the application of Paraquat to a grass sward at a

rate of 5.8 Kg/ha (5lb/acre), the herbage contained 1240 ppm of the herbicide. Even 4 months after treatment they detected residues of 136 ppm of Paraquat in the plant tissues. At such concentrations, herbicides have been shown to have significant selective effects on the colonisation of plant remains by pathogenic fungi (Wilkinson and Lucas, 1969a).

The reason for applying agrochemicals in agricultural, pastoral and horticultural practices is to control or eliminate undesirable target species. However, since the cellular processes upon which these compounds act may be common to many organisms, they may kill non-target species, including fungi, with resultant changes within the soil environment.

The 'climax' soil, which Kreutzer (1963) described, was an environment in which substrates for microbial colonisation are limiting and therefore potential energy for growth is low. The overall state is one of quiescence. Following the application of agrochemicals to the soil, available substrate will be released into the soil, either as a result of a portion of the biomass being killed, or through the biological utilisation of the biocide itself. Potential energy for the growth of tolerant heterotrophic organisms is therefore raised.

A consequence of this may manifest itself in changes within the microbial population and in associated biotic processes :

i) a reduction in the activity, or elimination, of cellulolytic organisms. Pugh and Williams (1971) noted that the accumulation of organic matter in turf, treated prophylactically with Verdasan, was accompanied by a reduction in fungal potential of the substrate, where potential has been defined by Pugh (1963) as mycelium plus propagules.

ii) detoxification of the agrochemical by a saprophyte which is normally a poor competitor. Williams and Pugh (1975) showed that *Chrysosporium pannorum* is able to detoxify Verdasan, and Kuthubutheen

(1977) isolated this species in increased frequency from green leaves treated with Verdasan. In the present study, Ulocladium botrytis formed the bulk of the mycoflora isolated from VERDASAN-treated leaf litter and further studies are needed to show whether this fungus is also able to detoxify Verdasan.

The present research has demonstrated that the leaf litter was colonised by a wide range of fungal species which included primary and secondary leaf colonisers. The number of species of primary colonisers was low, even though Cladosporium cladosporioides formed the bulk of the mycoflora isolated from CONTROL and treated leaves. Numbers of this species were reduced by CAPTAN, although they tended to be higher on VERDASAN-treated leaves.

Secondary colonists, such as Alternaria alternata, Epicoccum purpurascens, Fusarium spp., and Gliocladium roseum, were affected to differing degrees by the agrochemicals. Numbers of isolations of A. alternata were dramatically increased on fungicide-treated leaves but numbers of E. purpurascens were reduced.

There was a considerable reduction in the fungal potential of the leaf litter after CAPTAN application, both in numbers of isolations and in numbers of species isolated. With VERDASAN, the number of species was significantly reduced, although the number of fungal isolations was not reduced as compared with the CONTROL since Ulocladium botrytis was isolated at a higher frequency from VERDASAN-treated leaves than from the CONTROL.

The effects of the herbicides on the leaf litter mycoflora appear to be minimal, although the incidence of individual species was altered in some cases. The number of isolations of species of Fusarium was higher on leaves treated with the herbicides than on the CONTROL leaves. Also, the numbers of Ulocladium botrytis were higher on leaves treated

with MAZIDE and PARAQUAT than on the CONTROL. Wilkinson and Lucas (1969a) showed that Paraquat sprayed onto potato haulm altered the outcome of competition between *Trichoderma viride* and *Fusarium culmorum* for these tissues in favour of *F. culmorum*.

Thus, the presence of herbicide residues in or on plant material may have a significant effect in determining which species are capable of colonising the material. From a practical standpoint, such effects assume particular importance when pathogenic organisms are involved. For example, *Gaeumannomyces graminis*, the fungus responsible for "take-all" in wheat and barley, overwinters in infected stubble, and its survival is inversely related to the activity of saprophytic micro-organisms in the stubble. Therefore, herbicides which are able to effect qualitative changes in the mycoflora which colonises and decomposes plant material, may subsequently affect the saprophytic survival of pathogens, either through direct antagonism or through changes in the rate of decomposition of the infected material (Wilkinson and Lucas, 1969a).

While qualitative and quantitative changes in the fungi which are isolated by the normal techniques following agrochemical application are relatively easily monitored, the assessment of changes taking place within the Basidiomycete population of leaf litter or soil, still remains difficult.

Warcup (1957) used his hyphal isolation technique to recover Basidiomycetes and other sterile hyphae from the soil, and subsequently Warcup and Talbot (1962) developed several techniques which enabled them to identify some of the sterile forms which they isolated. However, the identification of sterile mycelium of suspected Basidiomycetes is still a problem confronting mycologists. Methods which have been used in the past to promote fruiting of sterile cultures include the wheat chaff method

(Warcup, 1959), the wood block method (Tamblyn and Da Costa, 1958), and the soil method (Flentje, 1956). However, a laboratory approach has advantages when studying the interactions of agrochemicals with soil fungi in some known species of Basidiomycetes which have been isolated from sporophores.

In the present study, pure culture studies have demonstrated the sensitivity of several Basidiomycetes to agrochemicals and has indicated their potential sensitivity under field conditions. MAZIDE, PARAQUAT, CAPTAN and VERDASAN were able to inhibit the growth of mycorrhizal and non-mycorrhizal Basidiomycetes, at concentrations less than their recommended field rates of application. The cellulase activity of a litter decomposing species was also shown to be inhibited by the fungicide VERDASAN.

In addition, PARAQUAT, SODIUM AZIDE and VERDASAN were shown to increase the amounts of potassium, phosphate and amino acids which leaked from both mycorrhizal and non-mycorrhizal Basidiomycetes. In general, an unidentified Basidiomycete 235M, was more tolerant of both PARAQUAT and VERDASAN in growth studies than either *Boletus variegatus* or *Cyathus stercoreus*. It also displayed lower rates of leakage. The net losses of potassium, phosphate and amino acids from 235M in the presence of PARAQUAT and VERDASAN were less than from the other two species. In addition, *B. variegatus* leaked the greatest amount of phosphate and amino acids in the presence of the three agrochemicals, although the amounts of potassium leaked were not so drastically increased as compared with the CONTROL.

The rate of release of significant amounts of individual metabolites may be related to the tolerance of the fungi to the agrochemicals : significant amounts of potassium were lost from *C. stercoreus* and *B. variegatus* in the presence of VERDASAN after only one hour, while a

corresponding loss occurred with 235M in the presence of VERDASAN only after 8 hours and at subsequent sampling times. Similarly, with the loss of amino acids, the most susceptible species, B. variegatus, lost significant amounts after 1 hour of incubation in the presence of VERDASAN, while the more tolerant C. stercoreus and 235M lost significant amounts after 4 hours and 8 hours respectively.

The rapidity with which significant amounts of metabolites were lost from the fungi may also give an indication of the toxicity and modes of action of the different agrochemicals. The metabolites were generally lost at the fastest rate in the presence of VERDASAN and at the slowest rate with SODIUM AZIDE. In field studies there was a 36% reduction in the number of species isolated from VERDASAN-treated leaves immediately after spraying, which is another indication of the rapidity with which this fungicide acts.

The mode of action of VERDASAN is ascribed to the direct interaction of the mercury with sulphydryl groups in the membrane structure of the fungus, leading to a molecular stress which may result in a generalised breakdown of the membrane and the concomitant release of cellular constituents (Rothstein, 1959). PARAQUAT does not directly interact with cellular membranes although the reduction of the bipyridylium ion in the fungal cell may lead to its reoxidation by water with the production of hydrogen peroxide which will subsequently react with cell membranes causing loss of cell compartmentalisation. The loss of metabolites from the fungal hyphae in the presence of SODIUM AZIDE was generally low and the amounts lost were small. This chemical does not affect the fungal membranes but inhibits nuclear phosphorylation (Mettler, 1972). As the levels of ATP drop, the metabolites diffuse down a concentration gradient into the surrounding medium and when all available

ATP has been utilised no further ions will be taken up by the transport system.

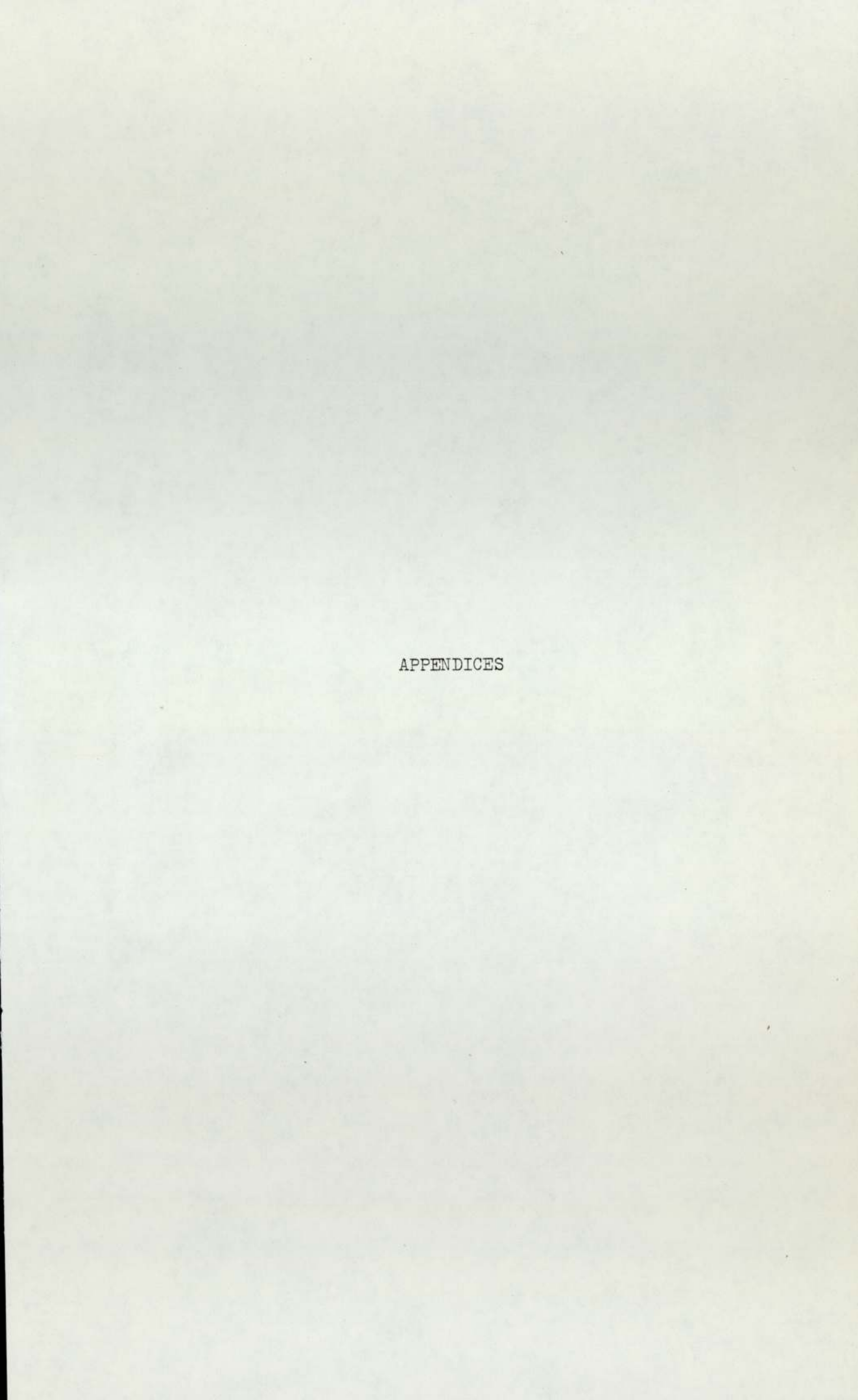
In general, the growth and physiology of the mycorrhizal species were more adversely affected by the agrochemicals than were the non-mycorrhizal species. The differences between the growth rates of mycorrhizal and non-mycorrhizal fungi, and their abilities to grow in increasing concentrations of agrochemicals, were suggested by Pugh and MacDonald (1980) as a possible criterion for distinguishing between these two ecological groups of fungi. These findings were complemented with further physiological work in this study, and the results suggest that differences in membrane permeability between the two groups of fungi in the presence of agrochemicals may be a further criterion which should be considered.

When further techniques become available, research can be carried out on the Basidiomycetes under more natural conditions. Ibbotson and Pugh (1975) used a fluorescent antibody technique to study Arthroderma uncinatum in the soil. The successful application of this technique to the study of selected Basidiomycetes in leaf litter and in the soil would greatly contribute to our scant knowledge of the activities of these organisms in vivo (Pugh, 1980). Also, Anderson and Domsch (1978) have utilised selective inhibitors to eliminate parts of the soil biomass in order to obtain an estimate of the total fungal living biomass, and Smith and Pugh (1979) have used a dehydrogenase assay as an indicator of soil microbial activity. A further refinement of these techniques may provide us with a method whereby selective inhibition could be used to determine the origin of the microbial activity. In addition, group-specific enzymes could give a direct indication of the activity of individuals within a mixed population, such as would be found in leaf

litter, since Wood (1975) has quantified the mycelial growth of *Agaricus bisporus* growing on wheat straw, by using an assay of extra-cellular phenol oxidase.

This study demonstrates that the agrochemicals tested have distinct and significant effects on the ecology and physiology of the fungi inhabiting the litter layer. Since the chemicals were randomly chosen, it may be assumed that other biocides will affect these fungi in a similar way. Further studies are needed to evaluate the ecological consequences, if any, resulting from the important use of agrochemicals in an age when there is increasing use being made of these compounds. It is important to ascertain the precise physiological responses of the ectomycorrhizal fungi in the light of Harley's suggestion (1976) that organic acid production by these fungi is responsible for the solubilis-ation of phosphate within the host root environment. If agrochemicals decrease the amounts of organic acids produced by the fungal symbiont, and indeed other litter inhabiting Basidiomycetes, there may be a reduction of phosphate uptake by the plant, and possible secondary effects on other soil and leaf litter micro-organisms with which they were in competition.

The implications of agrochemical contact with the litter layer may be a reduction in organic matter decomposition, and, with more and more litter reaching the surface, an organic matter build-up will occur (Williams, Pugh and Morris, 1972) : waterlogging and anaerobiosis of the underlying soil layers may then ensue if the drainage of water from the upper layers is decreased, leading to an overall decrease in fungal activity and biomass.



APPENDICES

APPENDIX 1 . The Agrochemicals

1. CAPTAN

Active ingredient: N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide (50% w/w)

Uses: Primarily as a foliar protectant on fruits, vegetables and ornamentals, seed treatment, drench against damping off, bird repellant on seeds.

CAPTAN is a broad-spectrum fungicide ineffective against rusts and downy and powdery mildews. The activity of CAPTAN appears to be associated with the destruction of thiol groups of matrix proteins in sensitive fungi (Richmond and Somers, 1966). The lipoprotein structure of fungal membranes is not thought to be affected (Kottke and Sisler, 1962).

CAPTAN hydrolyses to non-toxic breakdown products and has a half life of 2 - 4 days (Burchfield, 1959).

2. MAZIDE

Active ingredient: 1,2-dihydro-3,6-pyridazinedione (36% w/v maleic hydrazide as amine salt).

Uses: As a grass growth regulator on roadside verges, railway embankments, cemeteries etc; to prevent sprouting and prolong storage life of onions, potatoes and other root crops; to permit controlled growth

of flower crops and ornamentals.

MAZIDE is a synthetic plant growth inhibitor with auxin-like properties, and its mode of action is thought to be due to the disruption of nucleic acid production and protein synthesis.

3. PARAQUAT

Active ingredient: 1,1'-dimethyl-4,4'-bipyridylium (25% w/v)

$$CH_3-N^{+}\langle\bigcirc\rangle-\langle\bigcirc\rangle N^{+}-CH_3$$

Uses: As a total weedkiller and defoliant; as a dessicant of haulm of beans, peas, potatoes and other root crops to facilitate harvesting; control of plants in an aquatic environment.

PARAQUAT is a total non-selective weedkiller, destroying all vegetation with which it comes into contact. Its primary mode of herbicidal action is associated with the ability of the molecule to accept an electron in the light from photosystem I of the photosynthetic pathway, giving rise to a free radical. Subsequent reoxidation yields hydrogen peroxide (Davenport, 1963) which damages the fatty acid component of plant membranes and destroys the compartmentalisation of the cell.

4. SODIUM AZIDE

Uses: Agriculturally as a biodegradable herbicide and insecticide in the past. It is well known as an inhibitor of nuclear phosphorylation and has certain pharmaceutical uses, including the preservation of laboratory reagents and biological specimens.

