THE BIODEGRADATION OF QUATERNARY AMMONIUM COMPOUNDS BY MOULD FUNGI

by

Yu Zheng

B.Sc. (1990) and M. Sc. (1993), Fujian Normal University, P. R. China

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Department of Wood Science

The University of British Columbia
Vancouver, Canada

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ABSTRACT

Didecyldimethylammonium chloride (DDAC) has been described as one of the most effective quaternary ammonium compound (QAC) wood preservatives, based on laboratory experiments. Subsequently, QAC-treated stakes failed rapidly in field tests. It was suggested that the degradation of QACs by mould and stain fungi had reduced the efficacy of QACs. This thesis describes a study of the role that moulds play in limiting the performance of DDAC.

Fungi were isolated from DDAC-treated stakelets placed in a field test site at UBC. Other fungal strains were obtained from culture collections. A comparison was made of their tolerance and ability to degrade DDAC. Experiments were performed both in solid malt agar and Vogel liquid media with, and without, DDAC. It was found that tolerance and ability to degrade DDAC shown by the fungal strains were common properties of many of the moulds studied.

The metabolites produced by the action of moulds on DDAC were extracted by methylene chloride and analyzed by high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). The results showed that DDAC had been degraded by mould fungi, either by fission of the C-N bond or ω-and β-oxidation of the decyl group.

Electrophoresis was performed to try to identify additional proteins arising from the presence of DDAC. New proteins bands were obtained in the extracellular supernatant. The DDAC concentration in liquid media was decreased when inoculated and incubated with the

extracellular supernatant recovered from *Verticillium bulbillosum* grown in liquid media containing 250 ppm DDAC. No new proteins were obtained in the intracellular extract, and no degradation of the DDAC was recorded when the intracellular supernatant was incubated with DDAC. This suggested that DDAC degradation took place outside the fungal cell wall.

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LIST OF ABBREVIATIONS

AAC	Alkylammonium compound
ACA	Ammonium copper arsenate
BSA	Bovine serum albumin
BTMA	Benomyl-tetracycline-malt agar
CCA	Chromated copper arsenate
DDAC	Didecyldimethylammonium chloride
DDA	Decyldimethylamine
GC-MS	Gas chromatography mass spectra
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
MA	Malt agar
PAGE	Polyacrylamide gel electrophoresis
PAHs	Polycylic aromatic hydrocarbons
PCP	Pentachlorophenol
QAC	Quaternary ammonium compound
SCNR	Starch-casin-nitate-rose bengal
SDS	Sodium dodecyl sulphate

1. INTRODUCTION

Wood being a naturally biodegradable product, is often vulnerable to attack by biological organisms, which can degrade it and cause loss in value. Fungi of various kinds can grow in wood, resulting in stains and decay. In general, the basic requirements of deteriorating organisms are: oxygen, a suitable temperature (15-30 °C) and pH, water, and a source of nutrients. If one or more of the basic requirements can be controlled, wood decay could be prevented. Under normal exposure conditions the oxygen and the environmental temperature can not be controlled. One of the simplest preservation approaches is to keep wood dry. However, various uses of wood can not eliminate all the decay risk situations. So to prevent colonization by sapstain, mould and wood-destroying fungi, wood can be protected by application of chemical preservatives, and this is the most common method of protecting wood used in biologically hazardous situations.

Each year the industry in Canada treats approximately 2.0 million cubic meters (70.1 million ft^3) of wood. In 1992, 51.5% of the treated wood went to the consumer lumber, 21.3% to pole production and 24.1% to industrial lumber. In 1992 the value of the treated lumber was $547 million. The value of the total volume of treated wood installed in Canada annually is in excess of $10 billion (Stephens *et al.* 1994). The wood preservation industry is therefore an important contributor to the Canadian economy.

1.1 The limitations of currently used wood preservatives

Wood preservatives can be divided into two categories: those which are oilborne and those which are waterborne. The traditional oilborne preservatives include creosote, pentachlorophenol, bis(tributylin)oxide, copper-8-quinolate and copper naphthenate. However, of the five preservatives, only creosote and pentachlorophenol have been widely used in Canada.

Creosote was the first commercially successful preservative and it is still used today. It is produced by the high temperature carbonization of bituminous coal in the absence of air. The principal product of the process is coke. One of the by-products is coal tar, which is distilled to produce creosote. Creosote is neither a pure compound nor a well-defined mixture of compounds. The composition of creosote varies from one source to another, being also greatly influenced by the coking process. Many of the minor components in creosote remain unidentified. Further, creosote contains polycyclic aromatic hydrocarbons (PAHs), some of which may cause cancer or be mutagenatic. However, creosote is still the principal preservative used for railway ties in Canada and is also widely-used to preserve marine piling and timbers.

Pentachlorophenol the second generation oilborne preservative was developed in the mid 1930's. It is a pure crystalline organic compound, and is produced commercially by the chlorination of phenol, using an aluminum trichloride catalyst. However, dioxins, which are present as a minor impurity in technical grade pentachlorophenol have caused the use of this preservative to decrease, principally because one member of the dioxin family (2,3,7,8-tetrachlorodibenzo-p-dioxin) has been shown to be very toxic. This causes pentachlorophenol to have a poor image.

Waterborne preservatives are based upon formulations of inorganic chemicals that are solubilized in aqueous solutions. There are two fundamental types of waterborne preservatives. The first, chromated-copper formulations, is acidic in nature and relies upon the change in the oxidation state of the chromium compound to achieve fixation to the wood. This type of preservative system is widely used around the world, with chromated copper arsenate (CCA) being the most popular formation (Zabel and Morrell, 1992). The most commonly used formulation in North American, CCA type C, contains 47.5% chromium, 18.5% copper and 34% arsenate (expressed on an oxide basis). The fixation of CCA has been the object of considerable research but a complete understanding has still not been achieved. Only recently, it was discovered that a stable Cr(V) intermediate is formed during the fixation process (Ruddick, *et al.* 1994). CCA has been the most successful system used today because its stability in wood and effectiveness against a wide range of microorganisms. The second type of waterborne preservatives is that based upon basic copper solutions. The most widely used preservative of this type contains copper and another co-biocide dissolved in ammonium hydroxide [e.g. ammonical copper arsenate, (ACA)] (Zabel and Morrell, 1992). ACA is less appealing than CCA because of difficulty in handling ammonia and it is often considered to be more leachable than CCA. Both preservatives contain the toxic heavy metal arsenic.

In Canada, these traditional wood preservatives still dominate the use of treated wood. Of the total production volume, 79.1% was treated with waterborne preservatives while 20.9% was treated with oilborne preservatives (Stephens, 1994). Increasingly, it is becoming apparent that problems are arising with the currently used preservatives, because of two principal factors. They are: environmental concerns and the cost of oil and future energy

source, especially in regard to use of oil based preservatives. Of these two, environmental concerns dominate. Disposal of treated wood is a critical issue facing the industry today.

Environmental and economic pressures on currently used chemicals have led to a situation where considerable effort is now directed towards the development of new wood preservatives. Among the promising candidates resulting from the last two decades of research one group of compounds comprised of long chain quaternary ammonium compounds (QACs) and tertiary amine acid salts, [collectively known as alkylammonium compounds (AACs)] has received considerable attention.

1.2 Quaternary ammonium compounds

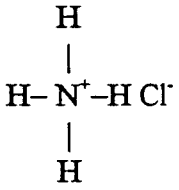
1.2.1 Industrial applications

AACs are derived from ammonium by successive replacement of the hydrogen atoms with hydrocarbon (alkyl) chains of varying length, thus making a wide range of compounds possible .

The following Figure 1.2.1.1 illustrates the structure of the main groups of alkylammonium compounds in relation to the simple structure of ammonium chloride. The figure shows only chloride compounds, although the anion can be selected from a wide range of chemicals including bromide, iodide and acetate.

There are two basic types of AACs that are of interest for wood preservation, namely quaternary ammonium compounds (QACs) and tertiary amine acid salts. Among AACs evaluated for use in the protection of wood from fungi, quaternary ammonium compounds with either two alkyl groups, or one alkyl and one aryl group, have been shown to offer promise as wood preservatives (Nicholas *et al.*, 1991 and Tsunoda and Nishimoto, 1983). The QAC [didecyldimethylammonium chloride (DDAC)] was chosen to be studied in this project.

QACs have been widely used as general purpose microbiocides for some 30 to 40 years, and are currently employed as microbiocides such as household disinfectants, eye-drops, nappy sanitisers, paint fungicides, swimming pool algaecides and surface wood treatment. Because of their surface active properties, they are also used as lubricants, antistatic agents, fabric softeners, hair conditioners and dye additives (Boething, 1984).



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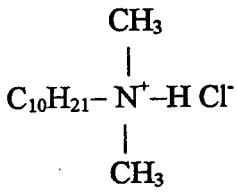

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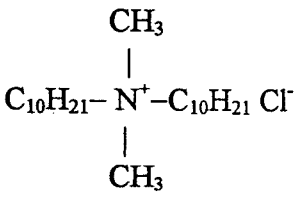

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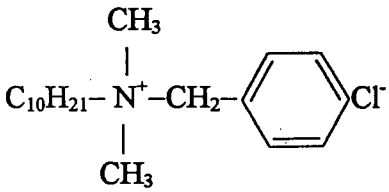

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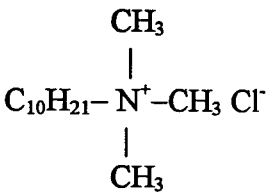


alkyltrimethylammonium chloride

Fig. 1.2.1.1 Diagrams of main types of alkylammonium compounds compared to ammonium chloride

1.2.2 Potential as wood preservatives

Based upon the extensive application of QACs for several decades without negative effects, as well as the toxicological data developed for specific QACs as required by regulatory agencies, it can be concluded that they are relatively safe compounds, readily available and economical for use by the preservation industry. For example, DDAC has an oral LD_{50} of 600-700 mg/kg (mouse) and an acute dermal LD_{50} of 4300 mg/kg compared with the of PCP oral LD_{50} of 50-140 mg/kg (rat) (Drysdale and Preston, 1982, Anonymous, 1984).

In general, the AACs showing the best potential as wood preservatives are of low toxicity. Also there is a long history of widespread and safe use of these compounds in commercial products. In particular, QACs appear to have the potential for use as wood preservatives, as they exhibit a broad spectrum on biocidal effectiveness. Their effectiveness is equal to, or higher than, that of currently used CCA (Butcher *et al.* 1977b).

QACs exhibit low volatility and are stable, colorless chemicals which are water and solvent soluble. They are environmentally more acceptable than presently used materials and reasonably close to being cost-competitive with the presently used preservatives such as CCA. These cationic materials are highly attracted to anionic surfaces such as wood, and do not pose problems for people handling treated wood.

1.3 Review of laboratory performance and field performance

1.3.1 Laboratory trials

The use of QACs as fungicides in paints may have initially suggested their potential as a wood preservative. Various QACs were screened in the laboratory in *Pinus radiata* against several types of wood decay fungi, such as *Postia placenta, Fomes gilvus* and *Chaetomium globosum.* Most of QACs had a toxic threshold of the order 3.2-6.4 kg/m^3 (Butcher *et. al.* 1977a and b). Further work showed that the effectiveness of QACs against basidiomycete fungi is equal to, or higher than, that of CCA (Butcher and Drysale, 1977). The same level of protection was obtained against several decay fungi when the retention of QAC 1-2 kg/m^3 was approximately half that of the CCA (2-4 kg/m^3) (Butcher, 1979a; Preston and Chittenden, 1982; Preston and Nicholas, 1982; and Nishimoto, 1983; Hedley *et al.* 1982). DDAC was found by several researchers to be most effective AAC, with threshold values of the order 1-2 kg/m^3 (Preston and Chittenden, 1982; Preston and Nicholas, 1982; Preston, 1983; Hedley *et al.* 1982; Tsunoda and Nishimoto, 1987).

1.3.2 Field trials

Parallel field trials did not reflect the initial laboratory observations. QAC-treated stakes showed signs of surface decay soon after being placed in the field and did not perform as well as CCA in a field trial during a 30 month period (Butcher *et al.*, 1979). The failure of stakes treated with dialkyldimethylammonium chloride (14.6 kg/m^3) after only one year exposure in the field (Tillott and Coggins, 1981) confirmed an earlier observation of stake failure (Ruddick, 1981) in which stakes were exposed to a mixed QAC formulation at a retention of 12 kg/m^3. In a subsequent field trial Ruddick (1983) also found that stakes

treated with DDAC (3.2 kg/m^3) failed after two years of field exposure. QAC-treated wood with retentions as high as 11 kg/m^3 had noticeable decay after six years of exposure (Ruddick, 1987; Morris and Ingram, 1988). Drysdale (1983a) found that dialkyldimethylammonium chloride (6.2 kg/m^3) treated and benzalkonium chloride (10.6 kg/m^3) treated *P. radiata* stakes were attacked by brown rot, soft rot and white rot fungi after four years in the field.

In 1978 because of the good performance in early above ground and laboratory studies in New Zealand. QACs were approved above ground use. This was subsequently restricted due to the failure of QACs treated fencing (Butcher and Greaves, 1982). These failures in above ground application mirrored the earlier failure of QAC-treated stakes placed in the field exposure trial (Ruddick, 1981; Tillott and Coggins, 1981).

1.3.3 Reasons for the poor field performance

The effectiveness of QACs against fungi in laboratory and field tests has varied widely. Several factors could account for this wide discrepancy in performance, including inadequate retentions being used, leaching of QACs from treated wood, uneven distribution and biodegradation of QACs. However, field trials with higher retentions also showed sign of decay (Ruddick, 1983, Morris and Ingram, 1988). So the use of too low a retention can not be the reason for the field performance.

Fixation of QACs in wood

QACs are surfactants. In aqueous solution, surfactants can be adsorbed onto a solid substrate by various mechanisms: physical adsorption by polarization or dispersion forces, ion pairing and ion exchange (Rosen, 1975). Wood has been described as having strongly

charged sites. Fixation of QACs to wood is a complex process, during which the adsorption of the solute may occur followed successively by ion-exchange, ion pairing and hydrogen bonding. Ion-exchange would appear to be the main mechanism for 'fixation' of QACs to wood. This was confirmed by the influence of pH on QAC retention in wood. Butcher and Drysdale (1978b) suggested that more acidic treating solution would retard fixation and result in a more uniform distribution, providing better protection of wood. An alkaline treating solution would create more negative binding sites and speed up fixation, which would result in higher loadings especially in the peripheral regions. Other studies confirmed that an increase in treating solution pH increase retention of DDAC in the wood, particular in the lignin component (Jin and Preston, 1991, Doyle and Ruddick, 1994). The most likely functional groups on the wood to react with QACs are negatively charged sites, such as the carboxylic and phenolic groups on lignin or hemicellulose components (Brezny *et al.* 1990). This was confirmed by the recent research which found that DDAC adsorbed onto lignin preferentially, while a small amount were adsorbed onto the cellulose (Doyle, 1995; Jin and Preston, 1991).

Distribution of QACs in wood

The erratic distribution of QACs in wood could be a factor contributing to their poor field performance. The QAC retention in outer zone of treated lumber is much higher than that of the core (Butcher and Drysdale, 1978a; Ruddick and Sam, 1982; Drysdale, 1983b; Nicholas *et al.* 1991). Drysdale (1983b) did not detect uneven distribution in the outer 6 mm of *P. radiata*. Recent studies found that earlywood retained more QAC than latewood (Nicholas *et al.* 1991; Doyle and Ruddick, 1994). However, the variation observed in these studies is not sufficient to explain the poor field performance.

Leachability of QACs from the treated wood

Leaching of QACs from treated wood has been proposed as one of the reasons for the failure in the field area. Initial research on QACs leachability using four softwood found that leaching was low and independent of the type of leaching solution used. The rate of depletion was dependent on the surface to volume ratio, but was not significant enough to account for the failure of QAC-treated wood in the field (Ruddick and Sam, 1982). Drysdale (1983b) found that 30% QAC leaching occurred from the outer 2 mm of wood samples during an 18 week leaching regime. The amount of QACs leaching from wood depends on the concentration of the preservatives, the sample size, wood species, the duration of the test and the type of medium used. For example, the higher the retention, the greater the leaching (Loubinoux *et al.* 1992). Water leaching studies showed that the AAC depletion was independent of the solution pH (Sundman, 1984, Ruddick and Lum, 1991). In addition, preleaching of QAC-treated blocks had little influence on their performance during soil block testing (Ruddick, 1986). Loss of DDAC from latewood during leaching in a soil substrates was 30-40%. In the study, the loss of DDAC from earlywood (35%) region was greater than the latewood region (20%) (Nicholas *et al.* 1991; Ruddick and Lum, 1991). In summary, All the experiments seemed to show that QACs do leach from the wood, but that the degree of depletion should not have led to the rapid failure of the treated wood in service.

Biodegradation of QAC

During the field exposure of QAC-treated wood, it was noted that it soon became heavily stained (Ruddick and Sam, 1982; Drysdale, 1983a). Ruddick (1986) carried out a study on the effect of pre-exposure of QAC-treated wood to staining fungi, on the toxic threshold of selected the decay fungi. The results showed that the toxic threshold for two of

the three fungi used, increased two times. Recent research sponsored by the NSERC/Industrial Chair in Wood Preservation at UBC, has demonstrated that non-wood destroying fungi can reduce the efficacy of AACs as wood preservatives (Doyle and Ruddick, 1993; Doyle, Chow and Ruddick, 1993). These studies showed that at least two fungi were very tolerant to DDAC, based on the ready fungal growth over media amended with high concentrations of the chemical. Further, it was noted that the DDAC concentration was greatly reduced in malt agar after exposure to the test fungi. The reduction of DDAC concentration showed that the fungi can degrade or modify DDAC.

2. OBJECTIVES

The principal objective of the current project was to determine the possible role of staining or mould fungi in degading QACs to reduce their performance as wood preservatives. This main objective was supported by three sub-objectives which were to:

- isolate and identify tolerant mould or staining fungi from treated wood;
- compare their tolerance and ability to degrade DDAC with similar fungi from stock cultures;
- confirm possible biodegradation processes in liquid culture by identifying metabolites.

When completed successfully, this QAC information can then be used to develop formulations with better all round field performance. It will also contribute to an understanding of the potential of these fungi for bioremediating QAC-contaminated waste.

3. LITERATURE REVIEW OF BIODEGRADATION OF QACS

The widespread application of QACs, particularly in synthetic detergents which were introduced in the 1960's, has resulted in their becoming increasingly detected in waste water, treated sewage and the receiving waters, due primarily to their excellent foaming properties. Typical concentrations in sewage plant effluent ranged from 5 to 10 ppm. Since QACs are toxic to a wide variety of aquatic organisms, including algae, fish, mollusks, barnacles, and shrimp (Taft, 1946, Vallejo-Freire *et al.*, 1954, Knauf, 1973, Kappeler, 1982, Waters, 1982, Lewis and Wee, 1983), the build up of QAC concentrations in waterways must be avoided.

Because of the potential for widespread exposure and the established toxicity of QACs, it is very important to understand the behavior of these compounds and how they are degraded by microbes in the environment. Consequently, it was anticipated that a review of the literature of the biodegradation of QACs would enable a good understanding of the relative ease of fungi and bacteria to degrade these chemicals. This information could then be used to formulate a strategy for evaluating the potential for mould and stain fungi to reduce the preservative efficacy of QACs through biodegradation. *Following a survey of the literature it is surprising to discover that all of the available research has been done on bacterial degradation of QACs, and that there was no information on the fungal degradation of QACs.* While a few reports of fungal tolerance have appeared in the literature no investigation detailing the biodegradation of QACs was discovered. Ruddick (1986) reported that pre-exposure of QAC-treated wood to a mixture of isolates recovered from failing field test material, substantially reduced its subsequent performance against three basidiomycete

fungi. Doyle and Ruddick (1993) have shown reported that three moulds isolated from discolored lumber, showed extreme tolerance to DDAC, growing on 2500 ppm DDAC in solid agar medium. The three fungi were identified as *Verticillium bulbillosum, Acremonium* sp. and *Gliocladium roseum.* High performance liquid chromatographic analysis of the amount of DDAC remaining either in the agar medium, or bound to the fungal hyphae, confirmed its disappearance.

Since there is no data in the literature on fungal degradation of QACs, this review will focus on the bacterial degradation of QACs which have been proposed or used as wood preservatives. It is understood that the mechanisms of fungal degradation of QACs may be the same or different from those reported to occur for bacteria. However, an important observation is that many of the QACs are degraded by bacteria, and that microbial action could well explain the significant reduction QAC in effectiveness as wood preservatives.

3.1 Definitions of biodegradation

The following is the generally accepted classification of various forms of degradation:

Biodegradation

According to Swisher (1987), biodegradation may be defined as the breakdown of chemical compounds as a result of the microbes. In this review, the organisms of interest are fungi and bacteria.

Primary biodegradation

Primary biodegradation may be defined as the disappearance of a compound to such an extent, that either specific characteristic properties are no longer evident, or it no longer responds to a compound specific, analytical procedure. The limitation of this approach is that it makes no allowance for the disappearance of the chemical by substrate adsorption or other physical processes.

Environmentally acceptable biodegradation

Environmentally acceptable biodegradation is often used to define the state at which a chemical has been partially degraded, yielding products that are deemed to have no harmful effect on the environment. Since the acceptability of chemicals for release into the environment often changes with time, this is not a good criterion for considering that the degradation has reached a suitable state. It also requires that the by-products of the biological action are known. Again no allowance is made for loss by adsorption processes.

Ultimate biodegradation or mineralization

Ultimate biodegradation is defined as the biological conversion of a chemical to the oxidized form of its basic components, i.e. carbon dioxide and water, together with the formation of mineralized salts and the biological waste products of the microbial action. It is often used interchangeably with mineralization. This is not strictly correct since in complete mineralization, even the carbon used by the organism to generate growth must be converted to carbon dioxide. Clearly this would take some time to accomplish and is a rather idealized concept.

3.2 Evidence of biodegradation of QACs

QACs can be divided into five groups according to the number and type of alkyl chains, attached to the central nitrogen. The five groups are: alkyltrimethyl-, alkylbenzyldimethyl-, dialkyldimethyl- alkylpyridinium, and trialkylmethylammonium salts. Only the first three of these have been considered as wood preservatives. A comprehensive listing of the literature on the bacterial biodegradation of types has been compiled by Swisher (1987), and is reproduced in Tables 3.2.1 to 3.2.3 below. In general two criteria prevail. They are that resistance to biodegradation increases with a) the number of long alkyl chains bonded to the nitrogen; and b) with increasing alkyl chain length.

3.2.1 Alkyltrimethylammonium salts, $C_nMe_3N^+$

This cationic surfactant group represents the most facile with respect to biodegradation and under appropriate conditions with a suitable organism, the QAC can be almost completely degraded (i.e. mineralized). Dean-Raymond and Alexander (1977) studied ten QACs and reported that of the ten, only decyl- and hexadecyltrimethylammonium bromide could be metabolized by the sewage microbes. Decyltrimethylammonium bromide was extensively degraded within 8-10 days at three concentrations tested (10, 25, 100 μ g/ml). By enrichment techniques, a mixed culture was developed which could live on 500 μ g/ml decyltrimethylammonium bromide as the sole carbon source. From the several species (at least five colony types), it was observed that while none of isolates could grow when

Table 3.2.1 Biodegradation data of alkyltrimethylammonium (RMe_3N^+) type quaternary surfactants (Swisher, 1987)

Substrate RMe_3N^+	Extent	Method	Time hours (h) or days (d)	Reference
C_2 Cl^-	100	In	72h;196h;215h	Ghisalba and Küenzi, 1983
C_{16} Br^-	0	BOD	5d	Sheets and Malaney, 1956a,b
	0.1	Wa	6h	Barden and Isaac, 1957
	100	CAS	8h	Pitter and Svitálková, 1961
	0	BOD	5d	Winter, 1962
	37;44	In	39d	
C_8 Cl^-	73	In	10d	Masuda *et. al.*, 1976
C_{10} Cl^-	91	In	10d	
C_{12} Cl^-	100	In	2d	
	63;90	In	10;12d	
C_{14} Cl^-	100	In	2d	
	59;82	In	10;12d	
C_{16} Cl^-	100	In	3d	
	35;57	In	10;12d	
C_{18} Cl^-	100	In	10d	
	0;22	In	10;12d	
	56	In	20d	
C_{16} Br^-	100;100	In	7d	Pitter and Svitálková, 1976
	93;94	In	7d	
EtO_2CC_{15} Br^- (Septonex)	91;79	In	7d	
	80;73	In	7d	
C_{16} Cl^-	≈100	Sew;Acc	7;2d	Baleux and Caumette, 1977
	≈100	RW	7-13d	
	100	In	65h?	Van Ginkel *et. al.*, 1992
C_{16} Br^-	≈100	Sew;Acc	4;2d	
C_{10} Br^-	≈100;0.62	In	3;10d	Dean-Raymond and Alexander, 1977
C_{16} Br^-	0.58	In	17d	
C_{16} Br^-	91-98	CAS	3h	Gerike *et. al.*, 1978
	107±19	CAS	3h	
	15;6	In	15h?	Mackrell and Walker, 1978
	109±19	CAS	3h	Gerike and Fischer, 1979
	53	BAS	14d	
	0-7	In	14-40d	
	0	In	14-40d	
	0	In	14-30d	
	0	In	28d	

Table 3.2.1 Biodegradation data of alkyltrimethylammonium (RMe_3N^+) type quaternary surfactants (contd.)

Substrate RMe_3N^+	Extent	Method	Time hours (h) or days (d)	Reference
Tallow Cl^-	0	In	10d	Itoh *et. al.*, 1979
C_8 Cl-	84	In	10d	Miura *et. al.*, 1979
C_{10} Cl^-	98	In	10d	
C_{12} Cl^-	100;88	In	2;10d	
C_{14} Cl^-	100;83	In	2;10d	
C_{16} Cl^-	100;46	In	3;10d	
C_{18} Cl^-	100;0	In	10d	
C_{12} Cl^-	98;99	RW	3;4d	Ruiz Cruz, 1979 Ruiz Cruz and Dobarganes Garcia, 1979
C_{16} Cl^-	99	In	4d	
C_{18} Cl^-	99	In	5d	
C_{16} Br^-	83,107	CAS	3h	Gerike *et. al.*, 1980
	91	BAS	19d	Gerike and Fischer, 1981
C_{16} Cl^-	70;92	In	0.26;0.22	Larson and Perry, 1981
C_{12} Cl^-	98	RW	4d	Ruiz Cruz, 1981
C_{18} Cl^-	81;<0	In	25d	Games *et. al.*, 1982
	99	BAS	1d	
(α-^{14}C)	96;65	BAS	7d	
(Me-^{14}C)	98;88	BAS	7d	
C_{16} Br^-	98	CAS	3h	Gerike, 1982
C_{18} Cl^-	57;0	In	20d	Itoh and Naito, 1982
*C_{18} Cl^-(α-^{14}C)	79	RW	21d	Larson and Wentler, 1982 Larson and Vashon, 1983
	74;0	In	25d	
	84	RW	23d	Larson and Vashon, 1983
C_{16} Br^-(α-^{14}C)	70	RW	23d	
C_{16} Cl^-	84	In	13d	
C_{18} Cl^-	0	In	25d	
C_{16} Br^-	98;107±19	CAS	3h	Berth *et. al.*,1984
*C_{12}	71	GW/Soil	36d	Larson, 1984

Table 3.2.2. Biodegradation data of alkyldimethylbenzylammonium ($RBzMe_2N^+$) type quaternary surfactants (Swisher, 1987)

Substrate $RBzMe_2N^+$	Extent	Method	Time hours (h) or days (d)	Reference
$R=C_{12}NHCOCH_2$- (Quartolan)	0	Wa	1d	Winter, 1962
CCBz Cl^- (Benzalkonium)	0	In	?	Lambin *et. al.*, 1966
$C_{14}Bz\ Cl^-$	95	SF	2d	Gawel and Huddleston, 1972
$C_{14}Eb\ Cl^-$	95	SF	2d	
$C_{14}Bz\ Cl^-$	63-79	CAS	3h	Fenger *et. al.*, 1973
$C_{12\text{-}16}$*Bz Cl^- (Hyamine 3500)	75;33	RW;Soil	20;64d	Krzeminski *et. al.*, 1973
	95;92	BAS	1d	
$C_xBz\ Cl^-$	100	In	11d	Arpino and Ruffo, 1976
$C_xC_{12}Bz\ Cl^-$	75	In	7d	
$C_8Bz\ Cl^-$	79	In	10d	Masuda *et. al.*, 1976
$C_{10}Bz\ Cl^-$	95			
$C_{12}Bz\ Cl^-$	97;100	In	10;2d	
$C_{14}Bz\ Cl^-$	83	In	10d	
$C_{18}Bz\ Cl^-$	0;62	In	10;25d	Masuda *et. al.*, 1976
$C_{16}Bz\ Cl^-$	96;88	In	20d	Pitter *et. al.*, 1976
	85;81	In	20d	
$C_{16}Bz\ Cl^-$	0	In	60d	Dean-Raymond and Alexander,1977
$C_{12}Bz\ Cl^-$	96;54±16	CAS	3h	Gerike *et. al.*, 1978
	83±7	CAS	3h	Gerike and Fischer, 1979
	0-95	In	14-40d	
	0	In	14-30d	
	50	In	28d	
CocoBz Cl^-	0	In	10d	Itoh *et. al.*, 1979
	94;0	CAS;BAnD	3h;70d	Janicke and Hilge, 1979
$C_{12}Bz\ Cl^-;Br^-$	99	In	4d	Ruiz Cruz, 1979 Ruiz Cruz and Dobarganes Garcia, 1979
$C_{14}Bz\ Cl^-$	99			
$C_{16}Bz\ Cl^-$	98	In	6d	
$C_{18}Bz\ Cl^-$	98	In	8d	
br-C_xBz	61	In	15d	
$C_{12}Bz\ Cl^-$	61	In	15d	
$C_{12}Bz\ Cl^-$	84	BAS	19d	Gerike and Fischer, 1981
$C_{12}Bz\ Cl^-$	97	RW	5d	Ruiz Cruz, 1981
	96	CAS	3h	Gerike, 1982
	83±7	CAS;BAS	3h;40d	Gerike *et. al.*, 1984
	90;75	In	30;28d	

Table 3.2.3 Biodegradation data of dialkyldimethylammonium ($R_2Me_2N^+$) type quaternary surfactants (Swisher, 1987)

Substrate $R_2Me_2N^+$	Extent	Method	Time hours (h) or days (d)	Reference
$(C_8)_2$ Cl^-	95	SF	2d	Gawel and Huddleston, 1972
$(C_{10})_2$ Cl^-	98			
$(C_{18})_2$ Cl^-	100	In	7d	Arpino and Ruffo, 1976
$(C_{10})_2$ Cl^-	50	In	19d	Masuda *et. al.*, 1976
$(C_{12})_2$ Cl^-	0;25-75	In	10;25d	
$(C_{14})_2$ Cl-	0;54			
$(C_{16})_2$ Cl^-	0;42			
$(C_{18})_2$ Cl^-	0;23			
$(C_{18})_2$ Cl^-	91-93	CAS	3h	May and Neufahrt, 1976; Neufahrt *et. al.*, 1976
$(C_{18})_2$ Cl^-	0	RW	30d	Baleux and Caumette, 1977
$(C_{12})_2$ Br^-	0	In	60d	Dean-Raymond and Alexander, 1977
$(C_{18})_2$ Br^-	0			
$(C_{10})_2$ Cl^-	95;0±38	CAS	3h	Gerike *et. al.*, 1978
$(C_{18})_2$ Cl^-	95;78	CAS	3h	
	108±9	CAS	3h	
$(Tai)_2$ Cl^-	0	In	10d	Itoh *et. al.*, 1979
	62-82	CAS	3h	Janicke and Hilge, 1979
	0	BanD	70d	
$(C_{12})_2$	20	In	25d	Miura *et. al.*, 1979
$(C_{14})_2$	30			
$(C_{16})_2$	40			
$(C_{18})_2$	25			
$(C_{10})_2$ Cl^-	98	RW	4d	Ruiz Cruz and Dobarganes Garcia, 1979
$(C_{12})_2$ Cl^-	90-92	RW	7d	
$(C_{10})_2$ Cl^-	99	RW	7d	Ruiz Cruz, 1981
$(C_{10})_2$ Cl^-	95;100	CAS;TF	3h;-	Gerike, 1982
	94±3	TF	-	
$(C_{18})_2$	100;86	TF	-	
	99±12	TF	-	
$(C_{18})_2$	90-96	CAS	3h	Topping and Waters, 1982
	77-100	CAS	5h	
	98-99	CAS	?	Vos and de Henau, 1982
	86	TF	-	
$(*C_{18})_2(U\text{-}^{14}C)$	66;43	RWg;RW	62;74d;	Larson 1983 and Larson and Wentler, 1983
$(C_{18})_2$ Cl^-	4	In	33d	
$(C_{18})_2$	>95	CAS	?	Neufahrt *et. al.*,1984
$(*C_{18})_2(\alpha\text{-}^{14}C)$ $(U\text{-}^{14}C)$	61;>31 81;>31	BAS	39d	Sullivan, 1983
$(C_{18})_2*Me_2$	77;>53			
$(C_{18})_2$	73-92	*CAS ?	HP	Wee, 1984

List of abbreviations used in Table 3.2.1-3.2.3

The Method column are as follows:

Acc	Special acclimation
BAnD	Batch anaerobic digester
BAS	Batch or semicontinuous activated sludge
BOD	Standard BOD bottle procedure
CAnD	Continuous anaerobic digester, usually fed in small daily or weekly increments(thus actually semicontinuous)
CAS	Continuous-flow activated sludge
GW	Groundwater
In	Natural or synthetic medium inoculated with acclimated or unacclimated organisms; although the shake-culture, BOD, and Warburg procedures fall in this category also, they have been designated individually.
RW	River water dieaway
Sew	Sewage dieaway
SF	Shake-flask culture
Soil	Percolation through a soil medium, usually intermittently, or dieaway in soil
TF	Trickling filter
Wa	Warburg respirometer, biodegradation may be estimated from other appropriate analysis besides O_2 uptake
*	Indicate field test or large-scale trials in sewage treatment equipment

inoculated by themselves onto decyltrimethylammonium bromide amended media, a mixture of two pure strains, a *Pseudomonas* and a *Xanthomonas,* were able to grow on the amended media using the QAC as a sole carbon source. Both of these bacteria were Gram-negative rods which gave a positive Kovac oxidase test. It was suggested that the *Xanthomonas* acted on the decyltrimethylammonium bromide, but since this bacterium could not grow on the amended media alone, the *Pseudomonas* was thought to provide one or more of the growth factors needed, while being sustained by partial degradation of the QAC by the *Xanthomonas.*

Metabolites extracted from culture by methylene chloride and identified by GC/MS included 9-carboxynonyl- and 7-carboxyheptyltrimethylammonium compounds. Their formation is consistent with the initial oxidation of the terminal carbon, followed by β-oxidation of the acetyl units, leading ultimately to the formation of lower molecular weight carboxylic acids. Cells from cultures of *Pseudomonas* and *Xanthomonas* were grown in the presence of decyltrimethylammonium bromide, trimethylamine, decanoic acid and other possible by-products. Both decyltrimethylammonium bromide and trimethylamine were rapidly and almost completely oxidized, while trimethylamine, methyldecylamine (40%) and decanoic acid (65%) were substantially degraded within 3 hours or less, with almost no lag phase. This was interpreted by Dean-Raymond and Alexander as suggesting the formation of trimethylamine upon completion of β-oxidation.