5. 2,4,5T

Active ingredient: 2,4,5-trichlorophenoxy acetic acid (50% w/v)

Cl

Cl — OCH_2COH (C=O)

Cl

Uses: As a clovericide; a total weedkiller and selective weedkiller for broadleaved plants depending on the concentration used.

2,4,5T is a highly selective systemic herbicide for woody shrubs (broadleaf weeds). Its mode of action is in its ability to disturb the plants metabolism or growth processes, affecting cellular division, phosphate metabolism, and modifying nucleic acid metabolism.

6. VERDASAN

Active ingredient: phenylmercury acetate (PMA) (2.5% w/w)

$CH_3—C(=O)—O—Hg—C_6H_4—OH \rightleftharpoons C_6H_5Hg^+ + O^-—C(=O)—CH$

Uses: Against turf diseases; as a seed dressing; as dormant sprays for fruit trees; as an industrial fungicide; as a mildewicide.

VERDASAN is an organo-mercurial fungicide, and its biological activity has been ascribed to the formation of ionic bonds between the PMA cation and anions present in living tissue, or by the formation of mercaptide with the thiol groups of living tissue.

HO, Hg (ring) $O—C(=O)—CH_3 + RSH \rightleftharpoons$ (ring) $Hg—S—R$ | $CH_3—COOH$

APPENDIX 2. Weather Data

Summary of temperature and precipitation from Edgbaston Meteorological Observatory, Birmingham.

Grid reference SP 043 908

	Total Rainfall (mm)	Mean Temp. °C min.	max.	Humidity % (mean 0900 hrs)	Grass min. temp. lowest °C
Nov 1977	65.6	3.9	8.2	82.0	-7.7
Dec 1977	76.5	4.2	7.3	88.3	-3.4
Jan 1978	91.4	1.6	5.0	87.8	-10.1
Feb 1978	60.3	0.6	4.3	91.0	-11.9
Mar 1978	47.8	3.6	9.8	81.0	-5.8
Apr 1978	44.8	3.2	8.9	82.0	-6.3
May 1978	33.1	7.4	15.8	75.4	-3.2
Jun 1978	37.0	10.1	17.1	71.0	-0.3
Jul 1978	83.7	11.3	18.6	76.0	-1.0
Aug 1978	76.6	11.7	18.1	79.5	-1.4
Sep 1978	40.1	10.7	17.4	76.9	-1.7
Oct 1978	13.9	9.1	14.7	84.0	-5.0
Nov 1978	39.7	6.3	10.7	86.0	-10.7
Dec 1978	160.2	2.1	5.5	90.0	-10.2

APPENDIX 3. Media

1. Cellulose agar (Eggins and Pugh, 1962)

$(NH_4)_2 SO_4$	0.5 g
L-asparagine	0.5 g
$KH_2 PO_4$	1.0 g
KCl	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2$	0.1 g
Difco Yeast Extract	0.5 g
Ball-milled cellulose	10.0 g
Agar	15.0 g
Distilled water	1 litre
pH 6.2	

2. Selective medium for isolation of Basidiomycetes (Hunt and Cobb, 1971)

Benomyl	8 mg (a.i.)
Dichloran	8 mg (a.i.)
Phenol	50 mg
Agar	15.0 g
Distilled water	1 litre

The phenol was dissolved in a 50% ethanol solution as recommended by Russell (1956), and benomyl and dichloran were added to this to make a stock solution of 10 mg/ml phenol, 1.6 mg/ml benomyl, and 1.6 mg/ml dichloran. Appropriate amounts of stock solution were added to cool, sterile water agar medium.

3. Hagem's agar (modified by Modess, 1941)

D-Glucose	5.0 g
Oxoid Malt Extract	5.0 g
NH_4Cl	0.5 g
KH_2PO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$FeCl_3$ (1% solution)	10 drops
Agar	15.0 g
Distilled water	1 litre
pH 4.6	

4. Basic liquid medium (modified from Palmer, 1971)

D-Glucose	10.0 g
Oxoid Malt Extract	20.0 g
NH_4Cl	0.5 g
KH_2PO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Thiamin	1 mg
Biotin	5 mg
Micro Elements[1]	2 ml
$FeCl_3$ (1% solution)	10 drops
Distilled water	1 litre
pH 4.6	

[1] Micro Element Solution (Lilly and Barnett, 1953):

$Fe(NO_3)_3 \cdot 9H_2O$	723.5 mg
$ZnSO_4 \cdot 4H_2O$	439.8 mg
$MnSO_4 \cdot 4H_2O$	203.0 mg

APPENDIX 4. Species isolated from Leaf Litter of *Agrostis tenuis*

PARAQUAT	J				F				M				A			
	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28
Acremoniella atra										1						
Alternaria alternata	2		3	2				2	1	2	2	1	3			2
Asteromyces cruciatus										1				1	1	
Bispora antennata																1
Botryotrichum piluliferum																
Botrytis cinerea											1			2		
Chaetomium spirale															1	
Chrysosporium luteum								1								
Chrysosporium parvum				2												
Cladosporium cladosporioides								8	6	7	24	23	23	23	14	24
Cladosporium herbarum								2	2							
Cladosporium oxysporum										5						
Drechslera biseptata														1		
Epicoccum purpurascens	4	6	1					6	4	6	6		6	3	4	3
Fusarium spp.	9	8	7	5				14	13	10	9	13	8	6	9	7
Gilmaniella humicola				1												
Gliocladium roseum	7	7	8	5				4	8	3	5	6	6	6	8	7
Gliocladium salmonicolor										1						
Gliomastix murorum				1						1						
Humicola fusco-atra								3	1		3		2			
Humicola grisea	2		4					1	1	1	2	1	1	1		
Monocillium indicum																
Mortierella vinacea												1	1	2	1	1
Mucor hiemalis		4						1	1	4	4	1	3	2	2	4
Paecilomyces elegans											1					
Papularia montagnei										5	2		1	5	2	3
Penicillium spp.		4	2	2				2	2	1	1		2	2	6	2
Phoma spp.								4		5	1	1	2	1		2
Pyrenochaeta decipiens											1					
Pythium sp.																
Rhinocladiella mansonii									1							
Rhinotrichum tenellum									1							
Stachylidium bicolor								1								1
Thermomyces lanuginosus		2	1							1	1					
Trichocladium asperum																
Trichoderma hamatum								1		1			1			
Trichoderma spp.		4						2			2	2				
Trichoderma viride			4	1				1	1		5		3	4	1	2
Ulocladium botrytis									2	2	1					
Verticillium lateritium									1		1					
Volucrispora aurantiaca									1							
Volutella ciliata													2	1		4
Pycnidial forms		1							2							
Basidiomycetes																
Total number of species	5	8	8	8				16	16	18	19	9	15	15	11	14
Total number of isolates	24	36	30	20				53	45	57	72	49	64	60	49	62

PARAQUAT	M				J				J				A			
	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28
Acremoniella atra													1			
Alternaria alternata		1	1		2	1	1		4	6	1	6	7	7	8	1
Asteromyces cruciatus																
Bispora antennata		1	1		2		1	1								2
Botryotrichum piluliferum					1											
Botrytis cinerea				1				1	1				2	1		
Chaetomium spirale																
Chrysosporium luteum														5	16	
Chrysosporium parvum														1	1	
Cladosporium cladosporioides	23	14	21	19	24	17	25	24	23	24	22	25	24	19	23	24
Cladosporium herbarum														5	16	
Cladosporium oxysporum	1						1			4	2	1	1			
Drechslera biseptata				2		1										
Epicoccum purpurascens	1	1	1	4	4	1	3	7	6	6	11	12	8	4	16	2
Fusarium spp.	4	6	12	4	8	5	5	6	10	9	7	5	6	6	8	12
Gilmaniella humicola	1				2							2				
Gliocladium roseum	10	10	5	7	4	8	1	3	5	3	7	10	12	8	3	9
Gliocladium salmonicolor																
Gliomastix murorum						1										
Humicola fusco-atra		2		1		2				2					1	
Humicola grisea	1	2	1			1				1			1	1		
Monocillium indicum				1				1			1					
Mortierella vinacea		2	4		2	1			1			1	1	1		1
Mucor hiemalis	5	2	2	3	2	2	4	3	8	10	9	11	15	13	3	9
Paecilomyces elegans					2										2	2
Papularia montagnei		2	1	1	5	6	4	1	1		1					
Penicillium spp.		2	3		1	1	1		2		1	1	4			1
Phoma spp.		3	1	2		1				1	4					
Pyrenochaeta decipiens																
Pythium sp.											2					
Rhinocladiella mansonii								1	1							
Rhinotrichum tenellum																
Stachylidium bicolor	1		1					1			1					
Thermomyces lanuginosus											1					
Trichocladium asperum				1					1							
Trichoderma hamatum					1	2			2		5		1	1		
Trichoderma spp.		1	5								1		2		1	
Trichoderma viride	5	1														
Ulocladium botrytis	1		2						1	1	1	1	4	[illegible]	1	1
Verticillium lateritium				1		1				1						
Volucrispora aurantiaca														1		
Volutella ciliata		1	3	2	3		10	4	2	2				2	3	
Pycnidial forms			2	2	2	1	2		1	2		2		2	2	1
Basidiomycetes							3							1		
Total number of species	11	16	17	15	16	17	12	12	16	14	17	12	15	18	14	13
Total number of isolates	53	51	66	51	65	52	61	53	69	72	77	77	89	80	103	66

PARAQUAT	S				O				N				D			
	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28
Acremoniella atra	1												1			
Alternaria alternata	6	6	8	6	11	12	8	3	2	14	6	10	3	6	4	2
Asteromyces cruciatus																
Bispora antennata	7	1				2	1	5	3		3		2		1	
Botryotrichum piluliferum																
Botrytis cinerea	1	5	1	2	1							1				
Chaetomium spirale																
Chrysosporium luteum	5	3	8	11	15	12	9	8	2	1	10	12	9	11	7	6
Chrysosporium parvum	2		1				1			3		1	1			
Cladosporium cladosporioides	20	11	3	4		2	3	1	2				1			
Cladosporium herbarum																
Cladosporium oxysporum																
Drechslera biseptata				1												
Epicoccum purpurascens	8	8	9	13	19	16	9	10	3	1	6	8	2	1	3	1
Fusarium spp.	10	4	16	15	12	3	7	6	3	10	1	8	7	6	7	3
Gilmaniella humicola																
Gliocladium roseum	6	9	9	5	2	6	4	10	15	7	10	10	9	7	8	6
Gliocladium salmonicolor																
Gliomastix murorum		6														
Humicola fusco-atra							1		2				1			
Humicola grisea						1		2	3		1		2			
Monocillium indicum								1								
Mortierella vinacea				1			1		6			1	1			
Mucor hiemalis	12	1	4	3	6	6	10	10	9	7	9	9	12	8	9	7
Paecilomyces elegans																
Papularia montagnei		4			2				1	1			2			1
Penicillium spp.				1	1	1	4	3	4	2	1	1	2	1		2
Phoma spp.		2				1		1	2	1			1		1	
Pyrenochaeta decipiens																
Pythium sp.																
Rhinocladiella mansonii					1											
Rhinotrichum tenellum																
Stachylidium bicolor																
Thermomyces lanuginosus		1						1	2		1	1				
Trichocladium asperum							1				1					
Trichoderma hamatum					1	1			2		2	2	1			
Trichoderma spp.							3	1			2	1	1		2	
Trichoderma viride																
Ulocladium botrytis	1		2	2	1			1	3	1			2		1	1
Verticillium lateritium																
Volucrispora aurantiaca																
Volutella ciliata	3						3	1	1		1		3			
Pycnidial forms	2	3	6	5	3	5	1		5	1		3				
Basidiomycetes						1				1						
Total number of species	14	14	11	13	13	14	16	11	19	13	14	14	20	7	10	9
Total number of isolates	84	64	67	69	75	69	64	67	70	50	54	68	63	40	44	29

CONTROL	J				F				M				A				M				J				J				A				S				O				N				D			
	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28
Acremoniella atra																													1		1																	
Alternaria alternata	3	2	1								2		1		2		3						1	6	5	3	2	5	6	5	4	5	6	8	5	2	3	5	7	1	1	5	4	3	3		3	2
Bispora antennata										1			2		1					2	1			1																3	2			1				
Botryotrichum piluliferum																												2											1								1	
Botrytis cinerea																				1						2												1										
Cephalosporium acremonium								1			1				1					1						1																						
Chrysosporium pannorum																										1																						
Chrysosporium parvum		1											1																																			
Cladosporium cladosporioides	1			1					5	13	21	14	14	25	24	25	25	21	13	21	23	24	25	25	25	25	23	25	25	25	24	24	21	18	15	12	1	1			4					2		
Cladosporium herbarum																							1						1		2	1		7	4	9	17	7	13	5	12		14	17	21	21	19	20
Cladosporium oxysporum																							1	1				3																			1	
Dachtylella minuta		1										1															1																					
Doratomyces stemonitis																																1																
Drechslera biseptata																																		4		3	1	2				2				1		
Echinobotryum atra																																									1							
Epicoccum purpurascens	7	6		3				2	1	1		1			4		2							8	9	2	8	9	8	7	11	9	11	19	15	9	8	14	12	15	18	4	5	3	4	5	11	7
Fusarium spp.	3	9	5	2				3	6	4	5	6	3	5	2	8	9	4	9	4	5	3	10	7	9	12	7	9	10	6	7	7	10	12	11	8	6	6	5	3	5	4	6	8	5	6	7	4
Geotrichum candidum																																1				1												
Gilmaniella humicola	2													2	1											1	1			1																		
Gliocladium roseum	4	2	2	2				2	1	2	3	1		3	5	12	8	4	1		2	1	1	5	5	1	4	5	5	2	6	3	4		6	7	6	5	4	2	9	3	3	5	5	3	6	4
Gliocladium salmonicolor															1										1																							
Gliomastix murorum											1				1	1																																
Graphium penicillioides								1																																								
Humicola fusco-atra			1	4				1	2	1	1	1	1	2	1	2	2	1		1	1		1	1			1			2										5	5	2					1	
Humicola grisea								1							3							1				1	1				1				1		1	1	1		4	1		2	1	3	2	
Mortierella vinacea										1	3	3	3	4	5	2	2	6		2	2	2	2	16	14	7	13	18	20	21	13	3	8	8	5	11	11	3	8	9	13	9	4	10	7	9	9	6
Mucor hiemalis	3	2	2	2				1	1	1	2				1														2		1	5		1		1					1		1					
Paecilomyces elegans																											1																					
Papularia montagnei	1			3				2		1	5	3	1	6	1	3	3	3		2	2	4	1	1	2	1			1			1	1	1	2	1	1	2	1	3	1	4		1				
Penicillium spp.	1	1	2	1				1	1		1	1	5	4	1	4	3	2		1	1	4	2	2	3	2	4	2	2	2	1		1		1	2			2	3	2	5	1	4	3	3	6	4
Phoma spp.			1	6				4	8	2	6	5	6	5	4	5	5	4	3			1		1				1												2		3		3				
Pythium sp.																			1								1																					
Rhinocladiella mansonii															1					1																					1					1		
Scopulariopsis brevicaulis																				1																												
Stachylidium bicolor									1	1	1			2	3							1		1																								
Thermomyces lanuginosus	2									1																										1	3			1	1	1	2	5		1	1	
Trichoderma hamatum																								1																	4	2	3	4				
Trichoderma spp.			2	1				1	1	6	4	3	4	9	1	2	1	1	1		1		1		2	1	14		1	1	2	2					2		2		1	2	1	1		2	2	1
Ulocladium botrytis			2					1	1	1			1			3	2	1		2	2	1		3	2			1						2				1		2		1		1		1		
Verticillium lateritium	1	1								1																1	1									1			1	1						1		
Volucrispora aurantiac.																																																
Volutella ciliata				1					1	1	1		4	2	4				2	7	4	4	5	1	2	2	1	1		3		8		8	10	7	6	2	4	1	1							
Pycnidial forms								1	1		1	3		1	4				1			2	1	1	3			2	2	2	1	4	3	2	1	3	4		1	1		1				1		
Basidiomycetes																						1	1	1						2				3		1	2	1				1						
Total number of species	11	10	8	11				14	14	16	16	11	13	13	21	11	12	11	8	13	11	13	14	17	13	16	16	13	14	13	13	14	9	13	12	17	15	14	14	16	19	17	11	15	8	15	13	8
Total number of isolates	28	27	16	26				22	31	40	59	39	46	70	71	67	65	48	31	46	41	49	53	82	82	63	79	83	85	[illegible]	74	74	65	93	76	79	72	51	61	57	86	50	44	66	49	60	69	48