Hampton and Zatman (1973) studied the metabolism of tetramethylammonium chloride by the bacterium 5H2. This Gram-negative, non-mobile, rod-shape organism isolated from soil, was described as being capable of growth on tetramethylammonium chloride and trimethylamine, when they were present as the sole source of carbon. The authors suggested

that the tetramethylammonium chloride was completely biodegraded via the intermediates of trimethylamine, trimethylamine N-oxide to methylamine.

Van Ginkel *et al.* (1992) reported that a bacterium, *Pseudomonas* strain B_1, isolated from activated sludge could utilize hexadecyltrimethylammonium chloride as a sole carbon source. Of the various alkylsubstituted quaternary ammonium salts (i.e. alkyltrimethylammonium, dialkyldimethylammonium etc.) tested, *Pseudomonas* strain B_1 could grow only on the monoalkyl compounds. It was able to utilize these compounds over a wide range of alkyl chain length (C_{12} to C_{22}). Van Ginkel and *co-workers.* also demonstrated that *Pseudomonas* strain B_1 could use a wide range of organic compounds as sole carbon sources, including acetate, ethanol, glucose, hexadecanoate, and hexadecanal. However, none of the amines tested (hexadecyldimethylamine, hexadecylamine, trimethylamine and methylamine) could serve as growth substrates for this bacterium and these amines were not oxidized by bacterial cells grown on hexadecyltrimethylammonium chloride. These observations suggested that hexadecanal and hexadecanoate were likely intermediates formed during the degradation of hexadecyltrimethylammonium chloride. However, the inability of the *Pseudomonas* strain B_1 to metabolize any of the amines, strongly suggests that this bacterium is only capable of cleaving and oxidizing the alkyl chain, and that the amines were not formed as intermediates in the biodegradation pathway. When the *Pseudomonas* strain B_1 was grown in liquid culture in the presence of silica gel, the stoichiometric formation of trimethylamine was confirmed by gas chromatography. These observations suggested that the ultimate biodegradation of hexadecyltrimethylammonium chloride must involve at least two species of bacteria.

In a very recent study, Nishiyama *et al.* (1995) have confirmed the de-alkylation of the alkyltrimethylammonium halide as the first step in the degradation using an inoculum of activated sewage sludge. They suggested that the tertiary amine initially formed underwent rapid demethylation. It was also proposed that the amines were present as a salt.

Using enrichment techniques, Ghisalba and Küenzi (1983) isolated a number of bacteria from the sludge of an industrial sewage treatment plant, which were capable of utilizing trimethylethylammonium salt. Nine of these bacteria, classified as *Pseudomonas* sp., were able to grow on a wide range of QAC and amine substrates as a sole carbon and nitrogen source, including methylamine, dimethylamine, trimethylamine, dimethylamine dimethylethylamine and tetramethylamine. Interestingly, the growth rate on the amines was more rapid than that on the alkyltrimethylammonium chloride. In shake cultures with an unbuffered medium (MV7) and 5g/l of trimethylethylammonium chloride as the sole carbon source, a decrease in the pH (from 7 to 3.5) was observed due to the formation of hydrochloric acid. It was found that the trimethylethylammonium salt was biodegraded and utilized without the accumulation of intermediate degradation products, but with the formation of biomass and CO_2 and the release of NH_4Cl and HCl.

In studying the biodegradation of $C_{18}Me_3N^+Cl^-$ using ^{14}C labeling of the carbons adjacent to the nitrogen, Games *et al.* (1982) observed that when placed at the first carbon of N-methyl bonding, the formation of $^{14}CO_2$ was significantly faster than when it was placed in the C_{18}-alkyl chain. This was interpreted as being due to the N-methyl carbon being used mainly for energy, while the C_{18} carbon was involved more in the synthesis of biomass.

3.2.2 Alkyl benzyldimethylammonium salts, $C_nBzMe_2N^+$

Alkylbenzyldimethylammonium salts have been thought as superior wood candidates as wood preservatives. Based upon the oxygen uptake in respirometric experiments Masuda *et al.* (1976) found that 80 to 100% of the linear $C_nBzMe_2N^+$ cationic surfactants could be degraded in 10 days, when the alkyl chain length varied from C_8 to C_{14}. However, longer alkyl chains, C_{16} and C_{18}, were much more resistant to degradation, with the oxygen demand falling to almost zero. The addition of an anionic surfactant enhanced the degradation of the hexadecylbenzyldimethylammonium chloride to about 45% in 15 days. Using a preformed activated sludge, in a continuous flow experiment, Fenger *et al.* (1973) were able to reduce the detectable amount of tetradecylbenzyldimethylammonium chloride by up to 85%. The debenzylated amine, as well as benzoic acid and acetic acid were identified by infra-red spectroscopy and gas chromatography, but products from further degradation (for example methylamine) were not detected. This would support the conclusion that the cleavage of the benzyl group occurs preferentially as the first step in the degradation. The authors also concluded that at concentrations in the range of 20 ppm, tetradecylbenzyldimethylammonium chloride could be degraded in activated sludge even when it is the sole carbon source. Examination of the adapted sludge revealed that the most resistant bacteria to the tetradecylbenzyldimethylammonium chloride were almost exclusively *Pseudomonas* and *Comamonas* bacteria. A similar observation was recorded by Gawel and Huddleson (1972) who found that tetradecylbenzyldimethylammonium chloride at a concentration of 10 mg/l was biodegradable in a shake culture. Confirmation of the disappearance of the QAC was based upon a colorometric and UV spectrometric analysis. Substitution of an ethyl group in

the benzyl ring caused the rate of degradation to slow, although 95% biodegradation was recorded in 2 days.

Krzeminski *et al.* (1973) examined the biodegradation of alkyl(C_{12-16})*$BzMe_2N^+$ (Hyamine 3500) by monitoring the generation of $^{14}CO_2$ from the ^{14}C tag in the methylene group of the benzyl substituent. They reported that more than 90% of the ^{14}C appeared as carbon dioxide with less than 10% being found in the effluent.

Gerike *et al.* (1978) investigated the biodegradation of dodecylbenzyldimethyl-ammonium chloride based upon the Confirmatory Test using an activated sludge developed by the Organisation for Economic Cooperation and Development (OECD). In this test, the elimination of the QAC was monitored by the disappearance of the QAC using a disulphine blue active complex formation. Such an approach is limited since even a small modification of the molecule would cause a reduction in the QAC detected. Therefore, the authors also compared the dissolved organic carbon (DOC) before and after biological treatment. Under these conditions, the dodecylbenzyldimethylammonium chloride concentration was shown to be reduced by 54%. It was concluded that this QAC was degradable at least at a primary level.

3.2.3 Dialkyldimethylammonium salts $(C_n)_2Me_2N^+$

Dialkyldimethylammonium salts are considered to have the best potential wood preservatives based upon laboratory evaluations. As anticipated, the addition of a second hydrophobic alkyl chain on the QAC decreases its aqueous solubility compared to the monoalkyl QAC. At the same time, it is rendered more bacteriotoxic. However, the same

strategies that were successfully used for biodegrading benzalkonium chloride, can be employed with the dialkyldimethylammonium chlorides. These include maintaining QAC concentrations below those levels that are toxic to the micro-organism as well as complexing the QAC with a suitable anionic surfactant. The usefulness of the latter strategy has been ably demonstrated by Masuda et al. (1976). Based upon the reduction in oxygen uptake in respirometric experiments, almost 50% of didecyldimethylammonium chloride was biodegraded in 10 days, while higher homologues were not degraded at all. Addition of sodium dodecyl sulphate allowed the biodegradation to reach almost 80% in 20 days.

Sullivan (1983) studied the degradation of ditallowdimethylammonium chloride using a "semi-batch" aerobic activated sludge, in which labeled surfactant was added at a concentration of 2 ppm to the initial charging of the system. In three replicate experiments, samples of ditallowdimethylammonium chloride were radiolabelled [^{14}C] at the N-methyl, the 1 position of the C_{18} chain, or uniformly in the chain. The approximate initial [^{14}C] ditallowdimethylammonium chloride concentration was 2.1 mg/l in each test reactor. The $^{14}CO_2$ formation was monitored to determined the susceptibility of the different positions to being metabolized. Although the ditallowdimethylammonium chloride rapidly disappeared (95%) from the solution, it had been adsorbed by the sludge. Subsequently, over the remaining 35 days, it was biodegraded with mineralization being demonstrated by the production of $^{14}CO_2$. Between 20 and 50% of the ditallowdimethylammonium chloride was mineralized by day 39, based upon the production of labeled carbon dioxide. This observation confirmed that each of the three major carbon positions in the molecule could be mineralized, and that ultimate biodegradation was possible for this QAC. The highest concentration of $^{14}CO_2$ was recorded for the N-methyl tagged molecule, suggesting that it was converted more

readily than carbons in the alkyl chain. A statistical analysis found no difference in the rate constants for the primary biodegradation, suggesting that all three labelled molecules were removed from the solution at the same rate.

Based upon radio thin layer chromatographic scans, Sullivan also reported that since the concentrations of any intermediates were very low during the biodegradation and they did not accumulate in the sludge, they must be short lived and the rate of degradation must be limited by the primary degradation process, during which the QAC is removed from the solution. This is consistent with the observations of Gerike *et al.* (1978) who by measuring the soluble carbon content in the effluent of the test reactors, reported the complete removal of ditallowdimethylammonium chloride and any breakdown products from the test solution. Similar experiments with didecyldimethylammonium chloride gave much less satisfactory results with almost no degradation being reported (Gerike *et al.* 1978). Subsequent studies on this same QAC using a trickling filter demonstrated 94% removal of didecyldimethylammonium chloride, based upon the analysis of dissolved organic carbon. However, such results should be treated with caution as adsorbed or precipitated QAC would not be separated from the biomass, leading to an overestimation of biodegradation.

The influence of the sediment on the biodegradation of QACs present in solution has been demonstrated by several researchers. Larson and Vashon, (1983b) confirmed the mineralization of dioctadecyldimethylammonium chloride in river water at 0.005 and 0.05 ppm, based upon the amount of $^{14}CO_2$ produced during biodegradation of the alkyl chains which were labeled uniformly with ^{14}C. Without any sediment the $^{14}CO_2$ production was much less (10 and 20% of theoretical) than that achieved when 5000 ppm of sediment was

present (65%). Thus under the right conditions the dioctadecyldimethylammonium chloride was biodegradable.

3.2.4 Alkylpyridinium salts $(C_n)py^+$

Without exception, studies of alkylpyridinium salts confirm that they are some of the most difficult QACs to biodegrade. Masuda *et al.* (1976) measured the degradation of $C_{12}Py^+$ at only 18% based upon the 10 day oxygen uptake. However, Pitter (1976) using the "die-away method" found that the rate of primary biodegradation for the pyridinium bromide was comparable to that of the benzalkonium chloride. Wright and Cain (1972) reported that a bacterium *Achromobacter* D, isolated from garden soil was able to utilize N-methylisonicotinic acid as a sole carbon source. The metabolic products were identified as, methylamine from the N-methyl group, succinate and formate from the pyridine ring, and carbon dioxide derived from the carboxylic group on the heterocyclic ring.

Barden and Isaac (1957) reported that 15 ppm of cetylpyridinium bromide was completely degraded by *Pseudomonas pictorium.* Baldwin *et al.* (1966) isolated a strain of *Lipomyces starkeyi* which was able to utilize paraquat and related pyridine compounds, as a source of nitrogen.

3.2.5 Trialkylmethylammonium salts $(C_n)_3MeN^+$

As expected these QACs are much more recalcitrant to biodegradation, although very few studies of their biodegradation have been reported. Ruiz Cruz (1979) studied the

biodegradation of tridecylmethylammonium chloride in river water at a concentration of 5 ppm. The time to 50% disappearance was 7.4 days, which is almost 3.5 times that of didecyldimethylammonium chloride.

3.3 Mechanisms of biodegradation

The mechanisms by which bacteria degrade QACs are still not fully understood. Examination of the literature failed to reveal clear support for any one of the proposed models. In general, two basic biodegradation processes have been advanced (Figure 3.3.1). In the first, the micro-organism initiates attack (ω-oxidation) at the end carbon of one of the long alkyl chains. Further degradation (β-oxidation) occurs at this long chain gradually removing carbon atoms. In the alternative biodegradation process, the initial step involves the breakage (fission) of one of the carbon-nitrogen bonds, leading to the formation of a tertiary amine and a residual alkan-1-al. A brief review of the supporting evidence for each of the mechanisms is presented below.

3.3.1 Oxidation of the long alkyl chain.

Cain (1976) has noted that the long alkyl chains of QACs are potentially susceptible to ω- oxidation at the terminal methyl position. Subsequent β-oxidation of this chain in the case of alkyltrimethylammonium chloride, results in the formation of betaine. Cain cites the fact that betaine can support the growth of selected bacteria, which demethylate it, forming first dimethylglycine and subsequently sarcosine (Figure 3.3.1.1). Sarcosine can be further demethylated to glycine or decarboxylated to form the secondary amine. The detection of the inducible dimethylglycine and sarcosine dehydrogenases by Frissell and Mackenzie (1962) was cited by Cain as providing supporting existence for this mechanism.

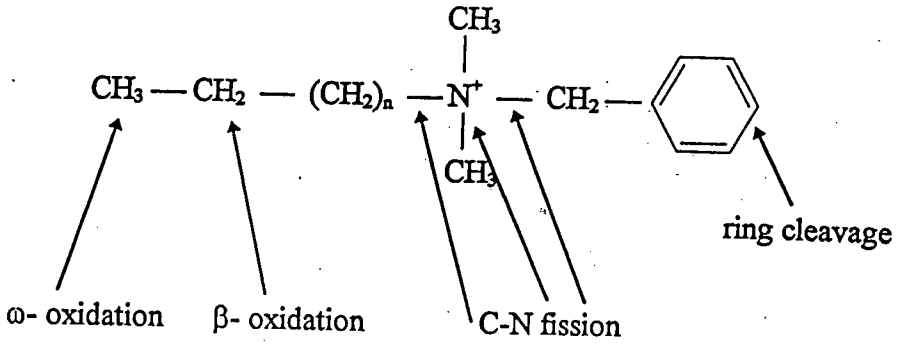


Fig. 3.3.1 Possible mechanisms of degradation of QACs by bacteria

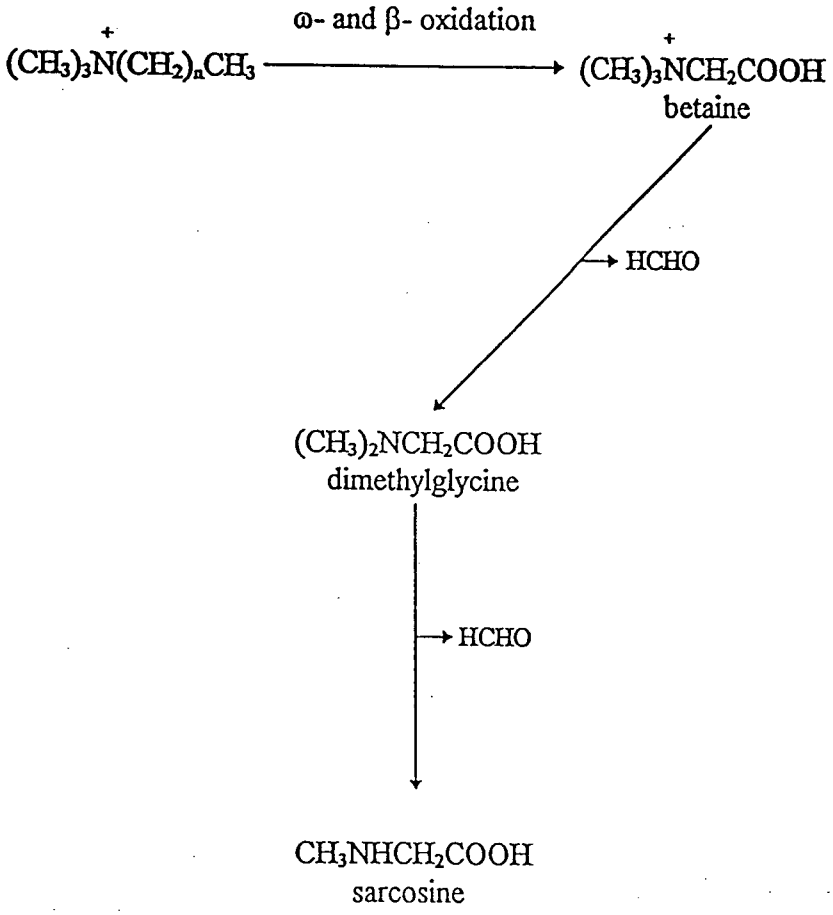


Fig. 3.3.1.1 Biodegradation of alkyltrimethylammonium chloride, initiated at the terminal methyl group of long alkyl chain, followed by β-oxidation of the chain with the formation of betaine. Subsequent cleavage of the methyl groups from the nitrogen yields sarcosine (Cain, 1976)

This biodegradation mechanism in which attack is initiated at the end of the long alkyl chain was also supported by Dean-Raymond and Alexander (1977). When studying the biodegradation of decyltrimethylammonium bromide they observed the accumulation of 9-carboxynonyl- and 7-carboxyheptytrimethylammonium as intermediates, consistent with the to ω-oxidation of the decyl group. Smaller amounts of lower molecular weight carboxylic acids derivatives were also possibly formed. The formation of such intermediates is consistent with an initial oxidation of the terminal carbon atom of the long chain by *Xanthomonas,* followed by stepwise cleavage of the acetyl units by β-oxidation.