CALTAN	J				F				M				A				M				J				J				A				S				O				N				D			
	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28
Alternaria alternata			2							2	2		2		3			1					1	8	8	20	20	18	17	23	14	13	9	19	21	18	19	17	18	15	14	23	17	18	19	18	8	12
Asteromyces cruciatus														7																																		
Bispora antennata											1					1	1		2	2																					1					1	1	
Botrytis cinerea																						1																										
Cephalosporium acremonium									1							1				1			1																									
Chrysosporium parvum																						1																										
Cladosporium cladosporioides								1	2	12	21	23	22	19	23	20	10	5	10	9	8	3	9	9	2	7	9	16	13	24	18	14	11	3	2	6	2		2	10				1		1		
Cladosporium herbarum																															3	2	1		2	1	1	1		1				5	3	5	1	
Cladosporium oxysporum													1	1	1			1		1	1										1																	
Drechslera biseptata																																				1	2								1			
Echinobotryum atrum																																											1					
Epicoccum purpurascens	8	5		4				6	5		5		2		2	2						1	1	1	1	1	1	4	2	7	5	2	2	3	4	5	2	2	6	2	2	2	5	1	3	2	1	
Fusarium spp.	7		5	2				2	5	3	3	2	2	3	1	1	5		1	3		2	4		1	1	1	3	3	1		1	1	3	1		1		1				1		3	6	4	1
Gliocladium roseum	3	3	5	3				2	6	2		1	2		1	3	5	3	1		3		1		1		3	4		2	2	1		2	3	3	1	2		7		5	1	4	1	1	5	2
Gliocladium salmonicolor														3				1																														
Humicola fusco-atra								2	5	1	2	3	2	2		1			1					1						1															1			
Humicola grisea	2								1	1																			1		1				1					2								
Mortierella vinacea	2								1	5	3	4		4	4	3	4	3	3	2	2	2	8	9	18	17	19	16	19	12	13	4	7	9	5	10	6	9	8	12	9	7	5	5	10	4	8	6
Mucor hiemalis			3							1	1																					5		1					1								1	
Paecilomyces elegans										1									1																													
Papularia montagnei								2	1	4	3	5	2	4	1	2	2	1	1	4			2									1												2	1		1	
Penicillium spp.	2		3	1				2	3	1	1	3	4	5	2	2	4	2		1	6	1	2	2			2	2	1									1				3	1	3	4	3	1	2
Phoma spp.			2	4				3	6	3	5	4	4	7	2	1	3	2	2		1		1	3																	1							
Polyscytalum sp.											2		1								1		2																									
Pythium sp.																			3								1																					
Scopulariopsis brevicaulis										1				1					1																													
Stachylidium bicolor								1	1			1			1		1			1		2		1																								
Thermomyces lanuginosus	1	1	1																														1		1			2	3	1	2	2	2	2	2	3	1	2
Trichoderma hamatum		2											1			1	2		1		1					5									4		1	1	1	1			1	2	1	1	4	2
Trichoderma spp.		3								1	1									1					2	3				2	1		1	1		1		1		3			1	1	1		1	
Trichoderma viride	3		2	1				1						1	1		2	1	1					1	1					2			4	1				3	4	1	4		3	1	1	6	1	
Ulocladium botrytis			3	1				1	1	3	1		2	1	1		2			1				1	1	8	7	7	14	12	2	1	1	2	2	2	5	5	4		1	4	3	2	4	1		
Verticillium lateritium	1															1						1	1		1											1						1						
Volucrispora aurantiaca										2			1	2	1	1			1						1			1			1	1															1	
Volutella ciliata											1	1	1		3	3	2		1	6	1		2	1			1				2	7		2		3	1		1	3			1					
Pycnidial forms								1	1						4	1	2		1	1	1	1	2	2	1	2		1	2		1	1			2	2	1			2						1	1	
Basidiomycetes																									1																	2						
Total number of species	9	5	9	7				12	12	16	15	10	15	14	15	16	14	10	16	13	10	10	14	12	13	9	10	10	9	10	13	12	10	10	12	12	12	11	11	13	8	9	13	14	15	14	16	17
Total number of isolates	29	14	26	16				24	39	43	51	47	49	60	51	44	45	19	31	33	25	15	17	39	39	64	65	72	71	86	64	52	38	46	48	52	42	42	49	60	34	49	42	48	55	52	40	27

VERDASAN	J				F				M				A				M				J			
	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28
Alternaria alternata	2	5	1	3				2		2	1							1						
Botryotrichum piluliferum																								
Botrytis cinerea																						1		
Brachysporium sp.		1																						
Cephalosporium acremoniella									1			1		1										
Chaetomium spirale									1												3			
Chrysosporium pannorum																			1					
Chrysosporium parvum										1					1	1				1				
Cladosporium cladosporioides								7	2	13	20	13	17	20	15	22	16	13	11	17	3	7	24	25
Cladosporium herbarum																								
Cladosporium oxysporum										5														
Clasterosporium carcinum																								
Cylindrocladium scoparium												1							3					
Drechslera biseptata	1																	3						
Epicoccum purpurascens	7	7	3	2				1	1	2					1	2	2			1				
Fusarium spp.	5	5	4	4				4	5	5	3	5	4	3		2	3	3	1	3		10	1	3
Gilmaniella humicola			1	1																				
Gliocladium roseum	3	5	2	3				4	3	1	1	4	3	1	3	4	3	2				1		3
Gliomastix murorum												1			1			1						
Humicola fusco-atra		1		3				4	3	4	3	1	2	2	3		1			1				
Humicola grisea		2							1	1	1	2	1					1						
Mammaria echinobotryoides																					1			
Monocillium indicum		1	1																					
Mortierella vinacea											1			3		1	1	1						
Mucor hiemalis	2							1	2	1	5	1	2	2	3	3	3	2	1	3			11	15
Oidiodendron sp.																1								
Paecilomyces elegans								1																
Papularia montagnei								3	1	1	2	2	2	1		2	1	1	1	1			1	
Penicillium spp.	2	2	2	1				1	2	1	3	2	2	2	4		2	2		2			4	2
Phoma spp.	6	5	7	5				4	6	3	5	4	5	10	5	3	4	3	6	1	1			
Rhinocladiella mansonii														2										
Rhizopus oryzae												1												
Scolecobasidium constrictum																			1					
Stachylidium bicolor											1	1		1			1			1				
Stemphylium vesicarium											1													
Thermomyces lanuginosus	2	1	2																					
Trichocladium asperum																								
Trichoderma hamatum																					1			
Trichoderma spp.								1																
Trichoderma viride		1	1						2	3				2	2	2		2			1			
Ulocladium botrytis			2						2	1		1	2		1		2	3		2	1	1	10	
Verticillium lateritium																	1			1				
Volucrispora aurantiaca									1	1	1	1				1			9	7	4			
Volutella ciliata															1	1								
Pycnidial forms			1						2	1	3	1			2		4		1		2	2		2
Total number of species	9	12	12	5				12	16	17	15	17	10	13	13	13	14	14	10	13	7	6	7	6
Total number of isolates	30	36	27	13				33	35	46	51	12	40	50	42	45	44	38	35	41	14	22	44	58

VERDASAN	J				A				S				O				N				D			
	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28
Alternaria alternata	1	3	3	6	6	11	9	10	5	12	14	15	18	14	12	16	19	15	15	14	13	10	21	17
Botryotrichum piluliferum			1																					
Botrytis cinerea																								
Brachysporium sp.																								
Cephalosporium acremoniella																								
Chaetomium spirale																								
Chrysosporium pannorum					2																			
Chrysosporium parvum	10		1										1			1	1						2	1
Cladosporium cladosporioides	22	24	24	21	24	24	23	23	23	18	22	24	20	23	3	20	10		2					
Cladosporium herbarum								1							4	1	13	2	18	21	24	17	17	14
Cladosporium oxysporum			1																					
Clasterosporium carcinum				1																				
Cylindrocladium scoparium																								
Drechslera biseptata					1																			
Epicoccum purpurascens		1		6	2	8	10	2	2	3	2	4	3	1	2	5	3	1	1	1	1	3	1	2
Fusarium spp.		1	6	5	5	6	5	3		2	3	2	2	1	3	5	2				1	1	3	1
Gilmaniella humicola						1																		
Gliocladium roseum	4	1	4	3	3	2	4	2	1	2	4	4	4	3	4	7	7	5	4	10	6	4	6	5
Gliomastix murorum																	1				1			
Humicola fusco-atra																3	1		1	1				
Humicola grisea			1		1		2										1			1				
Mammaria echinobotryoides					1																			
Monocillium indicum																								
Mortierella vinacea		1			2		1	1																
Mucor hiemalis	16	16	18	19	13	12	16	12	4	2	12	11	9	8	8	11	8	1	9	11	11	7	9	7
Oidiodendron sp.																								
Paecilomyces elegans																								
Papularia montagnei			1		1	1	1							1				2		2				
Penicillium spp.	2	1	2	1			1				1		2			1		5	1		3	1	5	2
Phoma spp.				1		2					1		1			1		2	1	3	1	2	2	1
Rhinocladiella mansonii													2								1			
Rhizopus oryzae																								
Scolecobasidium constrictum																								
Stachylidium bicolor																								
Stemphylium vesicarium																								
Thermomyces lanuginosus																				1				
Trichocladium asperum																								
Trichoderma hamatum														1										
Trichoderma spp.								1												1				
Trichoderma viride													1						2	2				
Ulocladium botrytis	13	12	12	11	16	8	11	10	16	15	22	21	19	23	22	18	23	19	21	22	21	22	22	20
Verticillium lateritium																		1						
Volucrispora aurantiaca						1				1											1			
Volutella ciliata																								
Pycnidial forms					1		3										1			1	1	1		
Total number of species	8	9	12	9	13	11	12	10	7	7	10	7	11	10	8	12	12	10	11	12	15	12	11	9
Total number of isolates	70	60	74	76	77	76	84	67	52	54	82	81	81	76	58	89	89	55	74	91	88	71	88	69

NAME	J				F				M				A				M				J				J				A				S				O				N				D			
	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28	0	7	14	28
Acremoniella atra										1																																						
Alternaria alternata	2	1	3							1		1			1	2		1	1	1			1	2				4	5	8	2	9	5	4	2	2	1	6	5	3	3	3	7		1	3	5	
Asteromyces cruciatus								1																																								
Bispora antennata												1		1				3			1				1								1	3	1	1					1		2	1	1			
Botryotrichum piluliferum																											1																					
Botrytis cinerea																				1	1				1		1																					
Cephalosporium acremonium														1	1			1			1						1																					
Chaetomium globosum										1																																						
Chrysosporium pannorum																			1																													
Chrysosporium parvum			3						1																							1																
Cladosporium cladosporioides								3	4	18	21	25	22	16	25	20	21	18	17	22	20	16	24	23	25	22	25	25	25	23	20	20	23	15	9	10	4											
Cladosporium herbarum																															1	1	3		1	6	14	9	6	9	7	3	2	16	15	11	21	7
Cladosporium oxysporum																						1				1	1		2	1																		
Clasterosporium caricinum																																								3	1							
Drechslera biseptata																																				2			1			1				1	1	
Epicoccum purpurascens	3	2						5		4	2	4			2	3			1				1	1	2	3	4	13	10	7	9	2	3	1	3	7	5	2	9	17	5	1	7	6	9	10	4	6
Fusarium spp.	5	6	4	6				8	11	7	1	2	3	2	1	2	2	8	8	2	2	2	9	10	11	10	8	10	11	13	16	1	10	8	14	20	16	12	15	5	7	10	7	6	11	9	10	7
Gilmaniella humicola																					1																											
Gliocladium roseum	2	2	6					2	5	2	2	2	2	1	3	2	5	4	3	3	1		1	1	4	4	2	7	11	2	7	6	6	7	7	4	7	7	3	10	8	11	3	3	10	9	10	8
Gliomastix murorum																																									1					2		
Humicola fusco-atra	2							1	1	3	2	2	2		1	3	2	1						1		2	1	2	2			1	1	1	2			1	1		4	1	2		3		4	2
Hammaria echinobotryoides																					1																											
Monocillium indicum									1																		3																					
Mortierella vinacea			3							1	1	1	4	2	1	1	1		1			1						3		4		2	1				1	1		2	3	2	1	1			1	
Mucor hiemalis	7	1						2	1	1	5	3	2	1	2	4	3	5	2	5	1	1	5	13	10	10	8	16	13	15	15	15	12	13	9	6	8	5	4	10	11	10	7	9	10	11	6	7
Oidiodendron sp.			1																																													
Paecilomyces elegans												1							1				1																									
Papularia montagnei	4									3	5	3	4	3	2	1	2	2	2	5	1	4	1		2			1							1	2			1		2	1	4	1	3		1	1
Penicillium spp.	2		3					2	1	1		2	8	1	2	2	1	1	1		1	1	3	1	2	2	4		2				1	2			1				2	3	5	4	5	3	3	2
Phoma spp.		2						5	3	4	2	7	4	3	6	4	2	6	1		2	1	5				1	1						1			1		1			1	4	1	4		2	
Pyrenochaeta decipiens																															1				1													
Pythium sp.																									2					1																		
Rhinocladiella mansonii																							1																	1								
Scopulariopsis brevicaulis													1																																			
Stachylidium bicolor										1	1												1			1																	1					
Stachybotrys atra																																																
Thermomyces lanuginosus	2		2					1	1	1								1																	3		1			1	1			1	1			
Trichocladium asperum									1																																							
Trichoderma hamatum																																			1						1					1	1	
Trichoderma spp.		1																																														
Trichoderma viride	1	2									1			2				1			3	2			1		1		1			2									2	1	2		3			
Ulocladium botrytis			1					1		4			4		4		1										1														1	1		3		1	3	
Verticillium lateritium	1									2	1	1	1	1				1	2		1		1	3	1	2	2	1	4	1	1	1	2	2			1	1		1	1	2	1	1		1		
Volucrispora aurantiaca		1							1					1				1						2	1		3				2						1		1			1						
Volutella ciliata																				2			1							1													1	1		1		
Zygorhynchus moelleri													1	4	5	1		4	6	7	5		4	1	2	1	2	1		2		2	1	5	2	8	7	7	2	1			1					
													1																																			
Pycnidial forms		1																																														
Basidiomycetes								1	2	1	2	1	1	2	3	2	1					2		1	1	2			1	6	2	2	1	1	2			1	2		1				2	1	1	
														3								1	1	2	2	3	1			1	1																	
Total number of species	11	10	9	1				12	14	18	13	15	15	16	15	13	12	16	14	9	15	11	16	12	16	13	18	12	12	14	12	14	14	13	15	10	14	11	13	12	19	16	17	14	14	14	15	18
Total number of isolates	31	19	26	6				32	33	56	46	56	60	44	60	47	42	58	47	48	42	32	60	61	70	63	69	84	87	85	77	64	70	63	58	67	58	58	51	63	62	52	57	54	78	64	73	40