3.3.2 Carbon nitrogen fission: cleavage of the alkyl chain

This mechanism has also been proposed by Van Ginkel et al. (1992) working with bacteria (Figure 3.3.2.1). They employed a *Pseudomonas* strain B1 to biodegrade hexadecyltrimethylammonium chloride, which was served as a carbon and energy source. However, while the bacterium could grow on hexadecyltrimethyl- ammonium chloride or related intermediates, it could not grow on amines. This observation formed the basis of the proposal by these authors, that a second bacterium was needed to achieve the ultimate biodegradation of this molecule. Confirmation for their hypothesis was obtained by the stoichiometric formation of trimethylamine when the bacterium was grown on hexadecyltrimethylammonium chloride. This proposal made by van Ginkel *et al.* (1992), that a consortium of micro-organisms would effectively lead to the ultimate biodegradation of QACs had not been identified earlier, but appears to be very plausible. The fact that the bacterium B1 could successfully grow on hexadeconal and hexadecanoate, indicates that both are intermediates during the degradation, supporting of alkyl chain cleavage as a key step in

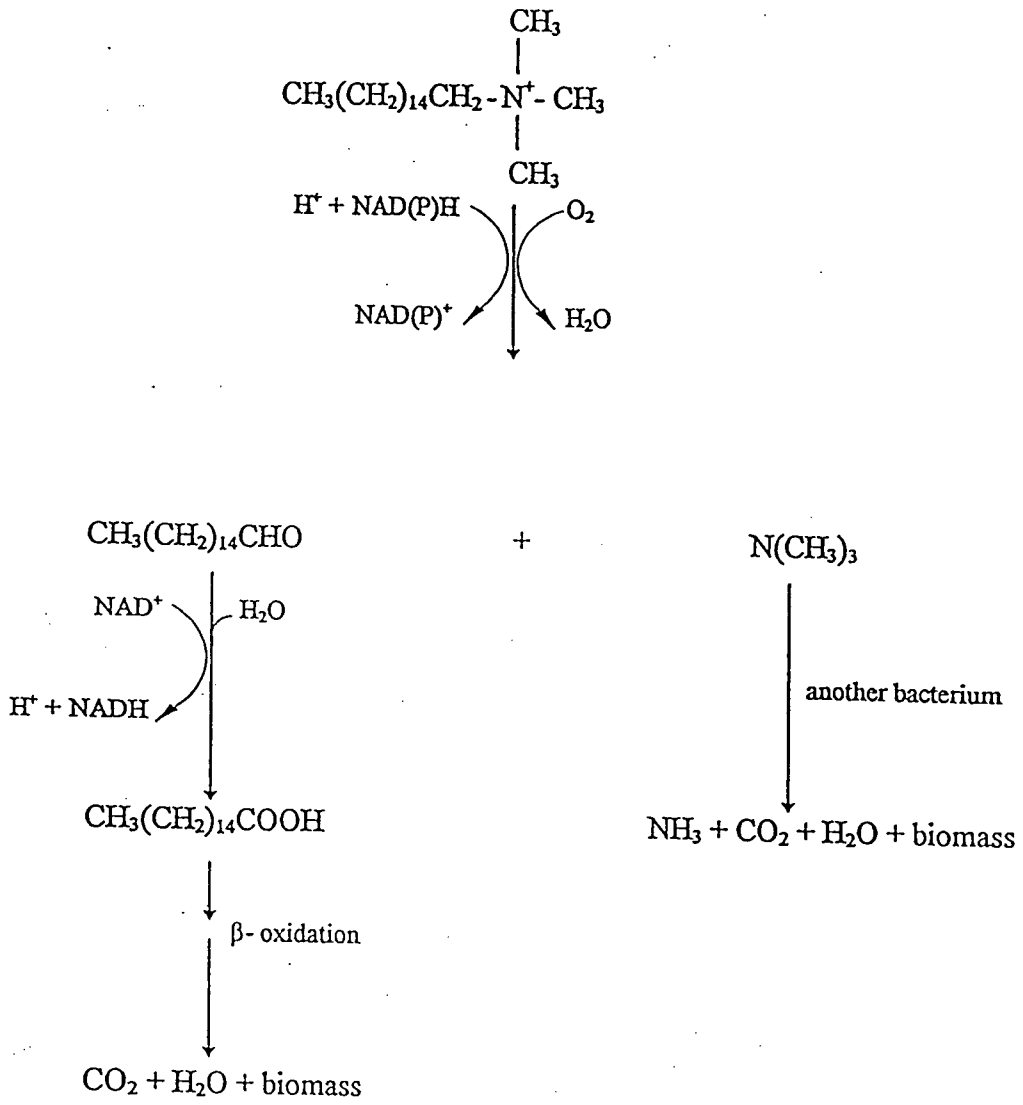


Fig. 3.3.2.1 Proposed biodegradation mechanism for hexadecyltrimethylammonium chloride by *Pseudomonas* strain B_1 (Van Ginkel *et al*, 1992)

the mechanism. The direct formation of trimethylamine is in agreement with the results reported by Hampton and Zatman (1973) who also observed the production of trimethylamine and methanol during biodegradation of tetramethylammonium chloride.

Ghisalba and Küenzi (1983) found that trimethylethylammonium chloride could be utilized by nine strains of bacteria, as both a carbon and nitrogen source. The pathways proposed by authors for the biodegradation of trimethylethylammonium chloride by *Pseudomonas* strains are depicted in Figure 3.3.2.2. They found that the *Pseudomonas* strains were able to grow much better on amines such as dimethylamine and dimethylethylamine than on either tetramethylammonium chloride or trimethylethylammonium chloride. This suggests that the first stage in degradation of the QAC, namely the loss of one alkyl group with the formation of a tertiary amine will be the rate limiting step in the biodegradation of the QAC. One question that remained unanswered was when the ethyl group was cleaved. Loss of the ethyl group during initial fungal attack would correspond to the left metabolic pathway in Figure 3.3.2.2. This resembles the biodegradation process for other alkyltrimethylammonium chlorides after the initial loss of the alkyl chain. The anticipated products from the first step would be trimethylamine and methan-1-al. In an alternative mechanism the ethyl group was cleaved at a later stage with the formation of methan-1 al and the corresponding amine acid salt. During fermentation studies, no significant accumulation of anticipated metabolites was detected. This would support the suggestion that the initial degradation of the QAC is the slowest step. However, it prevented any conclusion being made as to when the ethyl group was cleaved.

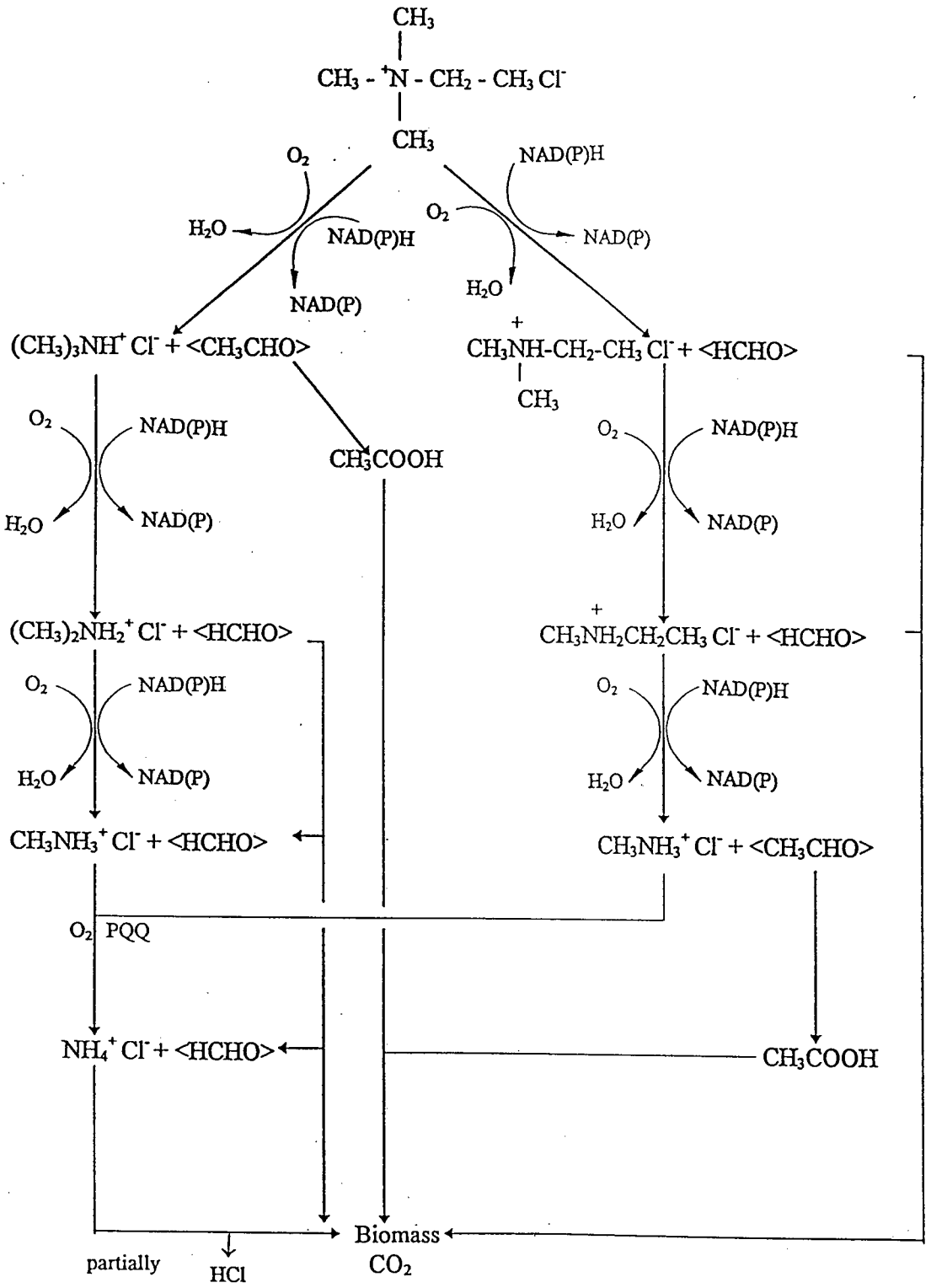


Fig. 3.3.2.2 Possible pathways for the biodegradation of trimethylethylammonium chloride by *Pseudomonas* (Ghisalba and Küenzi, 1983)

In a recent study Nishiyama *et al.* (1995) examined the biodegradation of several alkyltrimethylammonium halides by an activated sludge. Based upon analysis by ^{1}H-NMR the formation of trimethylamine (as an acid salt) after 40 hours incubation was positively identified for the decyl-, dodecyl- and tetradecyltrimethylammonium bromides. Subsequently, after 48 hours of incubation, the acid salt of dimethylamine was identified as being formed in the medium. The third step in the degradation pathway, the further demethylation with the formation of methylamine (as an acid salt) was confirmed by the NMR examination after 76 hours. Finally, following almost five days of incubation neither the parent QAC nor any of the degradation products could be detected. These observations clearly support the cleavage of the long alkyl chain as the initiating step for alkyltrimethylammonium halides. Interestingly, these authors also confirm the formation of acid salts of the amines in the media together with their gradual disappearance as the incubation time extends. These observations may have a bearing on the failure of other researchers to identify the formation of anticipated metabolites.

3.4 Factors affecting the biodegradation of quaternary ammonium compounds

A number of factors have been identified which can affect the biodegradability of QACs. Two of the most important are the length of the alkyl chain and the number of long alkyl chains present in the QAC. Based upon the body of research available, it is clear that the resistance of QACs containing linear hydrophobic chains to biodegradation increases in the order $RMe_3N^+ < RBzMe_2N^+ < R_2Me_2N^+ < RPy^+ < R_3MeN^+$ (Boethling, 1984). Ruiz Cruz and Dobarganes Garcia, (1979) recorded the time taken for 5 ppm of selected QACs to decrease by 50%. While this could be achieved for dodecyltrimethylammonium and decylbenzalkonium chlorides in 1 to 2 days, dialkyldimethylammonium chlorides required 2 to 3 days and trialkylmethylammonium compounds took approximately 7 days. Masuda *et al.* (1976) measured oxygen uptake after 10 days incubation of 20 ppm of various QACs. While the theoretical oxygen demand approached 100% for decyltrimethylammonium chloride, and decylbenzyldimethylammonium chloride, it was only 50% for didecyldimethylammonium chloride and 4% for decylpyridinium chloride.

Van Ginkel and Kolvenbach (1991) have reported the influence of structure and chain length on the biodegradability of selected QACs. They observed that as the alkyl chain length increased from C_{10} to C_{18} the oxidation rates of mono- and dialkylammonium chlorides decreased. However, the changes were less dramatic than those arising from an increase in the number of hydrophobic alkyl chains. This is consistent with the views presented by Boethling, (1984).

Ghisalba and Küenzi (1983) reported that *Pseudomonas* strains which were tolerant to tetramethylammonium chloride were not able to utilize tetraethylammonium chloride,

methyltrimethylethylammnium chloride and dimethyldiethylammonium chloride. Mackrell and Walker (1978) also found that their enrichment culture grown on tetramethylammonium chloride and adapted to trimethylethylammonium chloride was not able to grow on the higher homologue tetraethylammonium bromide. They suggested that this was probably due to the close-packed tetrahedral structure which protects the central nitrogen atom against biodegradation attacks. However, Ghisalba and Küenzi (1983) observed that ethylamine are not easily utilized by the bacteria and reasoned that the enzymes which demethylate the QAC may be specific for the methyl group. Conversely, triethylamine was reported to be a better carbon source than either the mono- or dimethylamines.

A third factor influencing the biodegradability of QACs is the presence of substrates that can adsorb or complex with the QAC thereby limiting the concentration present in the aqueous phase and/or reducing the surfactant properties of the QAC. There have been several studies which clearly demonstrate that the addition of sediment can encourage the biodegradation of QACs at much higher levels than can be biodegraded without the sediment (Krzeminski *et al.*, 1973; and Larsen and Vashon, 1983). In a similar manner, the addition of anionic complexing agents can be used to enhance the biodegradability of QACs (Gerike *et al.* 1978).

A fourth factor to be considered is the presence of tolerant micro-organisms. It is known that there are relatively few bacterial genera that can demethylate alkylamino-compounds, although their distribution is ubiquitous. Their presence can therefore enhance the biodegradability of QACs.

A fifth factor that will influence the degree of biodegradation will be the presence of a suitable consortium of micro-organisms that can undertake the degradation of the QAC and the metabolites that will be produced during the degradation process. Thus, van Ginkel *et al.* (1992) has proposed that at least two bacteria are required to biodegrade hexadecyltrimethylammonium chloride. Also of importance is the phenomenon of acclimation. Gawel and Huddleston (1972) reported that following acclimation, a second aliquot of a QAC was degraded much more rapidly than that present originally.

The germicidal properties of the cationic do not necessarily interfere with their ultimate biodegradation provided that operating concentrations are kept below the toxic threshold. Above that threshold, toxicity can be mitigated by neutralization of the cationic with an equivalent amount of anionic of ultimate biodegradation.

Finally, the biodegradation rates experience in the environment will also be influenced by factors other than chemical structure. These include such factors as microbial biomass, aeration, temperature, available nutrients, solar radiation and pH (Boethling, 1984).

4. MATERIAL AND METHODS

4.1 Evaluation of a rapid colormetric screening technique for identifying QAC degrading fungi

4.1.1 Chemicals

The quaternary ammonium compound used in the project was commercial grade, Bardac® 2280 (Lonza Inc.) which contained 80 % didecyl (C_{10},C_{10}) dimethyl-ammonium chloride (DDAC) active ingredient, 10% ethyl alcohol and 10% water. All amounts of DDAC are expressed in terms of the active ingredient only. Bromophenol blue used was supplied by Fisher Scientific Company, while the methyl orange indicator was purchased from Anachemia Chemical Ltd.