2,4,5T

	J 0	J 7	J 14	J 28	F 0	F 7	F 11	F 28	M 0	M 7	M 14	M 28	A 0	A 7	A 14	A 28	M 0	M 7	M 14	M 28	J 0	J 7	J 14	J 28
Acremoniella atra										1													1	
Alternaria alternata	2	1	4					1				1	1	1	2			1	1	1	2		2	3
Bipora antennata										5	1	2	2	1		2			2			1		1
Botryotrichum piluliferum																								
Botrytis cinerea										1					3	1					1			
Cephalosporium acremonium										1														
Chaetomium spirale																1								
Chrysosporium parvum		2							2															
Cladosporium cladosporioides								1	6	17	20	24	23	22	16	19	19	16	13	15	20	21	25	24
Cladosporium herbarum																								
Cladosporium oxysporum																			1					1
Clasterosporium caricinum												1		1										
Drechslera biseptata											1									1	1			
Echinobotryum atrum																								
Epicoccum purpurascens	4	7	3					3	3	1			1	1	1				1				1	1
Fusarium spp.	8	10	10	10				12	5	6	6	8	8	6	2	7	6	4	7	2	8	3	7	9
Geotrichum candidum																								
Gilmaniella humicola																			1					
Gliocladium roseum	3	2	3					6	2	5	9	2	3	3	3	1	5		2		4	3	2	3
Gliocladium salmonicolor											1			1		1				1				
Gliomastix murorum									1															
Humicola fusco-atra			1					2	5	3	2	3	4	5	2		2	2	1	2	1	1	1	
Humicola grisea	1							2		1	2	1	1					2						
Mammaria echinobotryoides																				1				
Monocillium indicum																							1	
Mortierella vinacea	2									1	3	1	2	2	1			2	1	3	1			
Mucor hiemalis	2	3						1	1	1	4					2	3	2	1				9	19
Paecilomyces elegans												1				1					2			
Papularia montagnei								3	1	5	4	1		6	4	9	5	5	4		4	3	2	1
Penicillium spp.	1	2	2					1	3	3	4		3	4	3	3		1	2	3	1	2		1
Phoma spp.	3	2	4					3	5	6	3	3	2	11	4	6	5	5	9	1	4	3	4	1
Pyrenochaeta decipiens								1						1						1	1			
Pythium sp.																								
Rhinocladiella mansonii													1	1	1									1
Scopulariopsis brevicaulis														1							1		1	
Stachylidium bicolor										1					1						1		2	
Sporotrichum aureum																								
Stachybotrys atra															1									
Stemphylium vesicarium										1	1													
Thermomyces lanuginosus									2		1										1			
Trichocladium asperum																								
Trichoderma hamatum														1										
Trichoderma spp.													2	1										2
Trichoderma viride	1	1	2							2		3	1				2				3		1	
Ulocladium botrytis			1					1	1					3	1	1	1		1				2	
Verticillium lateritium	1											1												
Volucrispora aurantiaca								6	1										1	3			1	1
Volutella ciliata									1				1	1	3	2	4	1	2				2	1
Pycnidial forms			2						4	1	1				1	1	14					2	1	5
Basidiomycetes																				2			1	
Total number of species	11	9	10	1				14	16	19	16	14	15	20	17	15	11	11	17	13	17	9	18	16
Total number of isolates	28	30	32	16				43	43	62	63	52	55	73	47	57	71	41	50	36	56	39	66	74

	J 0	J 7	J 14	J 28	A 0	A 7	A 11	A 28	S 0	S 7	S 14	S 28	O 0	O 7	O 14	O 28	N 0	N 7	N 14	N 28	D 0	D 7	D 14	D 28
Acremoniella atra					2	2																		
Alternaria alternata	2	1		13	6	5	5	5	5	8	3	3	6	4	5	1	3	5	4	2	1	1	3	2
Bipora antennata									2								1							
Botryotrichum piluliferum																							1	
Botrytis cinerea																								
Cephalosporium acremonium					1																	1		
Chaetomium spirale																								
Chrysosporium parvum																								
Cladosporium cladosporioides	24	23	25	25	25	23	24	24	23	13	7	7	9		1	2	2							
Cladosporium herbarum								3																
Cladosporium oxysporum				3	1		2																	
Clasterosporium caricinum																								
Drechslera biseptata						1		3	4	6	1	2	5	2	2		2	1						
Echinobotryum atrum																			1					
Epicoccum purpurascens		2	2	7	7	14	10	8	9	13	7	10	12	14	14	11	15	1	4	11	7	4	12	8
Fusarium spp.	8	5	2	9	12	11	10	9	10	14	13	14	12	17	14	3	4	6	12	4	4	10	13	11
Geotrichum candidum												1	1											
Gilmaniella humicola																								
Gliocladium roseum	4		3	7	5	6	9	1	1	9	3	2	9	4	2	8	7	3	7	5	4	4	6	7
Gliocladium salmonicolor																								
Gliomastix murorum																			1					
Humicola fusco-atra	1				1			1	1		1			1		1	2	2			1			
Humicola grisea		1	1	1	1			1	1				2		1	1	3	4		1		4	2	1
Mammaria echinobotryoides																								
Monocillium indicum	1							1				1												
Mortierella vinacea			1	2	2		1	1								3		3		3	2			
Mucor hiemalis	13	11	17	22	18	12	8	4	9	4	5	3	4	4	5	12	14	7	8	6	4	7	11	6
Paecilomyces elegans					1				1															
Papularia montagnei	1		1				1	3		1	3			1			1	1		4	1		1	
Penicillium spp.	2	2	1		1			2		1	1			1		1	2	2	2	6	2	3	1	2
Phoma spp.	4										1							5	1		5	2		
Pyrenochaeta decipiens						1																		
Pythium sp.			1																					
Rhinocladiella mansonii			1									1	1		1									
Scopulariopsis brevicaulis																								
Stachylidium bicolor																								
Sporotrichum aureum																					1			
Stachybotrys atra																								
Stemphylium vesicarium																								
Thermomyces lanuginosus		1										1					3	2		2	1	4	1	
Trichocladium asperum											1													
Trichoderma hamatum							1									2	1							
Trichoderma spp.	1	2														1			1		2			
Trichoderma viride			5	1							1					1	1	1		2		2		
Ulocladium botrytis	1	5			2	1				1		1							1		2			
Verticillium lateritium	1						1							1								1		
Volucrispora aurantiaca	2					3	2	3	7	2	10	14	15	17	5	14	6		20	17	18	20	23	19
Volutella ciliata			1			3	1			4	7	4	1	3	1		1							
Pycnidial forms	1	2	6	3	3	6	1	2	3	3	2			1	1	1	1							
Basidiomycetes						1	2																	
Total number of species	15	11	14	11	16	14	15	16	13	13	16	13	12	14	12	15	18	14	12	12	15	13	11	8
Total number of isolates	66	55	67	93	88	89	78	71	76	77	66	63	77	71	52	62	69	43	62	63	55	63	74	56

APPENDIX 4. List of Fungi Isolated from Leaf Litter

Acremoniella atra (Corda) Sacc.

Alternaria alternata (Fr.) Keissler

Asteromyces cruciatus F. and Mme. Moreau

Bispora antennata (Pers.) Mason

Botryotrichum piluliferum Sacc. et Marchal

Botrytis cinerea Pers. ex Fr.

Brachysporium sp.

Cephalosporium acremonium Corda

Chaetomium globosum Kunze

Chaetomium spirale Zopf

Chrysosporium luteum Constantin

Chrysosporium pannorum (Link) Hughes

Chrysosporium parvum Emmons et Ashburn

Cladosporium cladosporioides (Fres.) de Vries

Cladosporium herbarum Link ex Fr.

Cladosporium oxysporum Burk. et Curt.

Clasterosporium caricinum Schweinitz

Cylindrocarpon scoparium Morgan

Dactylella minuta Grove

Doratomyces stemonitis Corda

Drechslera biseptata (Sacc. et Roum.) Richardson et Fraser

Echinobotryum atrum Corda

Epicoccum purpurascens Ehren. ex Schlect

Fusarium spp.

Geotrichum candidum Link

Gilmaniella humicola Barron

Gliocladium roseum Bainier

Gliocladium salmonicolor Raillo

Gliomastix mumorum (Corda) Hughes

Graphium penicillioides Corda

Humicola fusco-atra Traaen

Humicola grisea Traaen

Mammaria echinobotryoides Cesati

Monocillium indicum Saksena

Mortierella vinacea Dixon-Stewart

Mucor hiemalis Wehmer

Oidiodendron sp.

Paecilomyces elegans (Corda) Mason et Hughes

Papularia montagnei

Penicillium spp.

Phoma spp.

Polyscytalum sp.

Pyrenochaeta decipiens Marchal

Pythium sp.

Rhinocladiella mansonii (Castell.) Schol et Schwarz

Rhinotrichum tenellum Szilvinyi

Rhizopus oryzae Went et Gerlings

Scolecobasidium constrictum Barron et Busch

Scopulariopsis brevicaulis (Sacc.) Bain

Sporotrichum aureum Link

Stachybotrys atra Corda

Stachylidium bicolor Link

Stemphylium vesicarum (Wallr.) Simmons

Thermomyces lanuginosus Tsiklinsky

Trichocladium asperum Hartz

Trichoderma hamatum (Bon.) Bain

Trichoderma viride Pers. ex Fr.

Trichoderma spp.

Ulocladium botrytis Preuss

Verticillium lateritium Berk

Volucrispora aurantiaca Haskins

Volutella ciliata (Alb. et Schw.) Fr.

Zygorhynchus moelleri Vuill.

APPENDIX 5. The Major Fungi that grew on or from Washed Leaf Litter Segments on CA at 25°C

Records of FR numbers are from a total of 450 leaf segments, and of 3 x FR are from a total of 700 leaf segments. The numbers, expressed as a percentage of CONTROL, are given in parentheses.

	FR						3 x FR					
	CON	C	V	M	P	T	CON	C	V	M	P	T
Alternaria alternata	14	12 (86)	16 (114)	14 (100)	23 (163)	16 (114)	96	385 (401)	262 (273)	81 (84)	139 (145)	98 (102)
Cladosporium cladosporioides	223	177 (79)	186 (83)	232 (104)	229 (104)	211 (95)	367	170 (46)	411 (112)	291 (79)	319 (87)	347 (95)
Epicoccum purpurascens	25	33 (132)	27 (108)	27 (108)	56 (224)	25 (100)	224	68 (30)	62 (28)	146 (65)	192 (86)	186 (83)
Fusarium spp.	85	38 (45)	56 (66)	78 (92)	148 (174)	117 (138)	183	38 (21)	71 (39)	263 (144)	202 (110)	243 (133)
Ulocladium botrytis	14	16 (114)	16 (114)	10 (71)	8 (57)	10 (71)	18	99 (550)	411 (2283)	31 (172)	24 (133)	16 (89)

APPENDIX 6a. Effect of Temperature on Linear Extension Rate of Basidiomycetes (means of 5 replicates)

Basidiomycete 235M

Temperature °C	Colony Diameter (mm) over 8 days						
	1	2	3	4	5	6	8
5	7.7	8.6	9.5	10.1	10.9	11.8	18.6
10	9.1	11.9	14.3	17.9	21.9	27.2	32.1
15	9.8	14.4	18.1	22.3	27.0	31.8	41.5
20	11.8	18.3	24.7	30.4	37.1	45.4	57.8
25	13.5	23.8	32.9	42.3	53.7	63.7	82.7
30	15.3	28.6	38.4	48.8	60.2	72.4	84.0
35	12.5	22.3	32.8	44.0		62.5	77.5

Boletus variegatus

Temperature °C	Colony Diameter (mm) over 22 days				
	4	8	12	17	22
5	-	-	1.2	2.8	4.1
10	0.65	5.6	13.7	21.9	28.9
15	0.25	5.0	11.1	18.0	33.2
20	3.45	14.0	28.4	42.9	58.3
25	7.7	15.8	29.1	42.1	52.9
30	-	-	-	-	-
35	-	-	-	-	-

Coprinus comatus

Temperature °C	Colony Diameter (mm) over 12 days							
	2	3	4	5	6	8	10	12
5	-	-	-	-	7.0	12.3	12.9	13.1
10	-	7.5	9.7	13.1	17.7	21.3	25.7	29.2
15	7.0	9.6	12.5	14.7	18.6	27.6	38.4	46.3
20	10.3	16.9	20.0	24.9	32.1	46.1	56.3	64.5
25	13.7	17.3	23.0	29.4	37.2	54.7	74.3	84.0
30	11.6	15.0	18.1	22.4	29.3	40.3	50.0	56.5

Cyathus stercoreus							
Temperature °C	Colony Diameter (mm) over 8 days						
	1	2	3	4	5	6	8
5	7.7	8.7	9.5	10.1	10.9	11.8	18.6
10	9.1	11.9	14.3	17 9	21.9	27.2	32.1
15	9.8	14.4	18.1	22.3	27.0	31.8	41.5
20	11.8	18.3	24.7	30.9	37.1	45.5	57.8
25	13.5	23.8	32.9	42.3	53.7	63.7	82.7
30	15.3	28.6	38.4	48.8	60.2	72.4	84.0
35	12.5	22.3	32.8	44.0	NT	62.5	77.5

Paxillus involutus						
Temperature °C	Colony Diameter (mm) over 30 days					
	8	12	16	20	24	30
5	-	-	-	3.3	7.0	7.5
10	3.3	6.6	12.0	20.4	26.7	34.5
15	2.1	4.4	11.6	18.3	28.4	43.6
20	-	3.9	11.5	2 .7	36.6	54.2
25	6.0	7.7	11.9	13.0	15.6	19.3
30	-	-	-	-	-	-

APPENDIX 6b. Mycelial Dry Weight, in mg, of Basidiomycetes at their Optimum Temperature for Growth.

(values are the means of five replicates)

Basidiomycete 235M

Days	1	2	3	4	5
Dry wt. in mg	8.3	20.1	41.6	75.2	92.4

Boletus variegatus

Days	4	8	12	16	20	24
Dry wt. in mg	3.7	3.3	26.9	49.9	62.9	62.5

Coprinus comatus

Days	3	6	9	12	15	18	21
Dry wt. in mg	3.9	7.2	19.1	42.4	47.0	48.5	62.8

Cyathus stercoreus

Days	2	4	6	8	10	12	14
Dry wt. in mg	7.8	32.7	44.9	67.6	71.1	71.5	72.5

Paxillus involutus

Days	4	8	12	16	20	24
Dry wt. in mg	4.3	7.2	10.0	30.9	48.9	64.2

Phallus impudicus

Days	4	8	12	16	20	24	28
Dry wt. in mg	0.9	1.9	6.2	18.9	30.1	43.6	46.3

APPENDIX 7. The Effect of Increasing Concentrations of Agrochemicals on Linear Extension of Basidiomycetes.

(values are the means of five replicates)

- denotes no growth

NT denotes not tested

Basidiomycete 235M							
Days		1	2	3	4	5	6
CAPTAN (ppm)	0	10.4	23.2	34.4	53.9	72.6	79.0
	10	9.3	19.3	32.4	51.2	68.7	79.0
	25	8.4	18.6	29.1	49.5	64.2	72.0
	50	7.2	16.4	23.0	44.9	52.4	63.9
	75	6.1	12.8	16.5	36.5	44.9	51.2
	100	4.3	9.2	12.4	28.7	34.6	39.4
Days		1	2	3	4	5	6
VERDASAN (ppm)	0	11.5	25.0	35.0	55.8	73.3	79.0
	0.1	10.4	23.0	32.0	55.3	72.5	79.0
	0.5	9.7	21.3	35.0	51.6	62.4	70.4
	0.75	5.0	13.3	23.0	14.3	16.4	18.4
	1.0	4.3	13.7	11.2	12.2	14.8	16.5
	2.5	-	-	-	-	-	-
Days		1	2	3	5	6	7
MAZIDE (ppm)	0	6.9	24.3	38.6	79.0	79.0	79.0
	100	6.0	22.0	35.7	79.0	79.0	79.0
	250	6.0	19.3	30.8	64.5	70.9	76.8
	500	5.1	16.9	27.5	49.0	53.1	60.8
	1000	4.3	14.4	21.5	32.6	33.2	35.0
	2000	3.2	10.1	15.5	21.0	21.7	22.9
	3000	-	-	-	-	-	-

Days		1	2	4	5	6	8
PARAQUAT (ppm)	0	8.2	13.4	24.4	30.0	37.3	48.2
	10	10.0	17.3	34.7	43.8	54.8	71.4
	25	3.1	6.8	18.0	23.2	28.9	39.5
	50	3.0	4.2	4.4	4.7	4.8	4.8
	100	1.7	1.7	1.7	1.7	1.7	1.7
	250	-	-	-	-	-	-
	500	-	-	-	-	-	-

Boletus variegatus						
Days		4	8	12	17	22
CAPTAN (ppm)	0	7.7	15.8	29.1	42.1	52.9
	10	6.2	13.4	26.2	38.3	48.6
	25	2.4	8.2	18.3	29.9	39.8
	50	2.8	8.6	18.3	27.0	36.0
	75	–	–	–	–	–
	100	–	–	–	–	–
Days		4	6	11	15	19
VERDASAN (ppm)	0	10.2	17.4	31.4	41.9	49.4
	0.1	5.9	8.8	18.4	24.3	29.4
	0.5	2.4	4.1	6.0	10.9	–
	0.75	–	–	–	–	–
	1.0	–	–	–	–	–
	2.5	–	–	–	–	–
Days		4	6	11	15	19
MAZIDE (ppm)	0	10.2	17.4	31.4	41.9	49.4
	100	7.7	12.7	27.3	35.5	41.0
	250	5.9	10.3	23.1	32.0	37.3
	500	3.5	7.2	14.1	17.9	21.0
	1000	2.2	4.8	11.4	15.7	19.4
	2000	0.5	2.3	5.9	7.8	9.7
	3000	–	–	2.3	2.8	3.9

Days		4	8	12	17	19
PARAQUAT (ppm)	0	7.7	15.8	29.1	42.1	58.6
	10	-	-	-	-	-
	25	-	-	-	-	-
	50	-	-	-	-	-
	100	-	-	-	-	-
	250	-	-	-	-	-
	500	-	-	-	-	-

Coprinus comatus						
Days		2	5	7	9	
CAPTAN (ppm)	0	11.3	41.0	62.0	72.0	
	10	11.2	36.0	57.7	58.5	
	25	9.7	31.0	47.7	60.9	
	50	6.9	17.4	33.0	41.2	
	75	6.3	11.7	15.1	17.0	
	100	6.1	11.4	12.1	16.2	
Days		2	4	6	8	11
VERDASAN (ppm)	0	7.0	17.1	28.2	37.5	51.5
	0.1	6.8	14.9	26.6	38.6	54.8
	0.5	3.5	9.4	18.3	30.5	46.2
	0.75	2.5	8.1	15.4	27.2	42.1
	1.0	2.1	7.4	13.2	22.5	37.1
	2.5	-	-	-	-	-
Days		2	4	6	8	9
MAZIDE (ppm)	0	5.7	19.7	33.3	49.0	56.2
	100	5.3	19.0	32.1	46.3	52.9
	250	5.0	19.4	31.5	47.1	54.4
	500	4.0	19.1	32.3	47.4	54.5
	1000	4.3	20.1	34.3	51.0	56.5
	2000	4.1	20.6	33.8	49.8	58.1
	3000	3.5	18.3	28.3	42.2	49.3

Days		2	3	5	7	10
PARAQUAT (ppm)	0	7.5	12.9	24.9	41.9	71.8
	10	6.5	12.0	26.3	46.7	79.0
	25	5.1	10.3	23.1	39.2	66.5
	50	3.1	5.4	12.7	21.8	40.7
	100	1.5	2.4	8.0	13.9	28.1
	250	–	–	1.5	3.9	10.8
	500	–	–	–	–	–

Cyathus stercoreus							
Days		1	2	3	5	6	7
CAPTAN (ppm)	0	6.0	16.0	26.6	46.3	56.5	66.0
	10	5.1	13.7	22.7	41.1	51.1	59.8
	25	2.7	9.8	16.5	33.2	43.3	52.0
	50	1.1	2.2	7.3	20.1	28.9	37.0
	75	0.7	1.6	4.1	12.2	18.6	24.3
	100	0.5	1.3	2.8	6.7	11.6	16.8
Days		1	2	3	4	6	8
VERDASAN (ppm)	0	4.6	13.0	22.6	32.7	53.4	72.9
	0.1	4.2	10.5	12.9	13.4	25.5	38.7
	0.5	3.6	8.1	9.4	10.8	16.4	21.2
	0.75	1.5	4.3	5.6	7.2	9.9	11.7
	1.0	-	-	-	-	-	-
	2.5	-	-	-	-	-	-
Days		1	2	3	4	6	7
MAZIDE (ppm)	0	5.8	14.5	25.7	36.5	57.2	70.9
	100	5.9	14.3	25.0	35.3	55.3	68.8
	250	5.3	13.7	24.0	33.9	52.2	64.4
	500	4.4	11.8	21.1	29.7	44.5	53.5
	1000	3.8	10.2	18.3	25.3	38.9	45.2
	2000	2.3	6.8	14.5	20.5	29.1	33.9
	3000	2.2	5.0	9.2	12.7	18.8	20.6

Days		2	3	5	6	7
PARAQUAT (ppm)	0	14.5	23.8	42.6	61.5	69.5
	10	6.1	12.9	24.2	33.7	40.3
	25	2.2	2.9	2 9	2.9	2.9
	50	1.3	1.5	1.8	1.8	1.8
	100	0.7	0.7	0.9	0.9	0.9
	250	-	-	-	-	-
	500	-	-	-	-	-

Paxillus involutus							
Days		4	8	12	17	22	
CAPTAN (ppm)	0	4.1	15.9	35.5	55.4	62.4	
	10	NT	NT	NT	NT	NT	
	25	0.6	3.1	6.6	16.3	19.3	
	50	–	–	1.1	2.1	4.4	
	75	–	–	–	–	–	
	100	–	–	–	–	–	
Days		4	8	13	16	20	28
VERDASAN (ppm)	0	7.7	10.8	13.8	16.4	18.1	23.1
	0.1	6.2	7.1	8.0	9.3	10.0	17.1
	0.5	–	–	–	–	–	–
	0.75	–	–	–	–	–	–
	1.0	–	–	–	–	–	–
	2.5	–	–	–	–	–	–
Days		7	11	16	23	30	39
MAZIDE (ppm)	0	8.2	11.5	15.0	21.1	26.7	34.7
	100	14.0	25.5	35.3	44.0	54.5	67.8
	250	6.3	9.2	13.8	21.9	29.9	39.3
	500	13.5	23.6	32.3	41.6	51.4	57.7
	1000	6.9	11.0	15.0	21.0	29.3	35.1
	2000	6.3	8.0	9.4	13.4	19.8	27.3
	3000	6.2	7.6	9.6	15.2	19.5	22.4

Days		4	8	13	16	20	28
PARAQUAT (ppm)	0	7.7	10.8	13.8	16.4	18.1	23.1
	10	-	-	-	-	-	-
	25	-	-	-	-	-	-
	50	-	-	-	-	-	-
	100	-	-	-	-	-	-
	250	-	-	-	-	-	-
	500	-	-	-	-	-	-

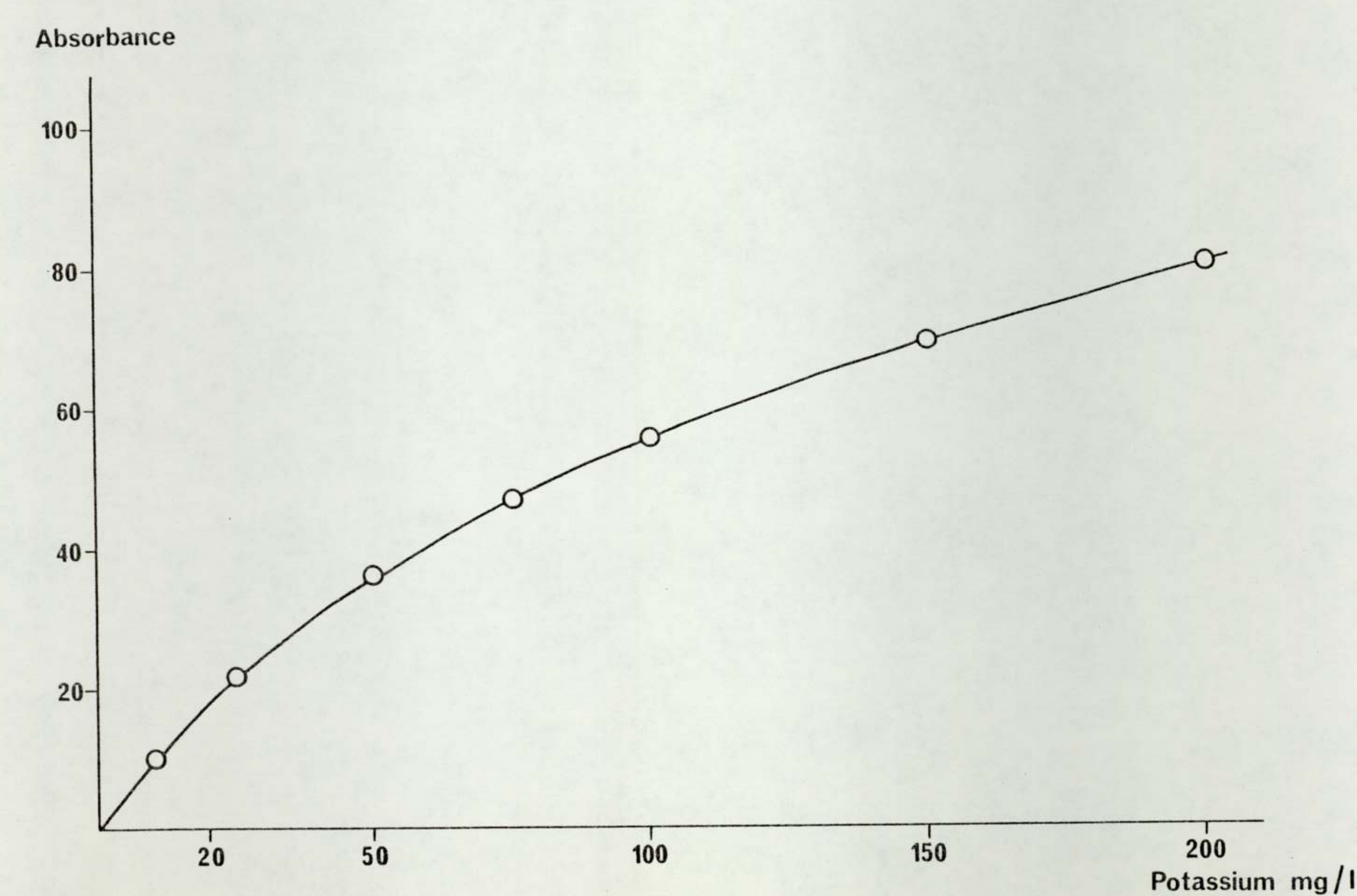
Russula emetica							
Days		7	11	16	26	30	39
CAPTAN (ppm)	0	3.1	8.4	14.7	29.2	36.6	49.8
	10	-	-	-	3.3	5.0	8.5
	25	-	-	-	-	-	-
	50	-	-	-	-	-	-
	75	-	-	-	-	-	-
	100	-	-	-	-	-	-
Days		6	11	16	25	32	39
VERDASAN (ppm)	0	-	6.5	15.1	29.8	39.2	49.3
	0.1	-	2.2	6.5	14.6	23.3	34.4
	0.5	-	-	-	3.3	8.5	13.5
	0.75	-	-	-	-	-	-
	1.0	-	-	-	-	-	-
	2.5	-	-	-	-	-	-
Days		7	11	16	25	30	39
MAZIDE (ppm)	0	3.5	8.8	14.4	28.2	34.2	48.0
	100	3.2	6.4	11.6	24.3	33.1	48.8
	250	3.6	7.9	12.9	25.4	32.4	48.8
	500	0.2	2.9	6.6	16.5	22.1	32.8
	1000	-	1.1	3.0	8.9	12.1	18.6
	2000	-	-	0.6	3.2	4.5	6.9
	3000	-	-	-	1.3	1.8	3.0

APPENDIX 8 . The Effect of Increasing Agrochemical Concentration on Dry Weight Production of Basidiomycetes

(means of 5 replicates)

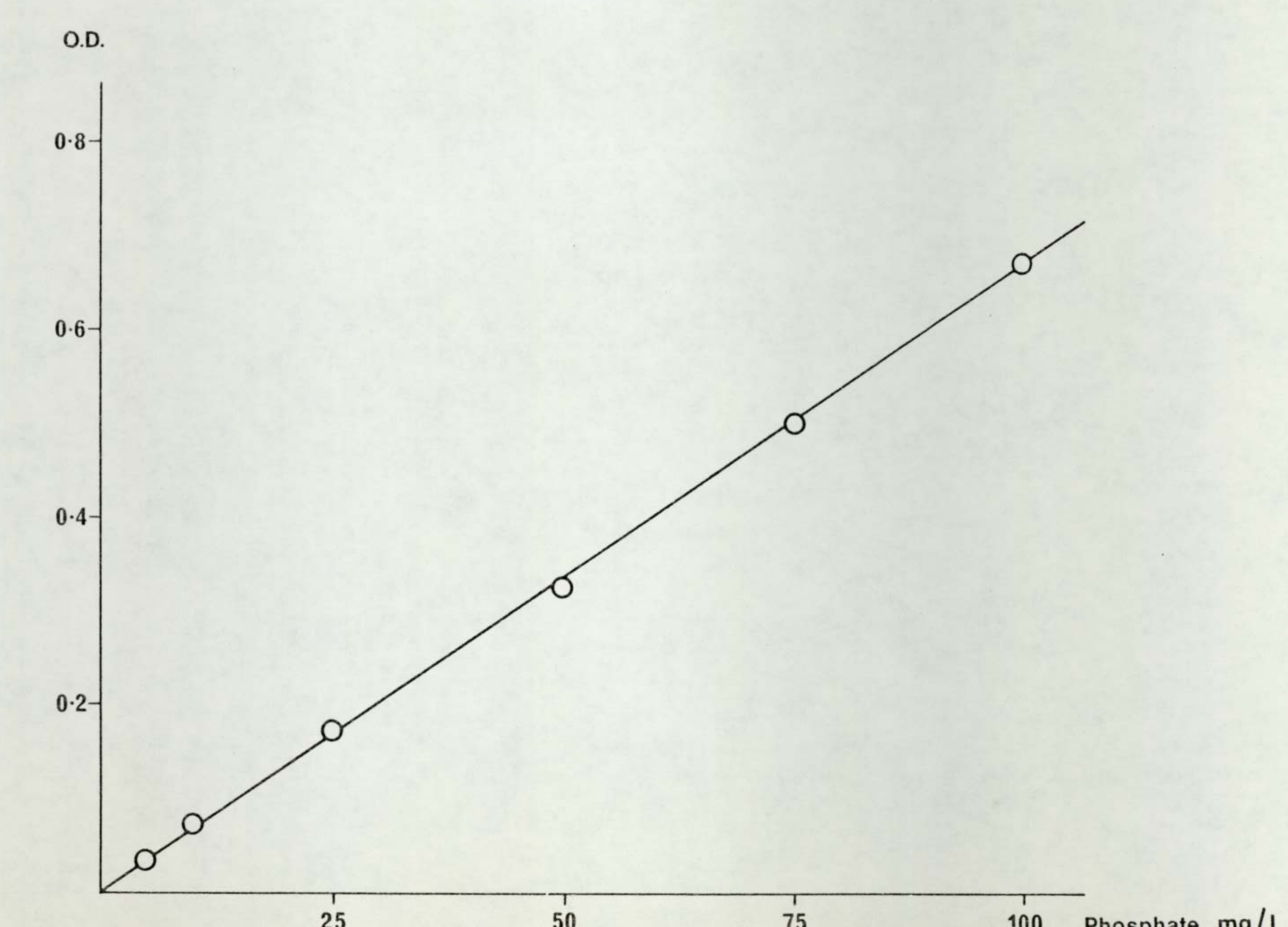
AGROCHEMICAL		Basidiomycete 235M	Boletus variegatus	Coprinus comatus	Cyathus stercoreus	Paxillus involutus	Phallus impudicus
MAZIDE (ppm)	0	53.4	48.2	34.8	39.1	42.2	34.1
	500	62.4	58.2	34.6	50.7	42.5	7.2
	1000	50.2	23.1	44.9	55.8	43.6	6.2
	2000	30.7	6.2	34.6	54.0	36.8	-
	3000	16.3	2.0	35.3	58.6	16.6	-
	4000	13.1	3.6	21.3	55.9	11.3	-
	8000	8.3	-	23.6	38.8	1.9	-
	F	38.8	91.8	5.0	25.2	52.7	145.0
	P	<1%	<1%	<1%	<1%	<1%	<1%
	L.S.D.(5%)	10.3	7.2	4.7	10.3	6.8	3.0
	L.S.D.(1%)	15.9	9.8	6.3	13.8	9.2	4.0
PARAQUAT (ppm)	0	41.7	73.8	37.4	33.0	27.4	36.1
	5	38.3	28.1	36.5	4.4	3.0	-
	10	23.0	-	30.0	-	-	-
	25	15.1	-	18.0	-	-	-
	50	11.2	-	16.8	-	-	-
	100	12.4	-	-	-	-	-
	250	-	-	-	-	-	-
	F	35.5	810.6	110.5	376.9	99.8	157.5
	P	<1%	<1%	<1%	<1%	<1%	<1%
	L.S.D.(5%)	7.4	2.9	4.3	1.8	3.0	3.2
	L.S.D.(1%)	9.9	3.9	5.8	2.5	4.0	4.2
VERDASAN (ppm)	0	69.5	52.2	33.7	33.9	41.0	19.9
	0.1	37.7	-	34.0	25.1	-	8.0
	0.25	30.6	-	25.2	8.3	-	1.2
	0.5	7.5	-	19.4	5.4	-	-
	0.75	6.6	-	10.8	2.3	-	-
	1.0	4.6	-	6.0	-	-	-
	2.5	2.6	-	3.9	-	-	-
	F	83.5	2088	77.4	88.3	3577	57.9
	P	<1%	<1%	<1%	<1%	<1%	<1%
	L.S.D.(5%)	7.9	1.3	4.1	4.1	0.75	2.9
	L.S.D.(5%)	10.6	1.7	5.6	5.6	1.0	3.9

APPENDIX 9. Standard Curve of Potassium Assay using Potassium Chloride (KCl).