4.1.2 Sample preparation

DDAC solution with a wide range of concentrations ($2.04x10^{-6}$ M, $2.04x10^{-5}$ M, $4.08x10^{-5}$ M, $2.04x10^{-4}$ M, $4.08x10^{-4}$ M, $4.08x10^{-3}$ M and $1.02x10^{-2}$ M) and one standard $2.04x10^{-4}$ M solution of bromophenol blue were prepared. For determination of the spectra test solutions containing both DDAC and bromophenol blue or methyl orange were prepared. The solution was adjusted using 1M sodium hydroxide or 1M hydrochloric acid to produce values of 1.80, 3.50, 6.80 and 12.00. All pH measurements were made with an Accumet pH meter 915 (Fisher Scientific).

It is generally expected that the stoichiometric relationship of a compound formed from monovalent ions of opposite charge will be 1:1. At high pH values (12.0), the phenolic

groups of bromophenol blue are completely dissociated, so that the possibility of two DDAC molecules combining with one mole of anionic dye exists.

For experiments under acidic conditions, 10 ml of bromophenol blue were added to 10 ml of each DDAC solution. For the alkaline pH of 12.0, 10 ml of bromophenol blue were added to 20 ml of each DDAC solution. The final solution concentrations tested were: $2.04x10^{-7}$ M, $2.04x10^{-6}$ M, $4.08x10^{-6}$ M, $2.04x10^{-5}$ M, $4.08x10^{-5}$ M, $4.08x10^{-4}$ M and $1.02x10^{-3}$ M of DDAC and $2.04x10^{-5}$ M bromophenol blue.

For the studies using methyl orange the procedure was identical to that employed for bromophenol blue, with the exception that pH values of 1.1 and 11.5 were studied.

4.1.3 Sample analysis

All the samples were analyzed by Cary UV-Vis spectrophotometer (200 900 nm) to determine the relative absorbances of the bromophenol blue-DDAC and methyl orange-DDAC compounds. Standard solutions of bromophenol blue and methyl orange were also analyzed to determine the changes due to the compound formation.

4.2 Isolation of tolerant fungi

In order to isolate fungi which are very tolerant to QACs or tertiary amines, small wooden stakelets treated with several retentions of DDAC and decyldimethylamine (Barlene 10S) were placed in a field test site at the University of British Columbia and recovered at selected time intervals. The treated stakelets were sampled to recover the tolerant fungi after different time intervals.

4.2.1 Wood treatment and exposure

Southern yellow pine (*Pinus spp*) sapwood was cut into small stakelets with approximate dimension of 12 x 10 x 102 mm. The small stakelets were vacuum treated with 0%, 0.3% and 0.6% solutions of DDAC or decyldimethylamine (Barlene 10S supplied by Lonza Inc.) and randomly placed in an experimental test site located at the University of British Columbia. Additional samples were installed in the fungal cellar at Forintek Canada Corp. (Western laboratory). Three replicates for each concentration were used. Each stakelet was completely driven into the soil.

4.2.2 Isolation of tolerant fungi from treated wood exposed in the field area

After intervals of six weeks and four months exposure in the field, representative stakelets were removed for observation. From those which showed visible fungal colonization representative samples were retained and sampled to isolate the early colonizing fungi. Selective media starch-casein-nitrate-rose-bengal (SCNR), benomyl-tetracycline-malt agar (BTMA) and preservative media (malt agar with DDAC or decyldimethylamine) were used to

isolate mould and stain fungi, basidiomycete, and fungi tolerant to either DDAC or decyldimethylamine, respectively.

Preparation of media

The SCNR media contained: starch 10.0 g/l, casein 0.3 g/l, KNO_3 2.0 g/l, NaCl 2.0 g/l, K_2HPO_4 2.0 g/l, $CaCO_3$ 0.03 g/l, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, agar 10.0 g/l, Rose Bengal 0.35 g/l, in one liter of water. The solutions of BTMA media contained: malt extract 20.0 g/l, agar 20.0 g/l, tetracycline 0.1 g/l in one liter of distilled water. Solutions of media containing DDAC or decyldimethylamine were prepared by dissolving: malt extract 20.0 g/l, agar 20.0 g/l, and 100 ppm of either DDAC or decyldimethylamine in one liter of distilled water.

Isolation from stakelets

Each stakelet was wiped with cotton or paper tower soaked in 70% ethanol before splitting. Small pieces (1 x 1 x 1 mm) were removed from the freshly exposed wood and placed on the selective media. The plates were incubated at 25 °C and the growth observed daily until no new fungi appeared on the plate (media). Any fungal growth which appeared in the selective media was subcultured onto malt agar containing 100 ppm chloramphenical to prevent bacterial growth. Once pure cultures were obtained through sub-culturing, all the isolates were grown on malt agar containing a threshold concentration of 250 ppm DDAC, to identify those which exhibited tolerance to this preservative.

To further limit the fungi to be used in subsequent experiment, they were inoculated (core diameter 6 mm) onto malt agar containing 750 ppm DDAC. After twenty days, only those fungi which grew on the agar with a colony diameter of 5.0 cm were retained for further experiments.

4.3 Comparison of the tolerance of isolates from the field experiment with those from culture collections

4.3.1 Tolerance of fungi in solid media

Fungal species

Strains of fungi obtained from the culture collection of Forintek Canada Corp., Ottawa included four strains of *Gliocladium roseum* Bain (321A, 321B, 321C and 321D), *Gliocladium viride* (623B), *Gliocladium virens** (258C and 258D), *Verticillium lecanii* (390A), *Verticillium dahliae* (163A). These fungal strains from the culture collections were compared with two strains of *Gliocladium roseum* (GRSB and GRTC), each of *Verticillium bulbillosum* and *Acremonium* sp. which were isolated by Dr. A. Doyle at the University of British Columbia and identified by the National Identification Service, Agriculture Canada. Five additional strains of fungi which were recovered from field stakelets treated with DDAC or decyldimethylamine were also studied. These isolates were coded as follows: S-1-1 and S-1-6 which were isolated in July, 1994; S-2-1 and S-2-11 which were isolated in October, 1994; and F-1-1 which was isolated from fungal cellar at Forintek Canada Corp. These fungi were identified by the National Identification Service, Agriculture Canada as *Fusarium avenaceum* (Fr.) Sacc. (S-1-1, S-2-1 S-2-11), *Zygorrhynchus moelleri* Vuill (S-1-6) and *Trichoderma virens* (Miller, Giddens & Foster) V. Arx (F-1-1). Since the tolerant fungal strains studied by Dr. A. Doyle were isolated largely from wood exposed out of ground contact (except for GRSB), the tolerances of the fungi isolated from the field exposure

* The two strains 258C and D were listed by Forintek Canada Corp. as *Gliocladium virens.* This fungus is now taxonomically more correctly identified as *Trichoderma virens.* However, the name G. virens will be retained for use in this thesis.

(described above) were also compared to that of the standard strains of *G. roseum, G. viride, G. virens, V. lecanii, V. dahliae.*

Preparations of different concentrations of DDAC

Aliquots of DDAC (Bardac 2280, Lonza Corp. which contains 80 % a.i.), were weighed and added to 2% malt agar solution to obtain the required concentrations of DDAC. Each solution was autoclaved and poured into petri plates (approx. 20 ml per plate) and allowed to solidify on sterile conditions. The nominal concentrations of DDAC were 0 ppm (control), 100 ppm, 250 ppm, 500 ppm, 750 ppm and 1000 ppm.

Inoculation and incubation of fungal cultures

Cores, 6 mm in diameter, were removed from the edge of colony on which the different strains had been grown for 10 days and inoculated onto the center of the test plates prepared as above. All fungi were incubated at 25 °C. Three replicates for all concentrations of DDAC were prepared with each test fungus. The growth rate was measured in two perpendicular directions from the center of the colony over 21 day period.

4.3.2 Tolerance of fungi in liquid media

Fungal species

Based on the tolerance of fungi on solid media, the following fungal strains were selected for a more detailed study in a liquid media test: a) four strains of *G. roseum* (321A, 321B, 321C and 321D), *G. viride* (623B), *G. virens* (258D and 258C), *Verticillium lecanii* (390A), all from the Forintek culture collection; b) from the previous investigation by D. Doyle (UBC) two strains of *G. roseum* (GRSB and GRTC); *V. bulbillosum* and

Acremonium sp.; c) four isolates made from stakelets exposed at the UBC test site: *Fusarium sp.* (S-1-1, S-2-11), *Zygorrhynchus sp.*(S-1-6), and *Trichoderma virens* (F-1-1)

Preparation of the liquid media

According to a previous study (Doyle, 1995), it was observed that none of three fungi studied (*G. roseum, V. bulbillosum and Acremonium sp.*) were able to grow in liquid media containing 100 ppm DDAC. In a preliminary experiment, three kinds of liquid media containing 0 ppm, 50 ppm, and 100 ppm DDAC were prepared. They were: 2% malt extract, Czapek and Vogel liquid media. The Czapek liquid media contained 30g sucrose, 2g sodium nitrate, 1g potassium phosphate, 0.5 g magnesium sulfate, 0.5 potassium chloride, 0.01 g iron sulphate, 0.01 g zinc sulphate and 0.005 g copper sulphate in one liter of distilled water. The Vogel liquid medium contained: $CaCl_2$ 0.4g, KH_2PO_4 1.0 g, Na_2HPO_4 0.8 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, NH_4NO_3 0.8 g, potassium hydrogen phthalate 3.0 g, 10 ml of 100 times concentrated micronutrient solution (Vogel 1956), 1 ml of filter-sterilized vitamin solution (Tan and Breuil 1986), 20 g of soluble starch (BDH) dissolved in one liter of distilled water. The pH of liquid media was adjusted to 5.8 ± 0.2 using 0.1M sodium hydroxide. Aliquots (50 ml) of liquid media were measured into 250 ml Erlenmeyer flasks, sterilized by autoclaving for 15 minutes at 103kPa and 121°C.

In the final experiment, Vogel liquid media was used. Aliquots of DDAC concentrate were weighed and added into the liquid media to obtain the required chemical concentrations. The Vogel liquid media containing 0 ppm and 250 ppm DDAC, which was the threshold concentration on solid media, were prepared.

Inoculation and incubation of fungi

Each flask was inoculated with a 6 diameter core of the pre-grown fungus grown on 2% malt agar plate. All the cores were from the edge of the colony. All the liquid culture were incubated for two weeks at 25 °C on a rotary shaker set at 200 rpm. Duplicate flasks were used for all fungi at all DDAC and decyldimethylamine concentrations.

Fungal biomass

The fungal biomass was measured after two weeks' incubation. In order to speed up the filtration, the liquid media after fungal growth was centrifuged at 3500 rpm for 20 minutes at 10 °C. The supernatant and the fungal mycelium was filtered under vacuum on and washed on preweighed glass fiber filters with hot water. Filters containing mycelia were oven dried at 103 °C to a constant weight, cooled in desiccator to room temperature, and weighed.

4.3.3 The growth curve of *V. bulbillosum*

Vogel liquid medium (prepared as described in Section 4.3.2) was added (50 ml) to 250 ml Elementary flasks, which were then was inoculated with a core (6 mm) removed from the growing edge of *Verticillium sp.* pre-grown on 2% malt agar. All the cores were removed from the similar distance away from the center of the colony. The growth rate was determined by measuring the biomass at selected 2 day time intervals for the first two weeks. Duplicate flasks were sampled at every time interval..

4.4 Assessment of fungal degradation of DDAC

4.4.1 Solid media

Fungal species

The fungal species were the same as that in section 4.3.1.

Preparation of media

The preparation of media was the same as that in section 4.3.1 but only one concentration of 750 ppm DDAC was employed.

Inoculation and incubation of fungal culture

The inoculation and incubation of fungal culture were the same as that in section 4.3.1.

Analysis of DDAC

After the fungal colony had covered approximately half of the plate (in area), the mycelium was carefully removed from the surface and oven-dried prior to weighing. Malt agar cores (0.2-0.5 g) were collected from beneath the center and edge of the hyphal growth of the fungal colony. Additional cores were removed from the perimeter of the plate which had not been colonized by the fungus. These additional cores were taken to provide a reference DDAC concentration. The cores at each location on a plate were collected together and weighed, in order to provide the minimum amount of agar needed to allow DDAC to be detected. Duplicate cores were removed from each position within each plate containing DDAC amended medium. In some cases duplicate plates were also sampled. Following weighing, the cores were placed in a tube to which was added either 1 or 2 ml of ethanol (pH = 5), acidified with formic acid. The amount of ethanol was adjusted for the mycelium mass to ensure good recovery of the DDAC. The solution containing the cores was sonicated in an

ultrasonic bath at 40 Hz for three hours. The solutions were centrifuged at 2,800 rpm at 9 °C for 25 minutes, after which the samples were filtered with 0.45 μm PTFE microfilter, and analyzed by HPLC. The HPLC spectrometer was fitted with an indirect photometric detector (Larson and Pfeiffer, 1983). The system consisted of a Spectra Physics 1200 series HPLC pump equipped with a 8 mm x 10 cm Partisil SCX column (Waters 85753) containing 10 μm particles and a Spectra Physics variable wavelength UV detector set at 262 nanometers. A solution containing 0.025 M of benzyltrimethylammonium chloride, 1% acetic acid and water:methanol mixture with a ratio of 5:1 was used as a mobile phase at a 2.2 ml/min flow rate. This method was also used for determining the amount of DDAC in mycelia.

DDAC concentrate was used to dissolve in ethanol (pH = 5) which was acidified with formic acid for DDAC standard. All DDAC peak was measured by peak height.

4.4.2 Liquid media

Fungal species

The strains of fungi used in this study were selected from those that had exhibited good tolerance in DDAC amended liquid culture, based upon their production of biomass. The fungi selected were: *V. bulbillosum, Acremonium sp. G. roseum* (TC-NP-16 and 321C), *Fusarium* (S-1-1), *and V. dahliae* (390A).

Preparation of media

The Vogel liquid media was prepared as described earlier (Section 4.3.2). The inoculation and incubation of the liquid cultures were described in section of 4.3.2.

Analysis of DDAC

Because there is no standard method available for recovering DDAC from the aqueous solution, it was necessary to develop one. Several different procedures were evaluated

Recovery of DDAC from liquid media

a. A known amount of DDAC (0.0098 g and 0.0092 g) was added to 50 ml of standard Vogel liquid media after which the solution was transferred to a separatory funnel. A 25 ml aliquot of methylene chloride was added to the separating funnel which was shaken for 5 minutes, after which the solution was allowed to stand for 20 minutes. The methylene chloride layer (lower) was collected. The procedure was repeated three times with 25 ml of methylene chloride, after which the four extracts were combined together, roto-evaporated to dryness, and redissolved in acidified ethanol (pH = 5) prior to analysis by HPLC.

b. Vogel liquid media (50 ml) containing a 0.00382g and 0.00592g of DDAC were roto-evaporated to dryness. Methylene chloride (25 ml) was added to the flask to dissolve DDAC. The flask was shaken and the methylene chloride transferred to another flask. This methylene chloride extraction was repeated three times and all four extracts were combined, after which they were roto-evaporated to dryness. The remaining solid was redissolved in acidified ethanol prior to analysis by HPLC. Since phosphate buffer was used during some experiment, the recovery of DDAC from pH 6.3 phosphate buffer was assessed using the same procedure. This experiment also produced some indication of the influence of inorganic salts on DDAC recovery.

c. The experiment described above (Section b) was repeated using acidified methylene chloride (250 ml of methylene chloride was acidified with 1 ml of formic acid). In addition,

0.01048g and 0.00984g of DDAC were incorporated in the Vogel liquid medium containing the pH 6.3 phosphate buffer.

d. Solutions of Vogel liquid media or pH 6.3 phosphate buffer alone containing a known amount of DDAC (0.0288g and 0.036g; 0.0294g and 0.0192g) were roto-evaporated to dryness. Acidified methylene chloride (250 ml of methylene chloride was acidified with 1 ml of formic acid) (25ml) was added to the flask which was then placed in an ultrasonic bath for 3 hours. The resulting solution was transferred to second flask. The remaining solid was then washed twice with 25 ml of acidified methylene chloride to recover any additional the DDAC. The acidified methylene chloride extracts were combined together and roto-evaporated to dryness. The resulting solid was redissolved in acidified ethanol and examined by HPLC.

e. Flasks containing standard Vogel liquid media were used to incubate *Verticillium sp.* After incubation for two weeks, a known amount of DDAC (0.0024g, 0.0023g and 0.0028g) was added to the resulting media which was then roto-evaporated to dryness. Acidified methylene chloride (250 ml of methylene chloride was acidified with 1 ml of formic acid) (25ml) was added to the solid and the flasks were placed in an ultrasonic bath for 3 hours. The resulting solution was transferred to another flask. The remaining solid was twice washed with 25 ml of acidified methylene chloride to recover any remaining DDAC. The three acidified methylene chloride extracts were combine together and roto-evaporated to dryness. The resulting solid was redissolved in acidified ethanol and examined by HPLC.