Absorbance
100
80
60
40
20
20
50
100
150
200
Potassium mg/l

APPENDIX 10. Standard Curve of Phosphate Assay using Potassium dihydrogen orthophosphate (KH_2PO_4) and the $H_2PO_4^-$ anion.

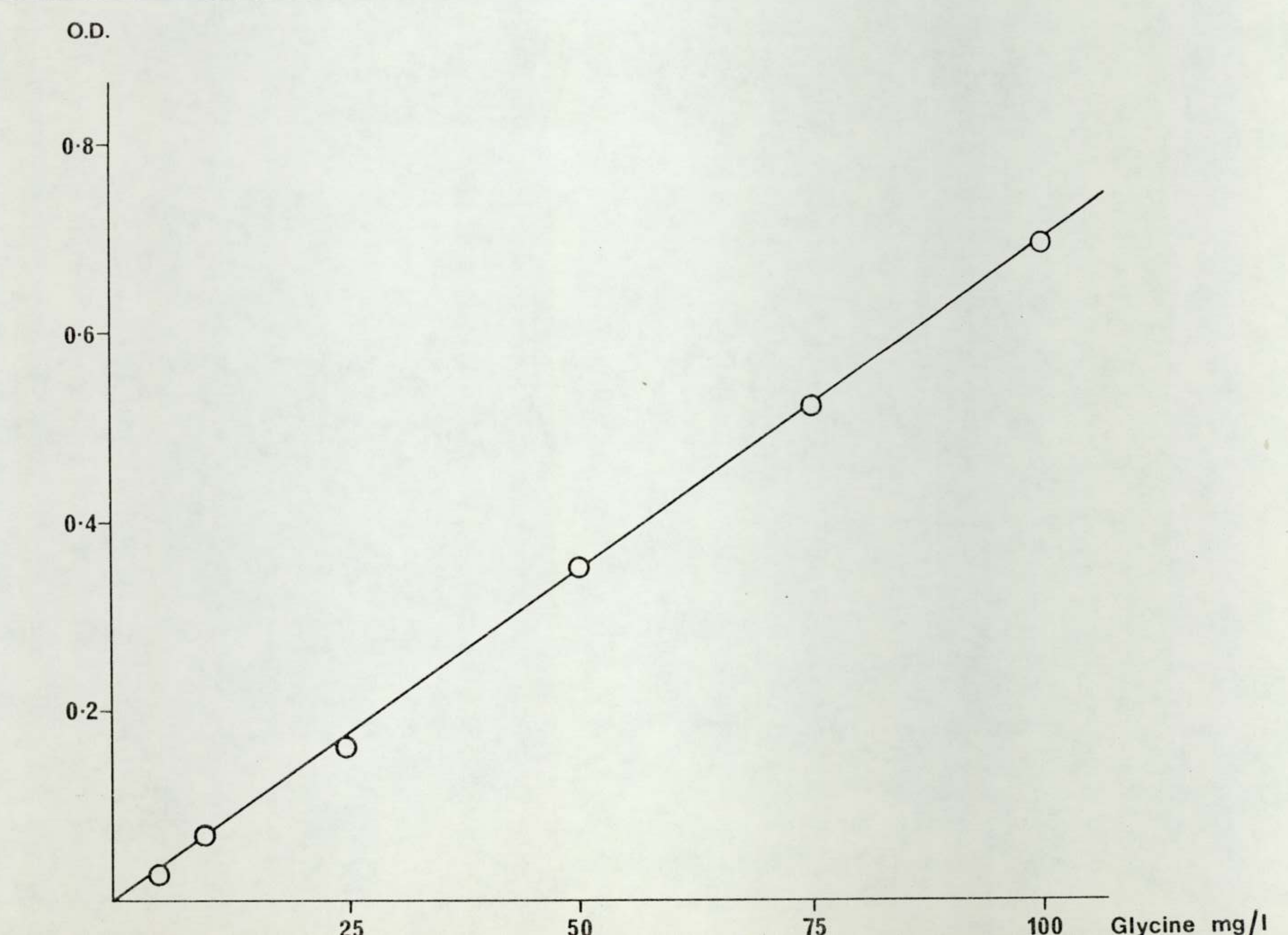

O.D.
0·8
0·6
0·4
0·2
25
50
75
100
Phosphate mg/l

APPENDIX 11. REMOVAL OF PARAQUAT FROM ASSAY SOLUTION PRIOR TO PHOSPHATE ANALYSIS

A modification of the method used by Calderbank and Yuen (1964) to remove PARAQUAT by cation exchange was used. 20 g of Zerolit 225 (RC 14) cation exchange exchange resin (BDH Chemicals) was washed with three changes of 100 ml of distilled water, and the water decanted off after each wash. The resin was then continuously stirred for 20 minutes, with 200 ml of 2N hydrochloric acid. After the acid was decanted off, the resin was again washed with three changes of 100 ml of distilled water and then stirred continuously for 20 minutes with 100 ml of 2.5% ammonium chloride solution. The charged resin was ready to use after three further washes with 100 ml of distilled water and was stored beneath a layer of distilled water.

2 ml aliquots of the assay solution containing PARAQUAT were each placed into test tubes, and a spatula-full (about 0.4 g) of charged resin added. The tubes were shaken every 5 minutes for 1 hour, after which 1 ml of the supernatant was removed for phosphate analysis.

APPENDIX 12. Standard Curve of Amino Acids Assay using Glycine as the Standard.



O.D.
0·8
0·6
0·4
0·2
25
50
75
100
Glycine mg/l

APPENDIX 13. The Effects of Increasing Concentrations of Agrochemicals on the Leakage of Metabolites from Basidiomycetes.

APPENDIX 13a. <u>The Effects of Increasing Concentrations of Agrochemicals on the Leakage of Potassium from Basidiomycetes</u>

Amounts of potassium leaked, in µM potassium/mg dry weight of mycelium, are the means of 5 replicates.

	Agrochemical concentration	Basidiomycete 235M	Boletus variegatus	Cyathus stercoreus
PARAQUAT (ppm)	0	22.4	6.3	9.4
	50	27.1	23.5	11.3
	100	28.5	29.6	14.3
	250	32.8	32.3	20.7
	500	33.7	33.0	21.1
	1000	32.9	32.5	26.5
SODIUM AZIDE (ppm)	0	12.3	5.9	2.0
	0.33	13.7	9.2	2.7
	0.65	14.7	15.9	5.2
	6.5	17.9	20.6	19.1
	32.5	15.6	21.3	20.3
	65.1	14.9	21.9	32.3
VERDASAN (ppm)	0	11.7	5.3	7.7
	0.1	10.8	14.6	9.4
	1.0	13.2	18.8	12.2
	5.0	23.1	29.9	15.3
	10.0	19.0	35.7	20.0
	50.0	16.0	37.3	21.6

APPENDIX 13b. Effects of Increasing Concentrations of Agrochemicals on the Leakage of Phosphate from Basidiomycetes

Amounts of phosphate leaked, in µM phosphate/mg dry weight of mycelium, are the means of 5 replicates.

	Agrochemical concentration	Basidiomycete 235M	Boletus variegatus	Cyathus stercoreus
PARAQUAT (ppm)	0	23.0	3.6	6.9
	50	27.7	35.5	7.5
	100	29.0	49.4	9.2
	250	31.2	55.6	12.9
	500	35.5	71.8	12.1
	1000	36.7	80.3	21.0
SODIUM AZIDE (ppm)	0	18.4	2.0	3.7
	0.33	20.7	11.4	4.7
	0.65	22.0	13.4	8.4
	6.5	28.0	27.8	14.1
	32.5	24.4	32.3	15.7
	65.1	24.0	34.4	20.2
VERDASAN (ppm)	0	7.2	2.4	7.6
	0.1	7.3	21.5	11.0
	1.0	9.3	33.9	12.4
	5.0	11.5	50.7	16.2
	10.0	11.9	50.8	18.1
	50.0	18.1	61.6	15.4

APPENDIX 13c. The Effects of Increasing Concentrations of Agrochemicals on the Leakage of Amino Acids from Basidiomycetes

Amounts of amino acids leaked, in µM amino acid/mg dry weight of mycelium, are the means of 5 replicates.

	Agrochemical concentration	Basidiomycete 235M	Boletus variegatus	Cyathus stercoreus
PARAQUAT (ppm)	0	7.9	1.8	0.603
	50	13.9	4.2	3.4
	100	23.4	13.2	5.9
	250	24.0	20.2	11.2
	500	33.3	32.0	11.9
	1000	44.6	47.3	21.0
SODIUM AZIDE (ppm)	0	6.3	1.6	0.0
	0.33	12.5	3.3	0.20
	0.65	15.9	10.1	0.40
	6.5	18.0	12.6	7.1
	32.5	16.0	15.8	7.5
	65.1	13.0	14.3	11.3
VERDASAN (ppm)	0	10 9	1.3	1.3
	0.1	15.1	1.7	1.9
	1.0	25.4	10.8	4.7
	5.0	56.2	39.4	9.8
	10.0	47.4	47.1	12.2
	50.0	58.6	29.7	18.6

APPENDIX 14. Time Course of Metabolite Leakage from Basidiomycetes in the Presence of Agrochemicals.

APPENDIX 14a. Time Course of Potassium Leakage from Basidiomycetes in the Presence of Agrochemicals

The amounts are expressed as μM potassium/mg dry weight of mycelium and are the means of 5 replicates. The amounts from CONTROL flasks are shown in parentheses.

	Time (hours)	Basidiomycete 235M	Boletus variegatus	Cyathus stercoreus
PARAQUAT (1000 ppm)	0	0.44(0.65)	1.4 (0.54)	3.3 (2.5)
	1	9.6 (10.7)	10.5 (5.4)	14.4 (8.9)
	2	13.9 (13.4)	13.6 (9.5)	17.5 (9.1)
	4	15.7 (15.3)	17.6 (10.4)	21.1 (10.3)
	8	12.7 (13.9)	22.8 (14.6)	26.2 (12.5)
	18	11.4 (10.7)	25.3 (13.2)	31.8 (11.3)
	24	12.9 (11.9)	25.8 (13.8)	27.4 (10.3)
SODIUM AZIDE (65.1 ppm)	0	0.24(0.65)	3.4 (3.0)	0.83(0.61)
	1	10.9 (10.7)	13.4 (9.1)	5.1 (1.5)
	2	12.8 (13.4)	14.6 (9.8)	8.0 (3.2)
	4	15.1 (15.3)	21.1 (12.5)	12.0 (4.1)
	8	12.9 (13.9)	20.2 (12.2)	15.6 (4.0)
	18	11.6 (10.7)	20.2 (15.3)	18.8 (3.1)
	24	12.0 (11.9)	15.7 (15.0)	20.2 (1.3)
VERDASAN (50 ppm)	0	0.0 (0.65)	1.5 (0.54)	4.1 (2.5)
	1	12.0 (10.7)	16.1 (5.4)	14.3 (8.9)
	2	14.6 (13.4)	22.0 (9.5)	15.2 (9.1)
	4	15.2 (15.3)	22.7 (10.4)	28.2 (10.3)
	8	20.6 (13.9)	23.0 (14.6)	23.7 (12.5)
	18	17.6 (10.7)	27.8 (13.2)	30.4 (11.2)
	24	13.8 (11.9)	28.3 (13.8)	27.6 (10.3)

APPENDIX 14b. Time Course of Phosphate Leakage from Basidiomycetes in the Presence of Agrochemicals

The amounts are expressed as µM phosphate/mg dry weight of mycelium and are the means of 5 replicates. The amounts from CONTROL flasks are shown in parentheses.

	Time (hours)	Basidiomycete 235M	Boletus variegatus	Cyathus stercoreus
PARAQUAT (1000 ppm)	0	0.0 (0.0)	0.091(0.0)	3.0 (2.9)
	1	13.8 (14.8)	6.16(5.5)	10.6 (9.9)
	2	22.3 (14.7)	12.7 (12.2)	14.6 (10.6)
	4	23.3 (17.3)	19.2 (15.4)	18.7 (13.0)
	8	21.1 (16.3)	23.6 (22.7)	23.0 (15.5)
	18	19.4 (14.9)	34 6 (19.7)	25.5 (15.5)
	24	20.8 (17.0)	41.0 (21.8)	26.4 (12.8)
SODIUM AZIDE (65.1 ppm)	0	0.0 (0.0)	2.2 (1.9)	0.0 (0.43)
	1	14.7 (14.8)	7.02(5.7)	2.4 (2.9)
	2	16.8 (14.7)	11.0 (8.1)	3.0 (4.1)
	4	23.6 (17.3)	20.0 (13.6)	7.4 (4.8)
	8	23.1 (16.3)	32.0 (15.3)	10.2 (4.5)
	18	18.9 (14.9)	31.6 (24.3)	15.3 (4.3)
	24	20.3 (17.0)	37.8 (21.1)	16.4 (3.4)
VERDASAN (50 ppm)	0	0.0 (0.0)	1.9 (0.0)	4.3 (2.9)
	1	13.9 (14.8)	12.3 (5.5)	12.9 (9.9)
	2	18.7 (14.7)	17.2 (12.2)	14.8 (10.6)
	4	20.7 (17.3)	26.3 (15.4)	21.1 (13.0)
	8	29.7 (16.3)	30.2 (22.7)	26.6 (15.5)
	18	26.8 (14.9)	32.5 (19.7)	29.6 (15.5)
	24	20.9 (17.0)	41.0 (21.8)	27.9 (12.8)

APPENDIX 14c. Time Course of Amino Acids Leakage from Basidiomycetes in the Presence of Agrochemicals

The amounts are expressed as μM amino acid/mg dry weight of mycelium and are the means of 5 replicates. The amounts from CONTROL flasks are shown in parentheses.

	Time (hours)	Basidiomycete 235M	Boletus variegatus	Cyathus stercoreus
PARAQUAT (1000 ppm)	0	0.0 (0.0)	0.40(0.24)	0.34(0.76)
	1	7.4 (7.8)	5.6 (3.2)	1.9 (2.7)
	2	8.9 (9.7)	8.3 (5.2)	2.7 (3.3)
	4	11.6 (9.8)	12.1 (5.5)	4.1 (3.0)
	8	10.5 (8.5)	17.9 (6.0)	6.7 (2.7)
	18	10.5 (8.0)	22.6 (1.8)	10.0 (1.0)
	24	13.5 (6.5)	31.3 (2.0)	13.0 (1.0)
SODIUM AZIDE (65.1 ppm)	0	0.0 (0.0)	1.3 (0.0)	0.18(0.0)
	1	7.5 (7.8)	4.2 (4.8)	2.7 (2.5)
	2	8.4 (9.7)	4.7 (5.3)	4.2 (3.3)
	4	12.5 (9.8)	5.5 (6.2)	8.2 (3.6)
	8	13.1 (8.5)	12.6 (4.9)	9.9 (3.0)
	18	10.8 (8.0)	14.7 (4.5)	15.3 (0.36)
	24	11.2 (6.5)	15.2 (4.6)	16.4 (0.0)
VERDASAN (50 ppm)	0	0.0 (0.0)	0.0 (0.24)	1.1 (0.76)
	1	5.6 (7.8)	13.4 (3.2)	3.5 (2.7)
	2	9.3 (9.7)	16.7 (5.2)	3.8 (3.3)
	4	9.9 (9.8)	37.0 (5.5)	5.5 (3.0)
	8	14.8 (8.5)	43.5 (6.0)	7.7 (2.7)
	18	13.7 (8.0)	42.4 (1.8)	14.3 (1.0)
	24	11.8 (6.5)	42.6 (2.0)	15.1 (1.0)

APPENDIX 14d. Time Course of Metabolite Leakage from Basidiomycetes in the Presence of Agrochemicals

Statistical data using analysis of variance method.