Based upon the biomass of *V. bulbillosum*, and the evaluation of the DDAC recovered from the different extraction regimes, the following procedure was adopted for the analysis of the residual DDAC content. The fungi were grown in liquid media (50 ml) for two

weeks, after which the culture was rotoevaporated to dryness. The dry sample was put in a desiccator overnight. Acidified methylene chloride (25ml) was added to the solid and the flasks were sonicated in an ultrasonic bath at 40 Hz for three hours. The resulting solution was transferred to a second flask. The remaining solid was washed three times to recover any remaining DDAC on the mycelium. All of the acidified methylene chloride extracts were combined and roto-evaporated to dryness. The resulting solid was redissolved in 5 ml of acidified ethanol (pH = 5), and filtered with a PTFE 0.45 μm microfilter and analyzed by HPLC.

4.5. Extraction and identification of metabolites in liquid media, following fungal growth in liquid media

4.5.1 Methylene chloride

Methylene chloride was chosen as the solvent for extraction. Since DDAC degrades when subjected to a GC-MS analysis, it was removed prior to analysis by passing the solution down a cationic ion exchange resin. *V. bulbillosum., G. roseum (TC-NP-16)* and *Acremonium sp.,* were grown in Vogel liquid media containing 0 ppm and 250 ppm DDAC (prepared as described in Section 4.4.2). In further experiments, *V. bulbillosum, G. roseum (TC-NP-16)* and *Acremonium* were grown for two weeks in 50 ml of liquid Vogel media containing 0 ppm and 250 ppm DDAC at 25 °C, on a rotary shake with a shaking speed of 200 rpm. The cultures and Vogel liquid media containing 250 ppm DDAC without growth of fungi, were extracted three times with 25 ml of methylene chloride. The extracts were combined together, roto-evaporated to dryness and re-dissolved in 5 ml of 100% anhydrous ethyl alcohol. The extract solution was added to a cationic ion-exchange resin (Bio-Rad AG 50 W x 4) which was then washed with 50 ml of 100% anhydrous ethyl alcohol. The wash solution was collected, evaporated to dryness using a roto-evaporator at 40 °C and re-dissolved in 5 ml of methylene chloride, prior to examination by (GC-MS).

4.5.2 Complex formation

Based upon the early data from the GC-MS results, the presence of the tertiary amine decyldimethylamine was not confirmed. Subsequently, it was found that the tertiary amine could not be washed off the cationic ion exchange resin under the experimental conditions used. Therefore the procedure had to be modified. The modified procedure was designed to

remove the DDAC, but not the tertiary amine. It was based upon the known ability of cationic surfactants to form compounds with anionic surfactants. The resulting compound is insoluble in both water and methylene chloride.

Verticillium sp. and *Acremonium* sp. were grown at 25 °C for two weeks in 50 ml of Vogel liquid media containing 0 ppm and 250 ppm DDAC with a shaking speed of 200 rpm. The cultures and liquid media containing 250 ppm DDAC without fungal growth were extracted three times with 25 ml of methylene chloride. The extracts were combined, roto-evaporated to dryness and redissolved in 10 ml of distilled water. Aliquots of 3.46×10^{-3} M anionic surfactant (sodium dodecyl sulphate) were added to the solution until it became turbid. The turbid solution was extracted three times with 5 ml of methylene chloride. The clear extract (lower layer) were combined together, roto-evaporated to dryness, and redissolved in 10 ml of distilled water. The procedure was repeated until addition of sodium dodecyl sulphate did not cause any turbidity at that point, the last solution was rotoevaporated to dryness and redissolved in 5 ml of methylene chloride for analysis.

4.5.3 Identification of metabolites by GC-MS

The methylene chloride solutions prepared as above were examined by GC-MS to identify the metabolites. The GC-MS was equipped with a VG Trio 1000 mass selective detector. The samples were analyzed on a 25 m long HP-5 capillary column (0.2mm ID, 0.32 μm film). The injector system was maintained at 250 °C, and the oven temperature was held at 50 °C for 2 minutes and then programmed to increase to 300 °C at a rate of 13 °C/min. The separated components were identified where possible by comparing their mass spectrum with those stored in the library. Samples of authenticated compounds of interest such as

decyldimethylamine were analyzed when the relevant compound spectrum was unavailable in the library.

4.6 Attempts to fractionate the proteins that degrade DDAC

4.6.1 Comparison of extracellular and intracellular protein in selected fungi exposed to DDAC and without DDAC, using electrophoresis

In the initial experiment, *Verticillium sp.* was grown at 25 °C in Vogel liquid media containing with 500 ppm DDAC and 0 ppm DDAC. The flasks were shaken at 200 rpm during incubation. After 15 days, the cultures were centrifuged at 3,900 rpm at 9 °C for 40 minutes to separate the supernatant from the mycelium. The mycelium was washed with 10 ml of 20 mM tris-HCl buffer at pH 8.0. The supernatant, which contained the extracellular protein, was concentrated by ultrafiltration (10,000 kDa) until the protein concentration was approximately 1 mg/ml. The hyphal cell wall was disrupted by grinding with a cell homogenizer to which was added sand, in order to recover the intracellular protein. The intracellular protein then was concentrated by ultrafiltration (10,000 kDa) until the protein concentration was approximately 1 mg/ml. Protein concentrations were measured by the Bradford microassay (Bio-rad), using bovine serum albumin (BSA) as the standard (Bradford, 1976).

Gel electrophoresis was used to analyze the extracellular and intracellular proteins. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing electrophoresis (IEF pH 3-9). SDS-PAGE and IEF gel electrophoresis were performed on the phastsystem (Pharmacia, Uppsala, Sweden). Separation of protein by SDS-PAGE was performed at a constant current of 10.0 mA for a total 70 V.h. Separation by IEF was performed at a constant current of 2.0 mA for a total of 431 V.h. The pI and molecular weight (MW) markers were provided in the Pharmacia kits for IEF and SDS-PAGE respectively.

Aliquots containing 0.25 μg/μl and 0.5 μg/μl of extracellular and intracellular protein were prepared, after which 4 μl of each sample was loaded onto the SDS-PAGE gel (gradient 8-25%). A silver staining method was used to show the location of the protein bands.

In a final experiment, *V. bulbillosum and Acremonium sp.* were grown in liquid cultures containing 250 ppm and 0 ppm DDAC. After two weeks' incubation growth, the supernatant was collected and concentrated by ultrafiltration until the protein concentration was about 2 mg/ml. Duplicate samples were prepared. The protein was diluted with 20 mM tris-HCl buffer, pH 8.0 to 0.5 μg/μl. Aliquots of 4 μl of extracellular protein solution were loaded onto SDS-PAGE (homogenous 12.5%) and IEF (pH 3-9) gels. The SDS-PAGE and IEF gel were run in duplicate and were silver stained for proteins according to the manufacturer's recommendations (Pharmacia) to show the locations of the protein bands.

4.6.2 Degradation of DDAC by supernatants from the cultures of *V. bulbilosum* containing 0 ppm and 250 ppm DDAC

The same source of supernatants and mycelia of *V. bulbillosum* as above without concentration were used in this experiment. The protein concentration was also measured.

A 0.02355 g sample of 80 % DDAC was dissolved in 100 ml of pH 6.3 phosphate buffer. Exactly 15 ml of this DDAC solution was added to 30 ml of supernatant in which the protein concentration was 0.075 mg/ml.

A 0.01286 g sample of 80 % DDAC was dissolved in 100 ml of pH 6.3 phosphate buffer. A 1 ml aliquot of this DDAC solution, was combined with 5 ml of pH 6.3 buffer and 0.5 ml of intracellular protein in which the protein concentration was 0.0017 mg/ml.

All of the flasks and tubes were incubated at 27 °C for 7 days with shaking at 200 rpm. After incubation, the samples were roto-evaporated to dryness at 45 °C. A 25 ml or 5 ml of acidified methylene chloride was added to each sample which was sonicated in an ultrasonic bath for three hours. The resulting solids were extracted three times with 25 ml or 5 ml of acidified methylene chloride. The methylene chloride extracts were combined and transferred to another flask for removal of the solvent. Acidified ethanol was added to the extracts and the solution analyzed by HPLC to determine the DDAC content.

4.7 The distribution of DDAC in starch, silica gel, and solution

A known weight (41.2 mg) of DDAC was added into the Vogel liquid media (50 ml) without starch but with 0.8 g of silica gel. Replicate samples were used. Aliquots of 38 mg or 35 mg of DDAC were added into the flasks containing cultures of *V. bulbillosum* grown in Vogel liquid media for two weeks. All of the samples were roto-evaporated to dryness. A 25 ml solution of acidified methylene chloride (250 ml of methylene chloride, acidified with 1 ml of formic acid) was added to the solid and the flasks were placed in an ultrasonic bath for 3 hours. The resulting solution was transferred to another flask. The remaining solid was twice washed with 25 ml of acidified methylene chloride to recover any remaining DDAC. The three acidified methylene chloride extracts were combine together and roto-evaporated to dryness. The resulting solid was redissolved in acidified ethanol and examined by HPLC.

Pre-determined amounts of DDAC (0g, 0.0163g, 0.0255g, and 0.0248g) were weighed and added to a 50 ml centrifuge tube which contained either i) 25 ml of Vogel liquid media (without starch) and 0.4 g of silica gel; ii) 25 ml of Vogel liquid media and 0.4 g of silica gel; or iii) 30 ml of standard Vogel liquid media. The samples containing starch were autoclaved at 103 kPa and 121 °C for 15 minutes. All the samples were prepared, shaken and allowed to stand overnight.

The next day, the samples were centrifuged at 3,900 rpm for 40 minutes. The clear solution and the solid were both collected. The DDAC was recovered from the clear solution by the procedure described above. The DDAC was extracted from the silica gel, and any undissolved starch (where this was present) by adding 8 ml of acidified ethanol and sonicating in an ultrasonic bath at 40 Hz for 2 hours.

In the final experiment 0.8 g of silica gel was added into 50 ml of Vogel liquid media containing 0 ppm and 250 ppm DDAC. Fungi *V. bulbillosum* and *Acremonium sp.* were used. The preparation of Vogel liquid media, inoculation, and incubation were described earlier (section 4.3.2). Duplicate samples were prepared. After two weeks, the cultures were centrifuged at 3,900 rpm for 40 minutes. The DDAC in the clear solution was recovered by the procedure described in the Section 3.4.2 (subsection 'e') using methylene chloride. The DDAC in the solid was extracted by adding 5 ml of acidified ethanol and sonicating in an ultrasonic bath at 40 Hz for 2 hours. All the samples were filtered with a PTFE microfilter (0.45 μm) and analyzed by HPLC.

4.8 Fungal growth in liquid media containing decyldimethylamine (Barlene 10S)

4.8.1 Fungal species

The following fungi were used in the first experiment : *V. bubillosum, Coniophora puteana,* and *Trametes versicolor.* Those in the second experiment were: *V. bubillosum, Acremonium sp., Fusarium sp.* (S-1-1) and *G. roseum* (TC-NP-16).

4.8.2 Growth conditions

Vogel liquid media was prepared and autoclaved as described above (Section 4.3.2). After the media were cooled to room temperature, decyldimethylamine was added to 50 ml of media in 250 ml Erlenmeyer flasks. The concentrations of the tertiary amine were: 1500 ppm and 800 ppm. To determine the ability of the fungus to use the tertiary amine as a sole N-source or sole C-source, the nitrogen or carbon sources were omitted from the medium during preparation of those specific samples. The various combinations of liquid media were inoculated with *Verticillium sp.* and incubated with shaking at low speed (140 rpm), at room temperature, for 12 days.

Vogel liquid media containing 1000 ppm decyldimethylamine was prepared and inoculated with *V. bulbillosum, C. puteana, T. versicolor* and incubated at room temperature for two weeks. Vogel liquid media containing 100 ppm decyldimethylamine were prepared and inoculated with *V. bubillosum, Acremonium sp. Fusarium sp. G. roseum* (TC-NP-16) and incubated at room temperature for two weeks with shaking at 140 rpm.

4.8.3 Fungal biomass

The measurement of fungal biomass was as described in section 4.3.2.

5. RESULTS AND DISCUSSION

5.1 Use of colorometric approach to monitor changes in DDAC concentration during fungal growth

It was anticipated that a large number of fungi may have to be screened for their tolerance to DDAC. In order to screen these fungi quickly and identify those that can degrade QACs, it would be helpful to develop a rapid technique that did not require the chemical analysis of QAC each time. Cationic surfactants such as DDAC, readily form stable colored complexes with anionic dyes such as bromophenol blue and methyl orange (Ballard *et. al.*, 1954; Zografi *et. al.*, 1964; Few and Ottewill, 1956; Waters, 1976). A screening trial was designed to investigate whether the colored complex formed was changed when the QAC was degraded. If the results were positive, this would confirm that the technique could be more generally used to identify those fungi which degrade QACs.

5.1.1 Bromophenol blue

At an acidic pH of 1.80 when the molar concentrations of DDAC were lower than the molar concentrations of bromophenol blue, the spectra of bromophenol-DDAC were the same as that of standard bromophenol blue. Increasing the molar concentration of DDAC so that it was equal to or higher than, that of bromophenol blue, caused an absorption peak observed around 610 nm due to the bromophenol blue-DDAC complex formed (Figure 5.1.1.1). However, no linear relationship between the intensity of the absorption and the concentrations of DDAC could be determined. At higher pH values of 3.50 and 6.80, there was no difference between the spectra of bromophenol blue-DDAC complex and standard

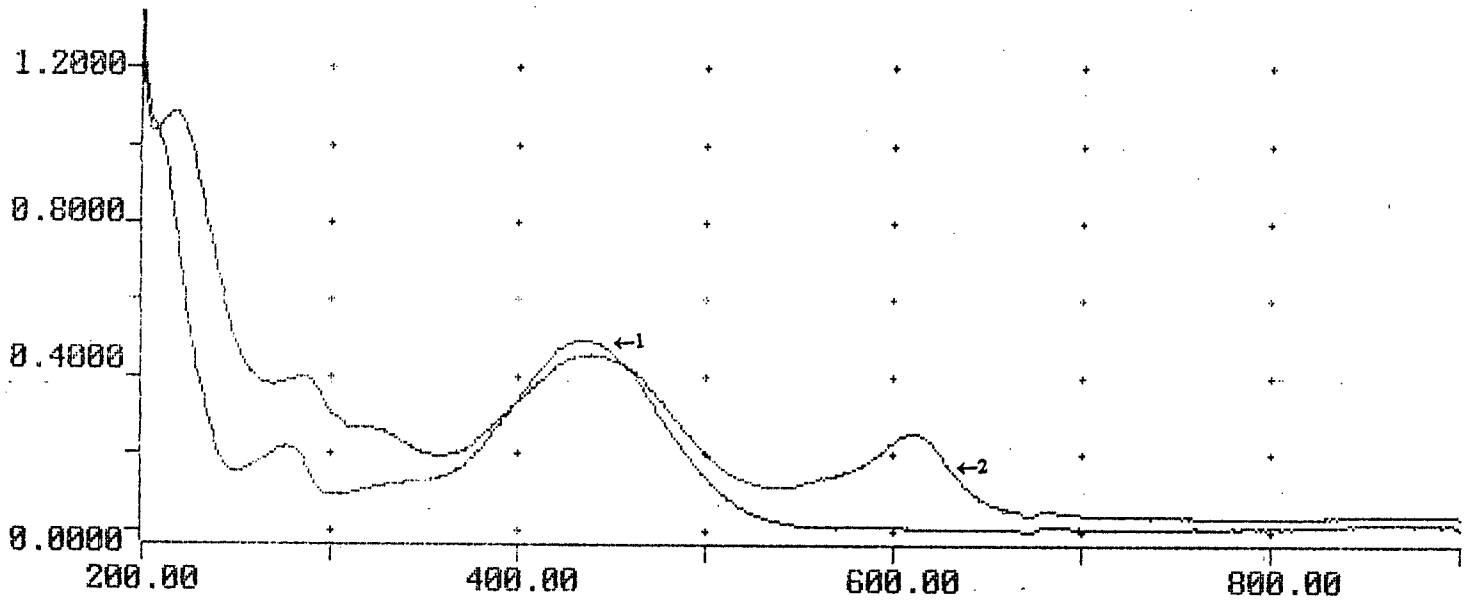


Fig. 5.1.1.1 Absorption spectra of 1) standard bromophenol blue (2.04×10^{-5} M) and 2) complex formed by bromophenol blue (2.04×10^{-5} M) and didecyldimethylammonium chloride (1.02×10^{-4} M) at pH 1.80

bromophenol blue, no matter whether the molar concentration of DDAC was equal to, higher than, or lower than, that of standard bromophenol blue. Increasing the solution pH to 12.00, failed to produce any difference between the UV-Visible spectra of the DDAC and the complex, when the molar concentration of DDAC was lower than that of bromophenol blue. If the molar concentration of DDAC was equal to, or higher than, that of bromophenol blue, the spectra were different (Figure 5.1.1.2), but there was no specific peak which could be assigned to the bromophenol blue-DDAC complex.

5.1.2 Methyl orange indicator

At the acidic pH of 1.1, there was no difference between the spectra of the methyl orange-DDAC complex and the spectra of standard methyl orange, when the DDAC molar concentrations were equal to, lower than, or higher than, that of methyl orange. Increasing the solution pH to 11.5, produced spectra of the methyl orange-DDAC complex and standard methyl orange that showed no difference, when molar concentration of DDAC was lower than that of standard methyl orange. However, when the DDAC molar concentration was higher than, or equal to, that of methyl orange, the spectrum was changed. However, it was impossible to establish a relationship between the absorption intensity of the peaks in the spectra of the complex and the DDAC concentration. The DDAC concentrations were lower than, or similar to the critical micelle concentration (cmc). Since fungal growth in liquid media containing DDAC will produce more complex solutions, the use of UV-Visible absorption spectroscopy of complexed DDAC to monitor its degradation does not appear fruitful. Potential difficulties may be identified as a) the range of DDAC concentration

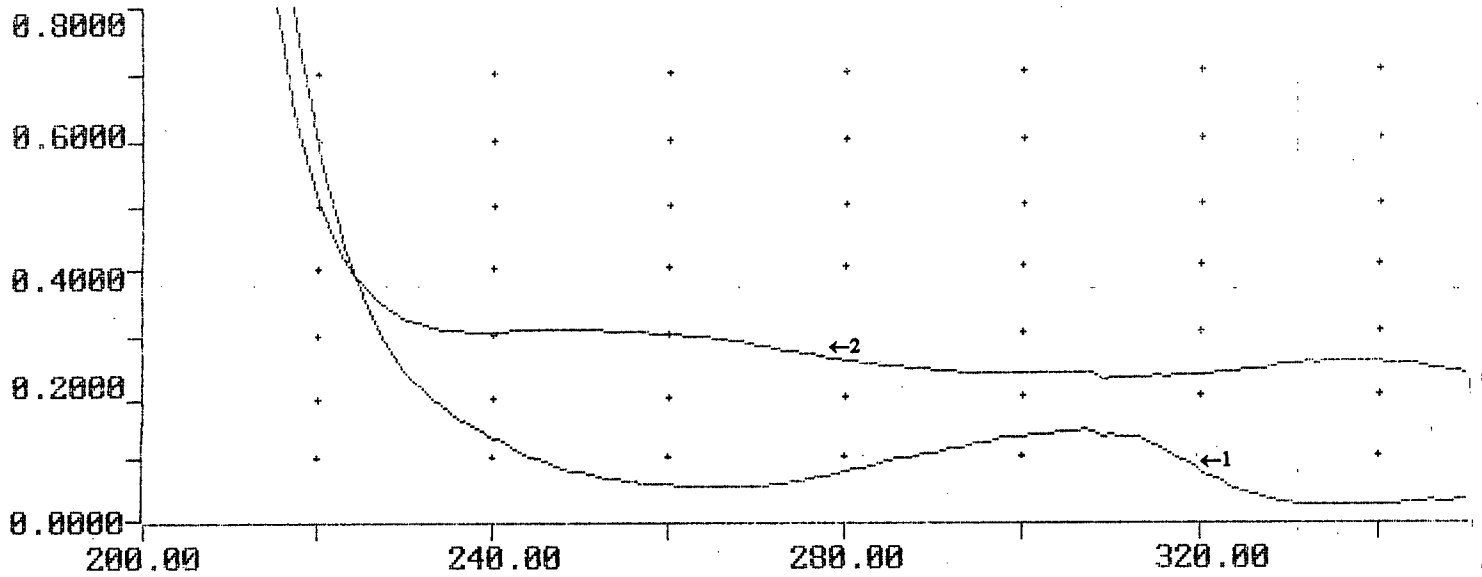


Fig. 5.1.1.2 Absorption spectra of 1)standard bromophenol blue (2.04×10^{-5} M) and 2) complex formed by bromophenol blue (2.04×10^{-5} M) and didecyldimethylammonium chloride (1.64×10^{-4} M) at pH 12.0

which may be studied by this method (i.e. below the cmc point); b) possible interference of fungal metabolites reacting with the dye; and c) lack of relationship between concentration and peak intensity.

5.2 Isolation and identification of tolerant fungal strains from field area

The survival and colonization of organisms in wood is dependent on (a) the moisture content; (b) aeration; (c) temperature; and (d) interactions between micro-organisms. All these factors and the constantly changing nutrient status of the wood will control the microbial successions in wood (Eaton and Hale, 1993). The general succession of fungal colonization during the decay of wood in soil contact is first mould and staining fungi during stage 1, soft rot and brown rot fungi during stage 2, and white rot fungi during stage 3.

Table 5.2.1 shows all fungi which were isolated in July, 1995 from pine sapwood stakelets installed at the UBC test site. A second set of stakelets were removed in October, 1995 and Table 5.2.2 describes all fungi which were isolated from the field area at that time. Table 5.2.3 shows all fungi which were isolated in October, 1995 from stakelets placed in fungal cellar. Five strains of fungi chosen for further experiments based on their growth rate on 2% solid malt agar containing 750 ppm DDAC, were identified as: *Trichoderma virens* (Miller, Giddens and Foster) V. Arx (F-1-1). *Zygorrhynchus moelleri* Vuill (S-1-6), and *Fusarium avenaceum* (Fr.) Sacc (S-1-1, S-2-11, S-2-2).

During the first four to six months, the progressive deterioration of wood will be in stage 1. Therefore it is anticipated that primarily mould and staining fungi will be isolated. This was confirmed by the low frequency of isolation of decay fungi.

Table 5.2.1 Fungi isolated in July from treated and untreated staklets placed in field test

Treated wood	Selective media	Reference No.	Pure culture	Tolerance		Code No.
				250 ppm	750 ppm	
0.3% DDAC*	SCNR*	1	+	+	-	
		2	+	+	-	
		3a	+	+	-	
		3b	+	+	-	
		4	-			
	BTMA*	5	-			
		6	-			
		7	-			
		8	-			
	Preservative media (DDAC)	9	+	+	-	
		10	-			
		11	-			
		12	-			
0.6% DDAC	SCNR	5	+	+	-	
		6	-			
		7	+	+	-	
		8a	+	-		
		8b	+	+	-	
	BTMA	9	-			
		10	-			
		11	+	-		
		12	-			
	Preservative media (DDAC)	1	-			
		2	-			
		3	+	+	+	S-1-1
		4	-			
0.3% DDA*	SCNR	5	+	+	-	
		6	+	+	-	
		7	-	-		
		8	+	-		
	BTMA	9	-			
		10	-			
		11	+	+	-	
		12	-			
	Preservative media (DDAC)	1	-			
		2	-			
		3a	+	-		
		3b	+	+	-	
		4	-			

*DDAC, DDA, BTMA and SCNR see LIST of ABBREVIATIONS on page xv.

Table 5.2.1 (contd) Fungi isolated in July from treated and untreated staklets placed in field test

0.6% DDA	SCNR	1	+	+	-	
		2	-			
		3	+	+	-	
		4	+	+	-	
	BTMA	9	-			
		10	+	+	+	S-1-6
		11	-			
		12	-			
	Preservative media (DDAC)	5	+	+	-	
		6	-			
		7	+	-		
		8	+	-		
Control	SCNR	1	-			
		2	-			
		3	-			
		4	-			
	BTMA	9	+	+	-	
		10	-			
		11	-			
		12	-			
	Preservative media (DDAC)	5	-			
		6	-			
		7	-			
		8	-			

*DDAC, DDA, BTMA and SCNR see LIST of ABBREVIATIONS on page xv.

Table 5.2.2 Fungi isolated in October from treated and untreated stakelets placed in field test site

Treated wood	Selective media	Reference No.	Pure culture	Tolerance		Code No.
				250 ppm	750 ppm	
0.3% DDAC	SCNR	1	+	-	-	
		2	+	+	+	S-2-1
		3a	+	+	-	
		3b	+	+	-	
		4	+	-		
	BTMA	13	-			
		14	-			
		15	-			
		16	-			
	Preservative media (DDAC)	9	-			
		10	-			
		11	+	+	-	
		12	+	+	-	
	Preservative media (DDA)	5	-	-		
		6	+	+	-	
		7a	+	-		
		7b	+	+	-	
		8	-	-		
0.6% DDAC	SCNR	1	+	-		
		2	+	+	+	S-2-11
		3	+	-		
		4	+	-		
	BTMA	5	-			
		6	-			
		7	-			
		8	-			
	Preservative media (DDAC)	9	+	+	-	
		10	-			
		11	-	+	-	
		12	+	-		
	Preservative media (DDA)	13	+	-		
		14	+	-		
		15	+	-		
		16	+	+	-	
0.3% DDA	SCNR	9	+	-		
		10	+	-		
		11	-	-		
		12	+	-		
	BTMA	13	-			
		14	-			
		15	+	+	-	
		16	-			
	Preservative media (DDAC)	5	+	-		
		6	+	-		
		7	+	-		
		8	+	-		
	Preservative media (DDA)	1	+	+	-	
		2	+	-		
		3	+	+	-	
		4	+	-		

*DDAC, DDA, BTMA and SCNR see LIST of ABBREVIATIONS on page xv.

Table 5.2.2 (Contd) Fungi isolated in October from treated and untreated stakelets placed in field test site

Treated wood	Selective media	Reference No.	Pure culture	Tolerance		Code No.
				250 ppm	750 ppm	
0.6% DDA	SCNR	1	+	-		
		2	+	-		
		3	+	-		
		4	+	-		
	BTMA	5	-			
		6	+	+	-	
		7	-			
		8	-			
	Preservative media (DDAC)	13	+	-		
		14	+	-		
		15	+	-		
		16	+	-		
	Preservative media (DDA)	9	+	-		
		10	+	-		
		11	+	-		
		12	+	-		
Control	SCNR	1	+	+	-	
		1	+	-		
		2	+	-		
		3	+	-		
		4	+	-		
	BTMA	5	-	-		
		6	-			
		7	-			
		8	-			
	Preservative media (DDAC)	9	+			
		10	-			
		11	+	-		
		12	+	-		

*DDAC, DDA, BTMA and SCNR see LIST of ABBREVIATIONS on page xv.

Table 5.2.3 Fungi isolated in October from treated and untreated stakelets placed in fungal cellar

Treated wood	Selective media	Reference No.	Pure culture	Tolerance		Code No.
				250 ppm	750 ppm	
0.3% DDAC*	SCNR*	1	+	+	-	
		2	+	+	-	
		3	+	+	+	F-1-1
		4a	+	+	-	
		4b	+	+	-	
	BTMA*	5	+	+	-	
		6	+	+	-	
		7	-			
		8	-			
	Preservative media (DDAC)	9	+	+	-	
		10	-			
		11	-			
		12	-			
0.6% DDAC	SCNR	5	+	+	-	
		6	+	-		
		7	+	+	-	
		8	+	+	-	
	BTMA	9	+	-		
		10	+	-		
		11	+	+	-	
		12	-			
	Preservative media (DDAC)	1	+	-		
		2	-			
		3	+	-	-	
		4	-			
0.3% DDA*	SCNR	5	-			
		6	+	-		
		6	+	+	-	
		7	+	+	-	
		8a	+	-		
		8b	+	-		
	BTMA	9	-			
		10	-			
		11	+	+	-	
		12	-			
	Preservative media (DDAC)	1	-			
		2	-			
		3	+	-		
		4	-			

*DDAC, DDA, BTMA and SCNR see LIST of ABBREVIATIONS on page xv.

Table 5.2.3 (contd) Fungi isolated in October from treated and untreated stakelets placed in fungal cellar

0.6% DDA	SCNR	1	+	-		
		2	+	+	-	
		3	+	+	-	
		4	+	+	-	
	BTMA	9	-			
		10	+	-		
		11	-			
		12	-			
	Preservative media (DDAC)	5	-			
		6	-			
		7	+	-		
		8	+	-		
Control	SCNR	1	+	+	-	
		2	+	-		
		3	+	-		
		4	+	-		
	BTMA	9	+	+	-	
		10	-			
		11	-			
		12	-			
	Preservative media (DDAC)	5	+	-		
		6	-			
		7	-			
		8	-			

*DDAC, DDA, BTMA and SCNR see LIST of ABBREVIATIONS on page xv.

5.3 Comparison of the tolerance of the isolates from field experiment with those from culture collections

It is also now well established that different fungal and bacterial species have significant variation in the chemical composition of the cell wall, which no doubt influences their ability to degrade QACs (Lawrence, 1970). The tolerance of these organisms to QACs has been postulated to be due to the presence of a lipid or lipid-protein material on the cell surface, which resists the disruptive surfactant forces of the disinfectant. Salton (1951) noted that bacteria treated with a cationic surfactant or other surface-active agents, could release nitrogen and phosphate compounds into the surrounding medium as a result of leakage of cellular constituents. The effect of DDAC on fungi is almost certainly similar.

When considering the action of QACs, two phenomena must be considered. The first involves chemical interaction of the QAC with the fungal hyphae either by i) adsorption of the QAC from the surrounding medium by the cell wall or ii) the penetration of the QAC through the membrane into the cell cytoplasm. The second phenomenon involves the reaction of the QAC with the cell protein. The QAC can disrupt bacterial or fungal activity in many ways, ranging from reducing the surface tension at the cell membrane causing leakage of the cell contents, to replacement of protons by ion exchange. Since the complex metabolic processes are finely balanced, even small changes can cause significant effects in the physiology of the organism, jeopardizing its viability. The tolerance and degrading ability of fungi to chemicals can be an inherent characteristic or may result from the development of resistant strain through adaptive behavior. The objective of these experiments was to examine whether the tolerance and DDAC degrading ability of the isolated fungi were common properties for

these fungi or whether they were particularly tolerant strains. This was done by comparing their tolerance to DDAC with that of strains obtained from culture collections.

5.3.1 Solid media

The growth rate of fungi on malt agar and malt agar containing DDAC was measured by two perpendicular directions through the center of the colony. This linear measurement is the simplest method of assessing fungal growth and is considered to be valuable as a rough estimate of growth. It is non-destructive and allows repeated observations of the same fungus. It fails to account for differences in aerial mycelium or mycelium submerged in the agar (Moore-Landecher, 1990), and does not distinguish between variations in hyphal thickness. Thus it may not relate well to mycelium spread over the medium and the total biomass production. Another potential problem is the production of non-uniform colonies. However, in the current strategy, the measurement of the diameter of the mycelial growth allowed a rapid comparison of the relative tolerance of strains of the same fungus.

All strains of fungi including isolates from treated and untreated stakelets exposed in the test site, culture collections and those stored at UBC following isolation by A. Doyle grew well on malt agar without DDAC (Figures 5.3.1.1 to 5.3.1.3). Figures 5.3.1.4 to 5.3.1.12 show the relationship between the growth rate of selected fungi on malt agar containing DDAC and incubation time. These results confirmed that, except for *V. dahliae* (163A) on malt agar with 1000 ppm of DDAC, this chemical did not inhibit the growth of the moulds over the concentration range studied. Increasing the DDAC concentration caused the fungal growth rate to decrease.