Potassium		235M	B.variegatus	C.stercoreus
PARAQUAT	F_1	0.11	354 **	378 **
	F_2	46.7 **	112 **	24.7 **
	$F_1 x F_2$	2.69	14.1 **	13.0
SODIUM AZIDE	F_1	0.03	183 **	1081 **
	F_2	23.3 **	10.6 **	58.7 **
	$F_1 x F_2$	1.0	2.67	42.1 **
VERDASAN	F_1	40.5 **	523 **	339 **
	F_2	36.6 **	19.3 **	40.7 **
	$F_1 x F_2$	20.9 **	1.6	22.9 **
Phosphate				
PARAQUAT	F_1	597 **	207 **	296 **
	F_2	108 **	5773 **	75.4 **
	$F_1 x F_2$	53.1 **	499 **	20.6 **
SODIUM AZIDE	F_1	496 **	4397 **	1047 **
	F_2	156 **	13.5 **	764 **
	$F_1 x F_2$	49.3 **	1.7	681 **
VERDASAN	F_1	329 **	252 **	201 **
	F_2	430 **	68.6 **	56.9 **
	$F_1 x F_2$	353 **	5.7 *	13.3 **
Amino Acids				
PARAQUAT	F_1	346 **	551 **	434 **
	F_2	30.4 **	62.7 **	63.4 **
	$F_1 x F_2$	56.8 **	94.3 **	143 **
SODIUM AZIDE	F_1	672 **	1751 **	19292 **
	F_2	35.1 **	219 **	126 **
	$F_1 x F_2$	34.1 **	301 **	305 **
VERDASAN	F_1	211 **	1351 **	748 **
	F_2	44.4 **	91.2 **	48.3 **
	$F_1 x F_2$	82.2 **	88.6 **	101 **

* significant at 1% level   ** significant at 0.1% level

F_1 denotes F ratio for time.

F_2 denotes F ratio for agrochemical treatment.

$F_1 x F_2$ denotes interaction between F_1 and F_2 (where treatment has different effects at different time periods).

APPENDIX 15. Reagents for Cellulase Assay

Cellulose Medium for C_x cellulase activity (Mandels and Reese, 1957)

KH_2PO_4	2.0 g
NH_4SO_4	1.4 g
Urea	0.3 g
$MgSO_4.7H_2O$	0.3 g
$CaCl_2$	0.3 g
$FeSO_4.7H_2O$	5.0 mg
$MnSO_4.H_2O$	1.6 mg
$Zn\ Cl_2$	1.7 mg
$CoCl_2$	2.0 mg
Difco Peptone	1.0 g
Whatman cellulose powder (CF 11)	10 g
Distilled water	1 litre

pH adjusted to 5.3 with 1N NaOH before autoclaving.

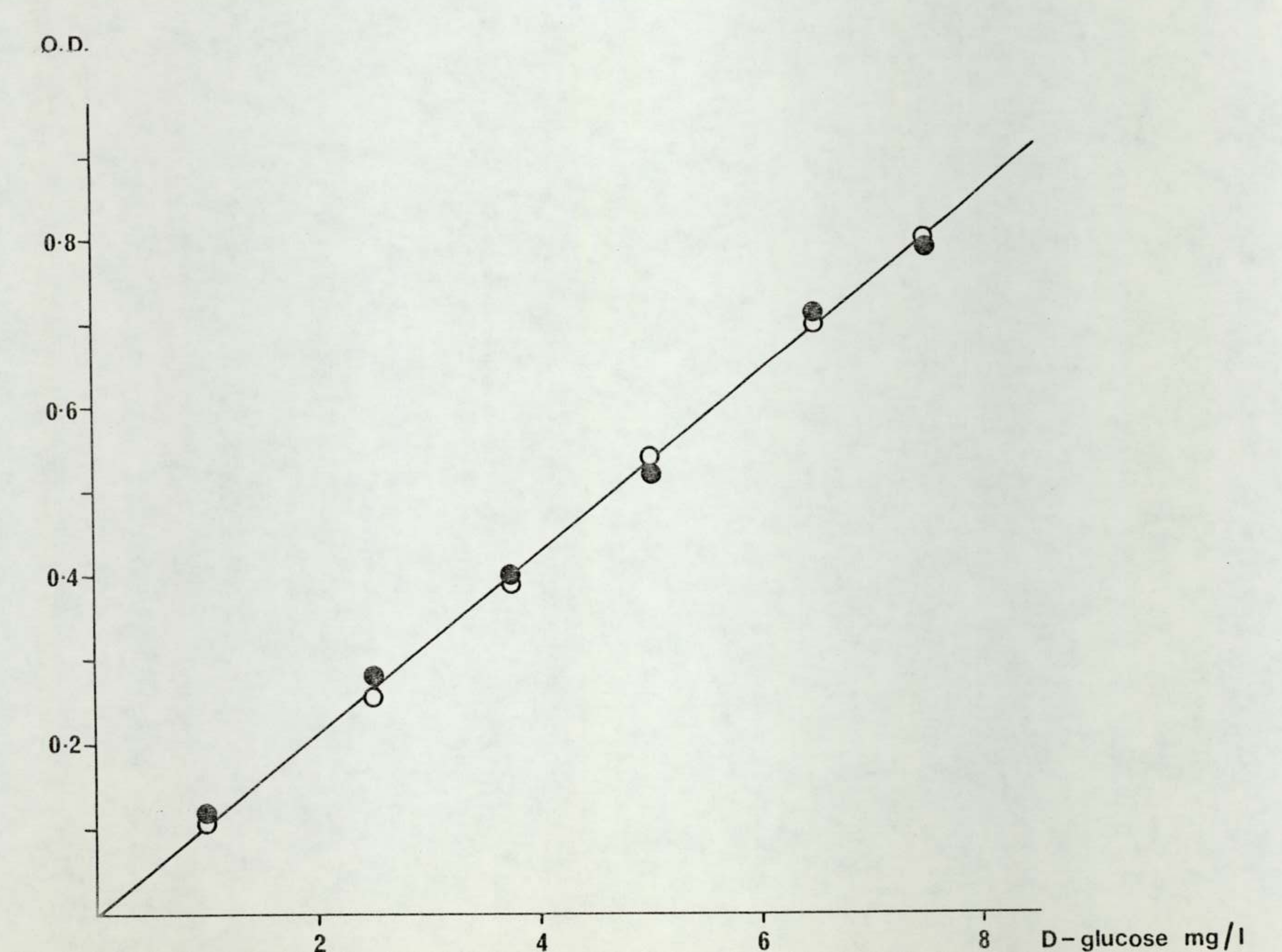
DNSA Reagent

3,5-dinitrosalicylic acid	1 g
Phenol	0.2 g
$Na_2(SO_4)_3$	0.05 g
NaOH	1 g
Sodium potassium tartrate	20 g
Distilled water	100 ml

APPENDIX 16. Standard Curve of Reducing Sugar Determination using D-glucose monohydrate.

○——○ standards without VERDASAN addition.

●——● standards with 50ppm of VERDASAN.



O.D.
0·8
0·6
0·4
0·2
2
4
6
8
D-glucose mg/l

APPENDIX 17. Effect of Temperature on Basidiomycete 235M C_x Cellulase Activity on CMC Substrate.

Amounts of reducing sugar produced (mg/l/day) are the means of 5 replicates.

Temperature °C	10	20	30	35	40	45	50	55	60	65
Reducing sugar	1.25	1.65	1.36	1.44	1.45	2.30	2.27	2.00	1.69	1.15

APPENDIX 18. Effect of pH on Activity of Basidiomycete 235M C_x Cellulase Activity on CMC Substrate.

Amounts of reducing sugar produced (mg/l/day) are the means of 5 replicates.

pH	3.5	4.3	4.6	5.0	5.4	5.8	6.2	7.0	7.5
Reducing sugar	0	0	0	0.9	1.97	1.96	1.93	1.34	1.33

APPENDIX 19 . Effect of VERDASAN on C_x Cellulase Activity of Basidiomycete 235M with CMC Substrate under Optimum Conditions of Temperature and pH

Amounts of reducing sugar (mg/l) are the means of five replicates.

VERDASAN concentration	15 min	30 min	1 hr	2 hr	3 hr	5 hr
0	2.2	6.3	12.3	20.4	26.4	31.7
0.1	1.9	3.6	7.8 *	14.8 *	19.6	25.4
0.5	1.8	4.8	9.3	15.8 *	21.3	26.7
1.0	1.5	4.3	8.8 *	16.2 *	19.9	26.3
2.5	1.3	3.9	7.3 *	14.7 *	19.6	25.0
5.0	0.97	3.3	8.5 *	16.2 *	19.6	24.3
10.0	1.2	3.9	6.3 *	13.7 *	17.1	23.6

F	5.9
P	< 5%
L.S.D. 5%	3.2
L.S.D. 1%	4.3

* significant at 5% level.

APPENDIX 20. Publication

GROWTH OF BASIDIOMYCETES IN THE PRESENCE OF AGROCHEMICALS

G.J.F. Pugh and M.J. MacDonald (1980)

In: Soil Biology as Related to Land Use Practices (Ed. D. Dindall). New York State University, (in press).

GROWTH OF BASIDIOMYCETES IN THE PRESENCE OF AGROCHEMICALS

Introduction

The role of members of the Basidiomycetes in decomposition processes has been studied by relatively few researchers. The ability of many soil Basidiomycetes to break down lignin, noted by Falck (1923, 1930), is common in many wood-destroyers. On the other hand, Melin (1925) remarked that the mycorrhiza forming Basidiomycetes are incapable of utilizing cellulose or lignin but depend for their nutrition on carbohydrates derived from their host trees. It was subsequently shown by Norkrans (1950) that even some of the mycorrhizal fungi are capable of decomposing at least cellulose.

Lindeberg (1944, 1946, 1948) studied the ability of soil inhabiting Basidiomycetes to decompose litter and its main constituents, cellulose and lignin. In addition, Norkrans (1944, 1950), Mikola (1954) and Fries (1955) indicated that the ability to decompose these constituents is very common among soil fungi. The most active litter decomposers have been found among the genera of *Marasmius*, *Mycena*, *Clitocybe*, *Collybia*, *Clavaria*, and *Stropharia*, and many wood destroying fungi found on decaying trees have decomposed litter effectively *in vitro*: *Armillaria mellea* and species of *Flammula*, *Hypholoma*, *Pholiota*, and *Polyporus* (Lindeberg, 1946; Mikola, 1954).

Thus the Basidiomycetes in soil represent a physiologically heterogeneous group and in order to determine their role in the soil, thorough investigations into the physiology of individual species are needed. The course of litter decomposition, including its speed and intermediate and final products, depends on three main factors: i) the physical and chemical properties of the litter, ii) the environmental conditions and iii) the organisms themselves. The factors in turn are interdependent. The significance of each individual factor in the decomposition of litter or wood is a matter for investigation. In addition, with the rapid escalation in biocide usage in agricultural and forestry practice, it is urgent that we evaluate the pressures which these compounds exert on the fungi responsible for decomposition processes. Biocides are considered to be indispensable aids in agricultural, horticultural and forestry practices, and a vast array of chemicals are applied directly to the soil. Other agrochemicals enter the soil as run-off from treated aerial systems, or from drifting sprays.

During the course of studies on soil fungi, members of the Basidiomycetes have often been overlooked because of difficulties of isolation and identification. Chesters (1949) referred to "the secret of the higher Basidiomycetes" : Warcup (1959), with his hyphal isolation technique, showed that Basidiomycetes can be recovered from the soil, and Warcup and Talbot (1962) were able to identify several species. However, these isolated studies give only a small indication of the work still to be done : other approaches, such as the cultivation of mycelia from sporophores, enable cultural studies to be carried out.

In the present study, three non-mycorrhizal species (235M, *Coprinus comatus* and *Cyathus stercoreus*), two known mycorrhizal species (*Boletus variegatus* and *Paxillus involutus*), and *Phallus impudicus*, a species of uncertain status, have been used to investigate the basic physiology and the reaction of each to the presence of agrochemicals. In this way comparisons can be made of the abilities of the nutritionally different species under a range of environmental pressures.

Materials and Methods

Species

The fungi used in this study include : two species isolated from sporophore tissue, *Coprinus comatus* growing on a grass lawn, and *Phallus impudicus* growing among mosses in deciduous woodland; an unidentified Basidiomycete isolated from leaf litter of *Agrostis tenuis*, designated 235M; two known mycorrhizal species, *Boletus variegatus* and *Paxillus involutus* (supplied by Dr. P. Mason, Institute of Terrestrial Ecology, Edinburgh) and the non-mycorrhizal fungus *Cyathus stercoreus* (supplied by Dr. P. Blakeman, Aberdeen).

Media

All species were maintained on a modified Hagem malt agar (HMA) (Modess, 1941) and contained per litre of distilled water : Glucose, 10 g; NH_4Cl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; malt extract agar, 50 g; $FeCl_3$ (1% solution), 10 drops.

The basic liquid medium (BLM) contained per litre of distilled water : Glucose, 10 g; NH_4Cl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; KH_2PO_4, 0.5 g; malt extract, 20 g; Thiamin, 1 mg; microelements (after Lilly and Barnett, 1953), 2 ml.

Inocula for liquid cultures were cut from the growing edge of Petri plate cultures on HMA. These inocula were transferred to the flasks and floated on the surface of the culture medium. All flasks were incubated as standing cultures.

Chemicals

Herbicides : Mazide 36 (as maleic hydrazide) - active ingredient (a.i.) 36% (w/v) 1,2-dihydro-3,6-pyridazynedione.

Paraquat - a.i. 25% (w/v) 1,1'-dimethyl-4,4'-bipyridinium.

Fungicide : Verdasan - a.i. 2.5% (w/v) phenyl mercury acetate.

The field application rates of the agrochemicals are as follows:

Mazide : approx. 4000 ppm
Paraquat : approx. 800 ppm
Verdasan : approx. 20 to 80 ppm

Effect of temperature on growth rate

Petri plates containing about 20 cm^3 of HMA were inoculated with a 5 mm disc cut from the margin of colonies growing on HMA. Plates were incubated at 5°, 10°, 15°, 20°, 25°, 30° and 35°C. The colony diameters were taken as the mean of two diameters at right angles to each other. Five replicate plates for each temperature for every fungus were used. Extension growth rates were calculated during the log phase of growth.

Dry weight increase

Flasks containing 20 ml BLM were inoculated with the test fungi and incubated at their optimum temperatures. Three replicate flasks for each consecutive sample for each fungus were used. For dry weight analysis flasks were removed, the mycelia harvested and dried to constant weights.

Effect of agrochemicals on growth

Appropriate amounts of herbicide stocks were added to BLM to give final concentrations of 500, 1000, 2000, 3000, 4000 and 8000 ppm (a.i.) for Mazide, and 5, 10, 25, 50, 100 and 250 ppm (a.i.) for Paraquat. Flasks were autoclaved for 15 min at 15 psi. Appropriate amounts of stock Verdasan solution were added to cooled sterile BLM to give final concentrations of 0.1, 0.25, 0.5, 0.75, 1.0 and 2.5 ppm (a.i.). BLM without agrochemical addition was used as control. Five replicate flasks for each concentration of agrochemical for every fungus were used, and dry weight analysis performed as above.

Results

Effect of temperature on growth rate

The results are summarised in Figure 1 and indicate the optimum growth temperatures. All six species grew at 5°C, the lowest temperature used. Four of the species investigated showed growth between 20° and 25°C Basidiomycete 235M, *Coprinus comatus*, *Boletus variegatus* and *Phallus impudicus*). *Paxillus involutus* showed a lower optimum temperature, around 20°C, while *Cyathus stercoreus* had its optimum near 30°C.

In general, the two mycorrhizal species (*P. involutus* and *B. variegatus*) and *Phallus impudicus* did not grow above 30°C, whereas the other three non-mycorrhizal species grew above this temperature.

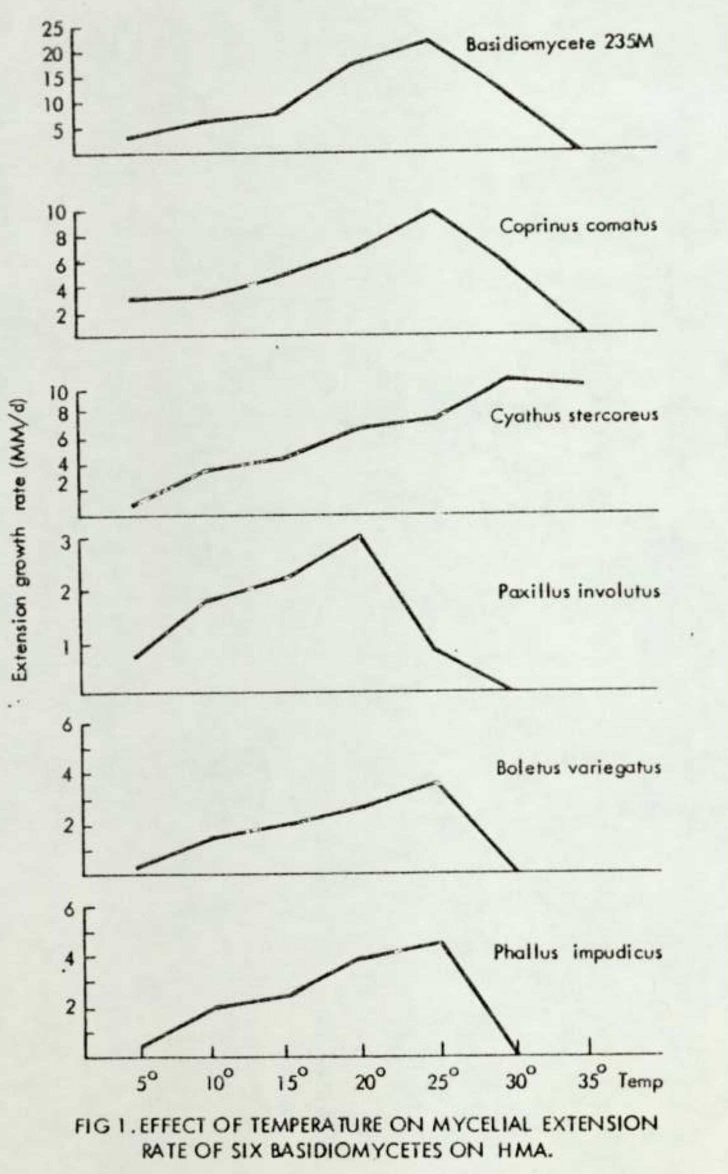


FIG 1. EFFECT OF TEMPERATURE ON MYCELIAL EXTENSION RATE OF SIX BASIDIOMYCETES ON HMA.

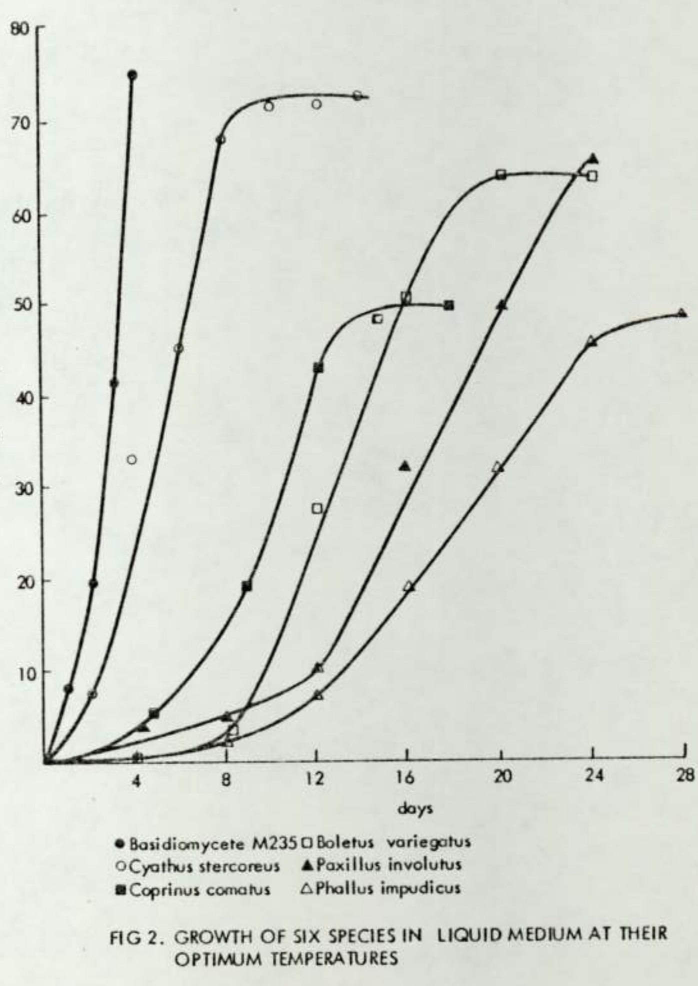


FIG 2. GROWTH OF SIX SPECIES IN LIQUID MEDIUM AT THEIR OPTIMUM TEMPERATURES

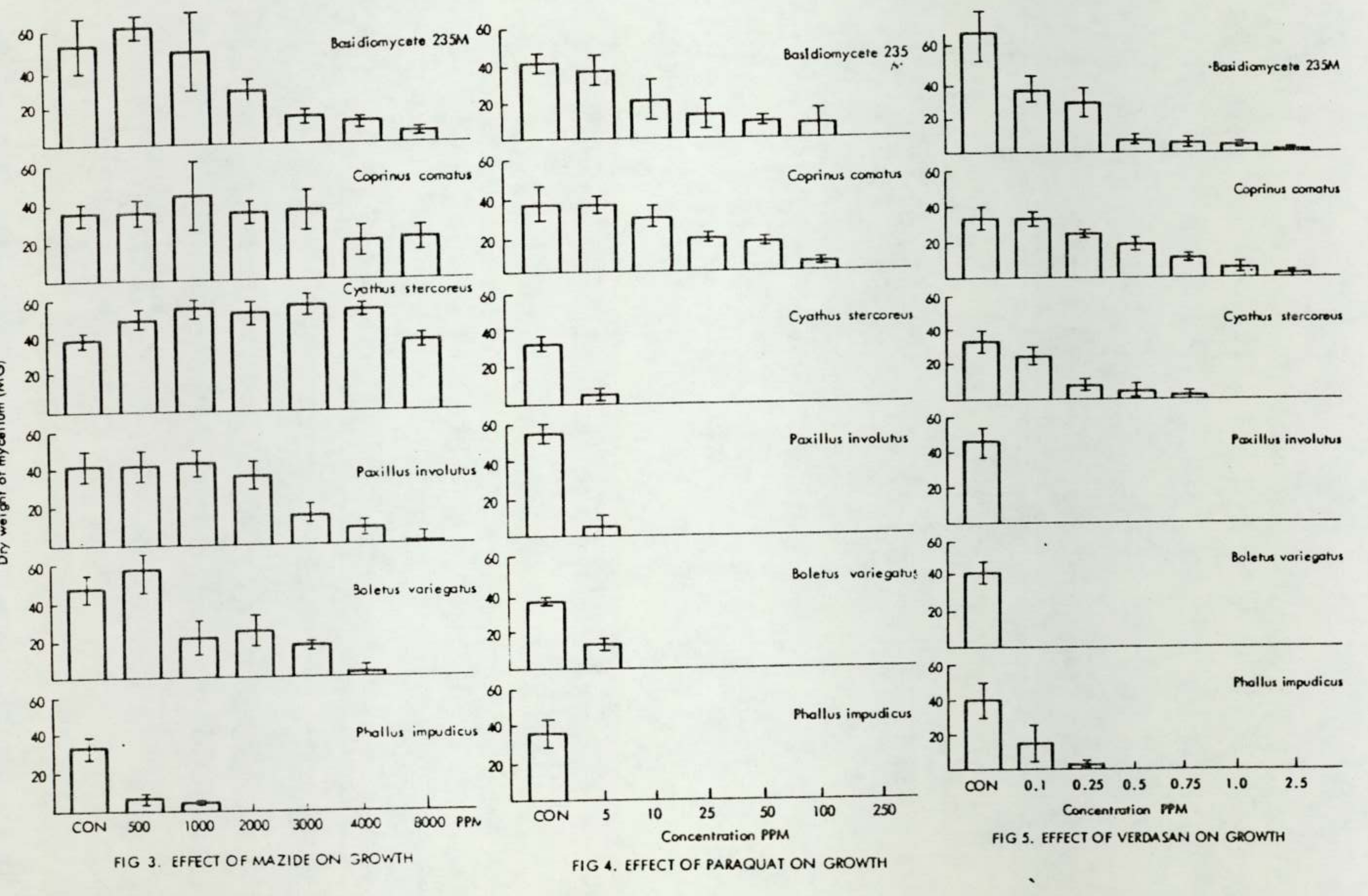


FIG 3. EFFECT OF MAZIDE ON GROWTH

FIG 4. EFFECT OF PARAQUAT ON GROWTH

FIG 5. EFFECT OF VERDASAN ON GROWTH

Dry weight increases

The three non-mycorrhizal species, 235M, *C. comatus* and *C. stercoreus*, showed relatively higher growth rates than did *P. involutus*, *B. variegatus* and *P. involutus* (Table 1).

TABLE 1. Growth rate (mg dry weight/24 hr) of Basidiomycetes at their optimum temperature for growth.

Basidiomycete 235M	33.50	*Boletus variegatus*	6.25
Cyathus stercoreus	9.38	*Paxillus involutus*	4.50
Coprinus comatus	7.67	*Phallus impudicus*	3.25

In addition, it is evident from Figure 2 that not only were growth rates slower in the two mycorrhizal species and *P. impudicus* but the lag phase of growth was, in general, considerably longer. Conversely, those species with a shorter lag phase also showed a faster growth rate.

Effect of agrochemicals on growth

The effects of the agrochemicals on the growth of the six Basidiomycetes are shown in Figures 3, 4 and 5. In general the fungi were relatively tolerant to Mazide at the concentrations used: only *Phallus impudicus* was inhibited at concentrations above 1000 ppm. Paraquat and Verdasan showed some similarities in their effects on growth at the concentrations used : 235M and *Coprinus comatus* were more tolerant than the other species. *Boletus variegatus* and *Paxillus involutus* were least tolerant, being inhibited at 10 ppm of Paraquat and 0.1 ppm of Verdasan. *Cyathus stercoreus* and *Phallus impudicus* showed growth patterns similar to the mycorrhizal species in the presence of Paraquat, but both were more tolerant than these species to Verdasan.

Discussion

In earlier studies on Basidiomycetes, most have been found to be mesophilic in their temperature requirements. Three mycorrhizal species of *Boletus* studied by Melin (1925) showed optimum growth at 25°C, while Mikola (1948) found a slightly lower optimum for species of *Amanita* and *Lactarius*. Norkrans (1950) found that mycorrhizal *Tricholoma* species had optima within the range 18° to 30°C, while Marx (1969) showed that *Pisolithus tinctorius* grew best at 30° to 35°C. Laiho (1970) reported the temperature maximum for eight strains of *Paxillus involutus* to be about 30°C, with all strains being killed at 32°C.

Of the various wood-destroying Hymenomycetes studied by Humphrey and Siggers (1933) and Bjorkman (1946) belonging to the genera *Polyporus*, *Stereum* and *Poria*, the great majority have maximum growth at temperatures of 28°C or higher On the other hand, several *Mycena* species grew best at 20°C (N. Fries, 1949). Treschow (1944) found the coprophile *Psalliota bispora* to grow best at a temperature range between 20° and 27°C with the optimum at 24°C. The coprophilic species of *Coprinus* investigated by Rege (1927) showed a high optimum of 30° to 35°C with good growth at 44C in one species.

In the present study, the six species used were also mesophilic, with *Cyathus stercoreus* showing he highest temperature range. All six species exhibited growth at 5°C, and there were indications that *Coprinus comatus* could probably grow quite well at lower temperatures. Melin (1925) found that three species of *Boletus* grew well at 10°C and two of them continued to grow at 6°C, while Lobanow (1960) reported that the temperature minimum of mycorrhizal fungi was 1°C to 5°C. However, temperature requirements can depend on the origin of the strain used: Moser (1958) showed that the minimum for a strain of *P. involutus* isolated from a valley was 2° to 8°C, whereas a mountain strain grew at -2°C to 4°C.

Soil inhabiting Basidiomycetes have long been regarded as slow growing organisms. However, Basidiomycete 235M is exceptional in showing a rate of growth comparable with many non-Basidiomycetes. *Phallus impudicus* and the two mycorrhizal species tested were not only slower growing than the other non-mycorrhizal fungi, but they also showed a much longer lag phase. Further studies are in hand to see whether the growth rate can be used as a criterion for distinguishing between mycorrhizal and non-mycorrhizal fungi.

In the presence of the agrochemicals used, there were again some differences in the tolerance shown by the mycorrhizal and non-mycorrhizal species. With the exception of *Phallus impudicus* the mycorrhizal species were less tolerant than the non-mycorrhizal species to the three agrochemicals used. At field concentrations, all species except *Phallus impudicus* were tolerant to Mazide. With Paraquat and Verdasan, however, all of the test fungi were inhibited below the field application rate.

The behaviour of *Phallus impudicus* indicates many similarities with the known mycorrhizal *Boletus variegatus* and *Paxillus involutus*. However, Grainger (1962) described it as growing saprophytically on leaf mould and decayed wood, while Trappe (1962) reported it as a possible mycorrhizal species.

Thus, the use of these agrochemicals could have more deleterious effects on mycorrhizal activity than on decomposition in general. This could be particularly important in those marginal situations where higher plant growth is dependent on mycorrhizal associations.

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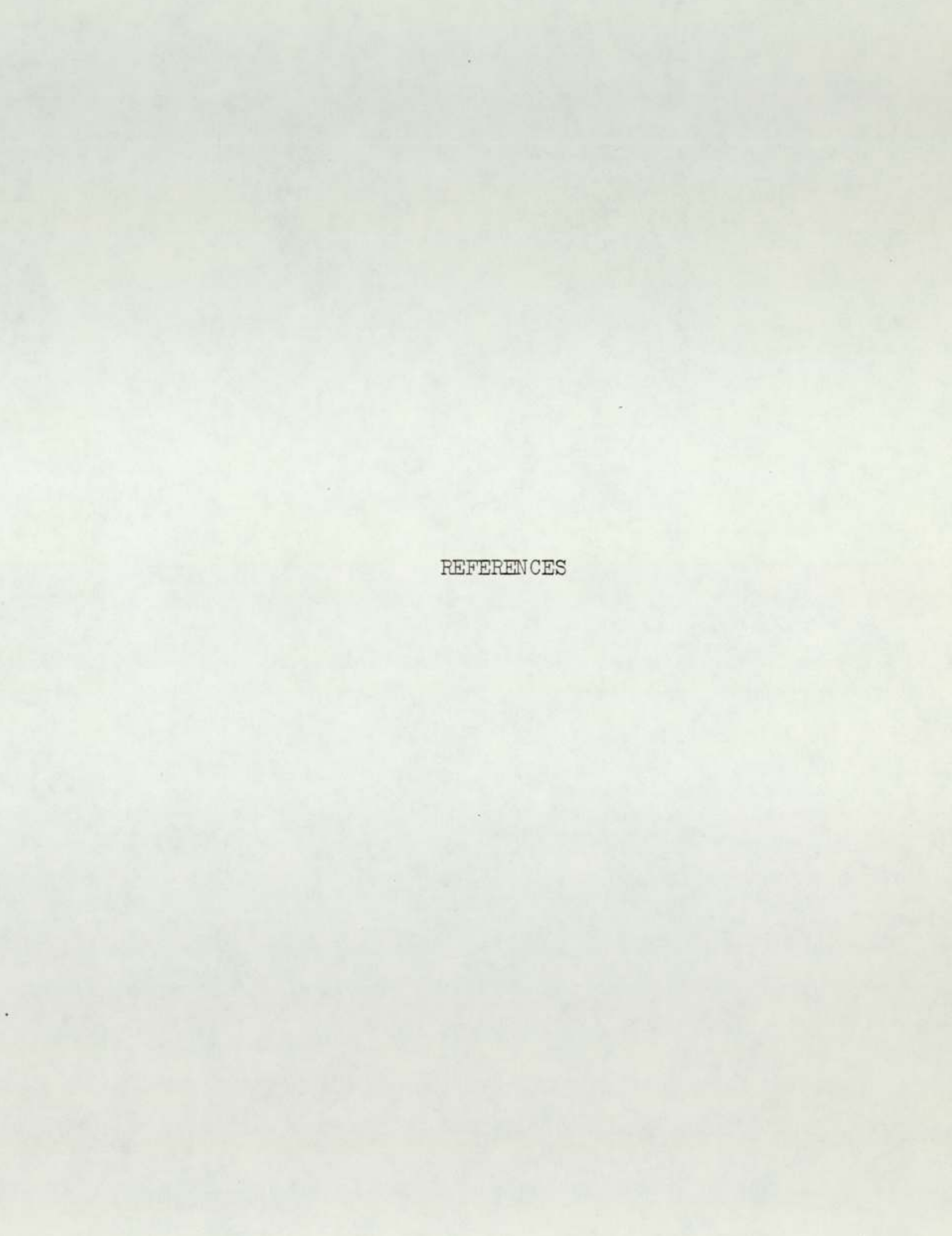

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