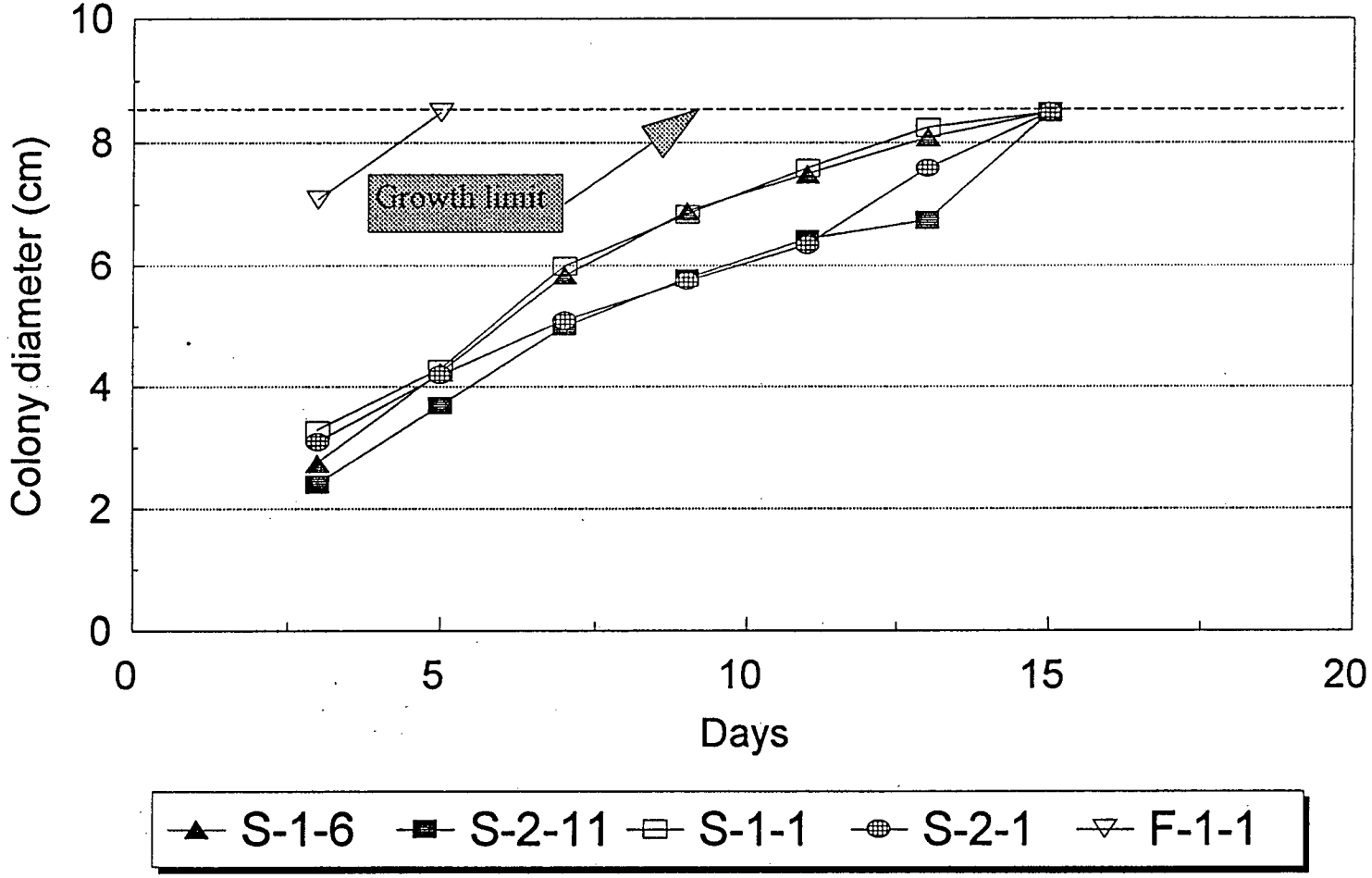


Fig. 5.3.1.1 Fungal growth on malt agar
(S-1-6 is *Z. molleri;* S-2-11, S-1-1 and S-2-1 are *F. avenaceum;* F-1-1 is *T. virens*) (Standard deviations ≤ 0.6 cm)

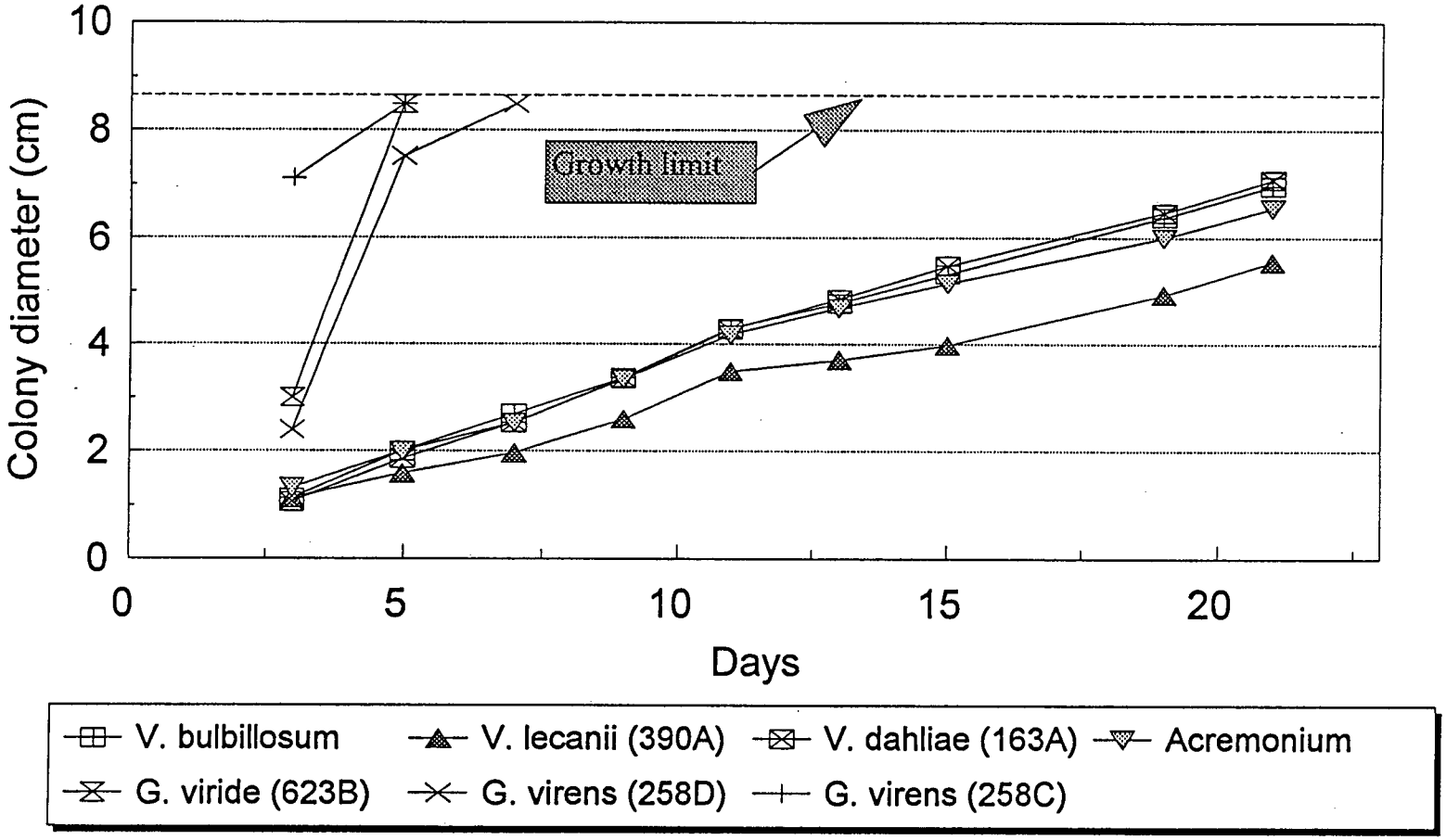


Fig. 5.3.1.2 Fungal growth on malt agar
(Standard deviations ≤ 0.6 cm)

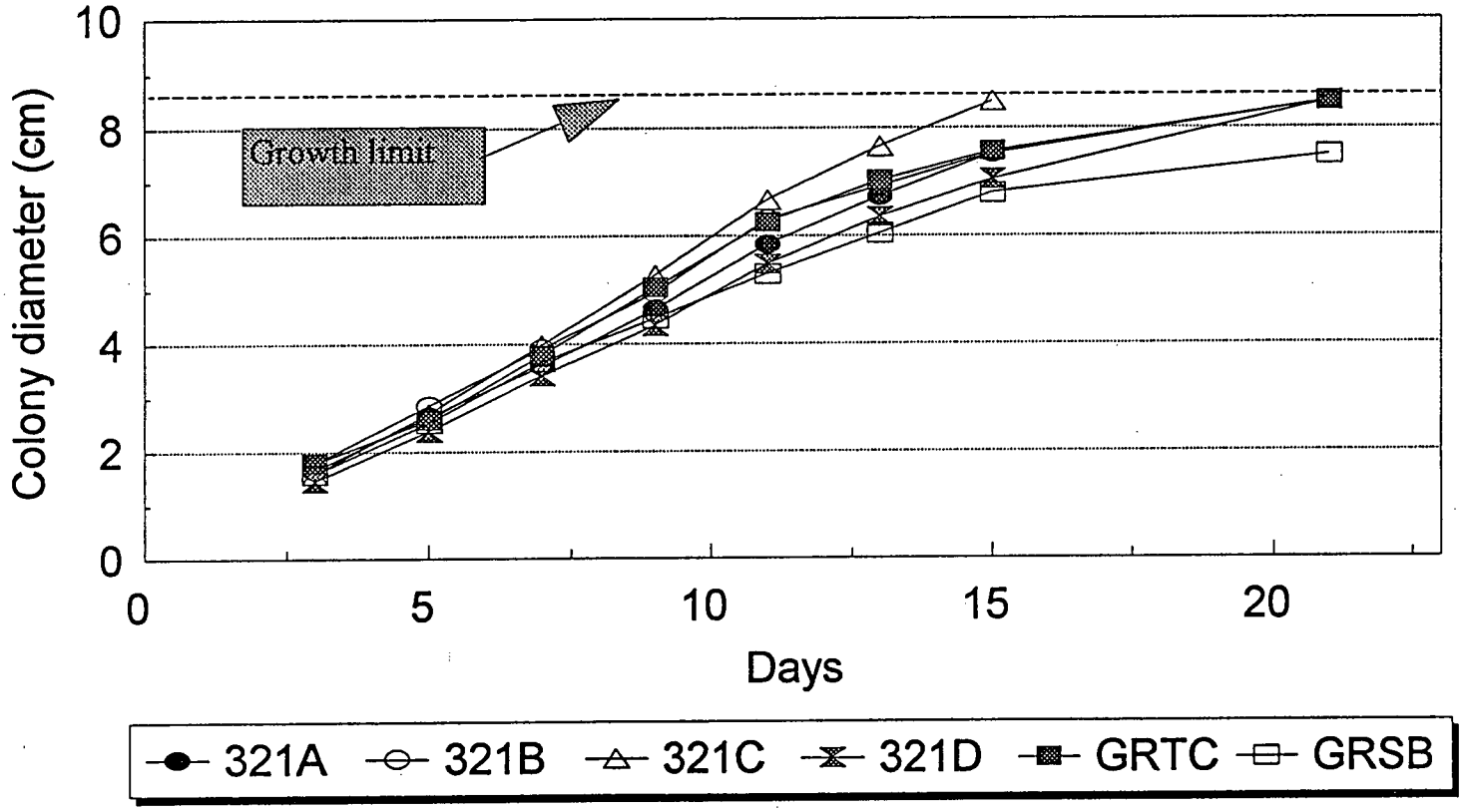


Fig. 5.3.1.3 Growth of *G. roseum* on malt agar
(Standard deviations ≤ 0.6 cm)

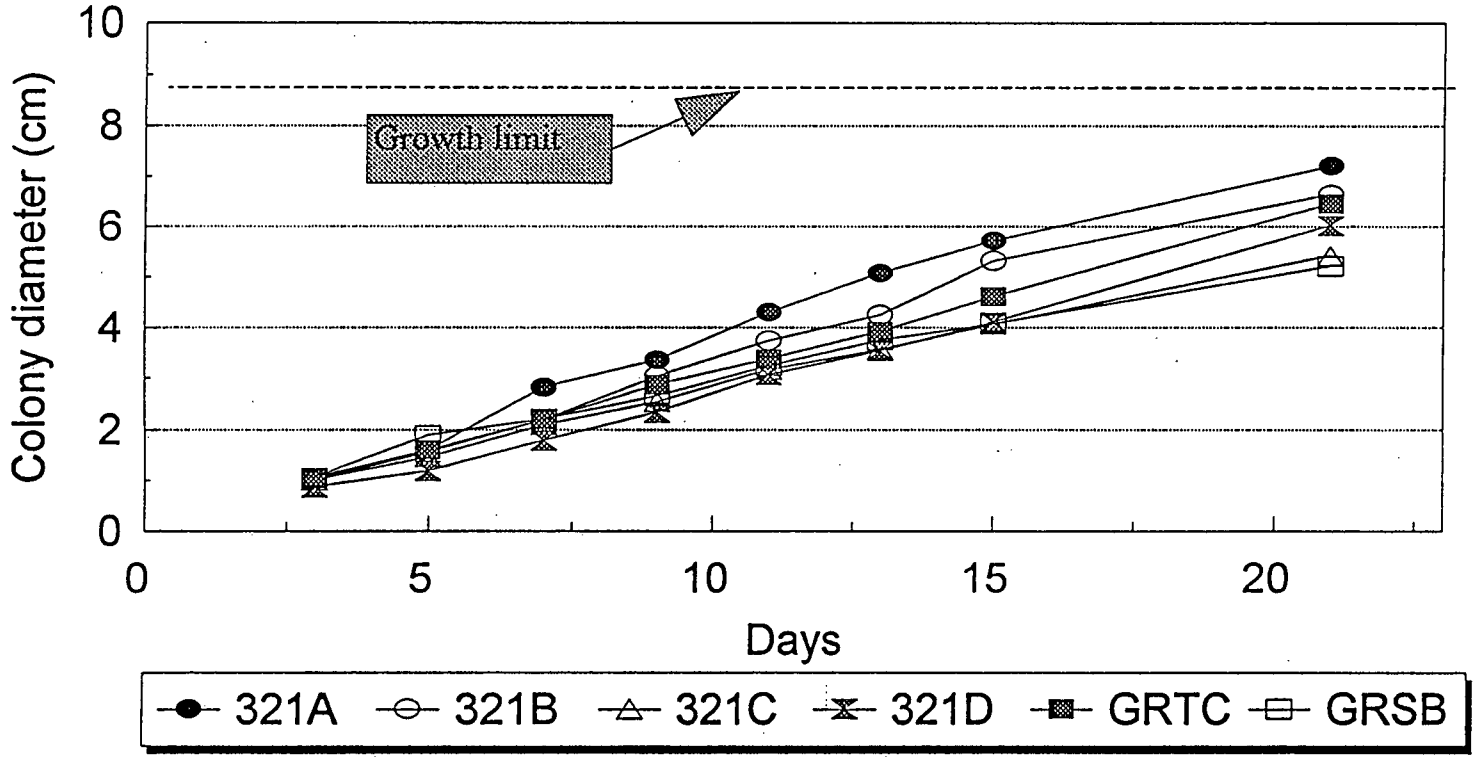


Fig. 5.3.1.4 Growth of *G. roseum* on malt agar containing 250 ppm DDAC
(Standard deviations ≤ 0.6 cm)

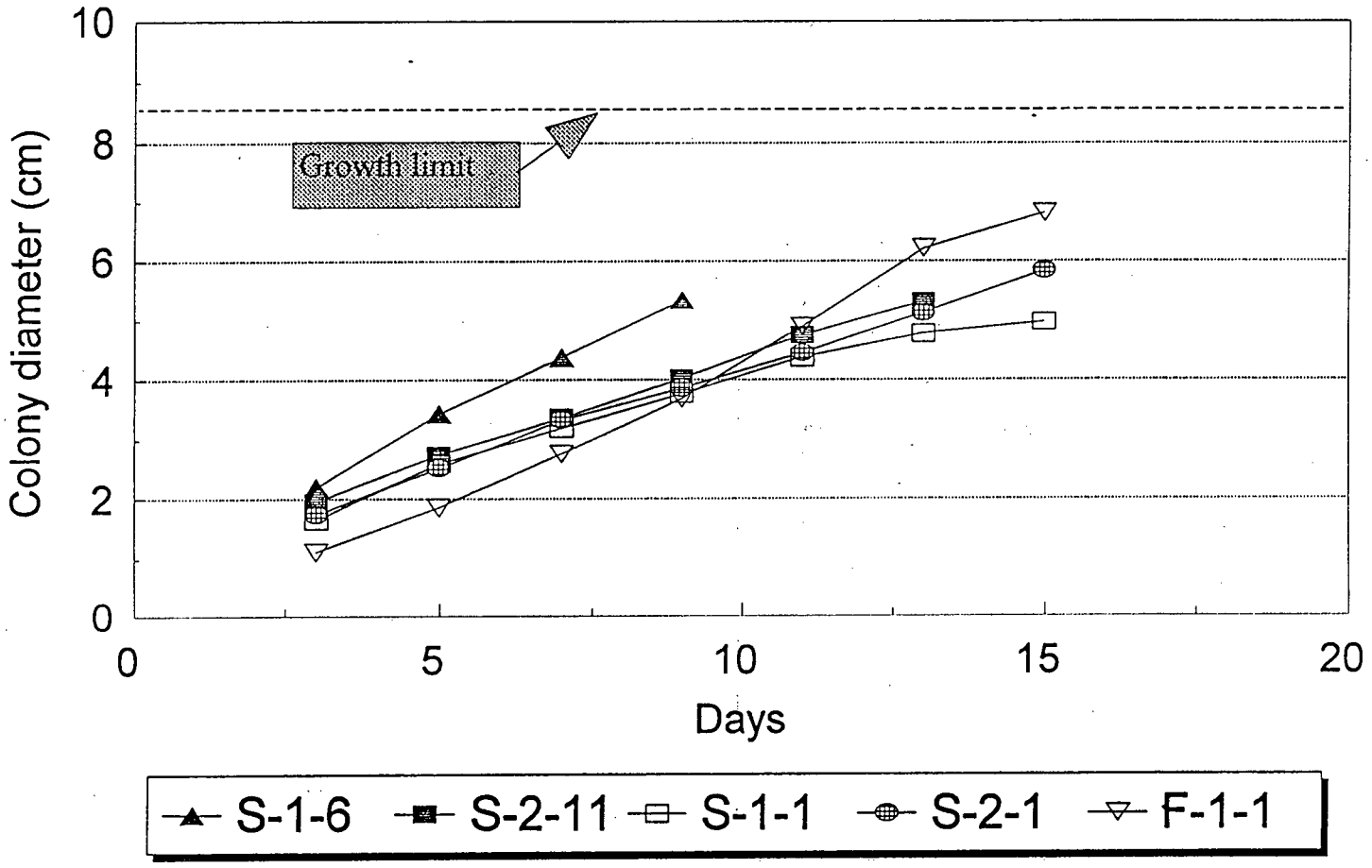


Fig. 5.3.1.5 Fungal growth on malt agar containing 500 ppm DDAC (S-1-6 is *Z. molleri;* S-2-11, S-1-1 and S-2-1 are *F. avenaceum;* F-1-1 is *T. virens*) (Standard deviations ≤ 0.6 cm)

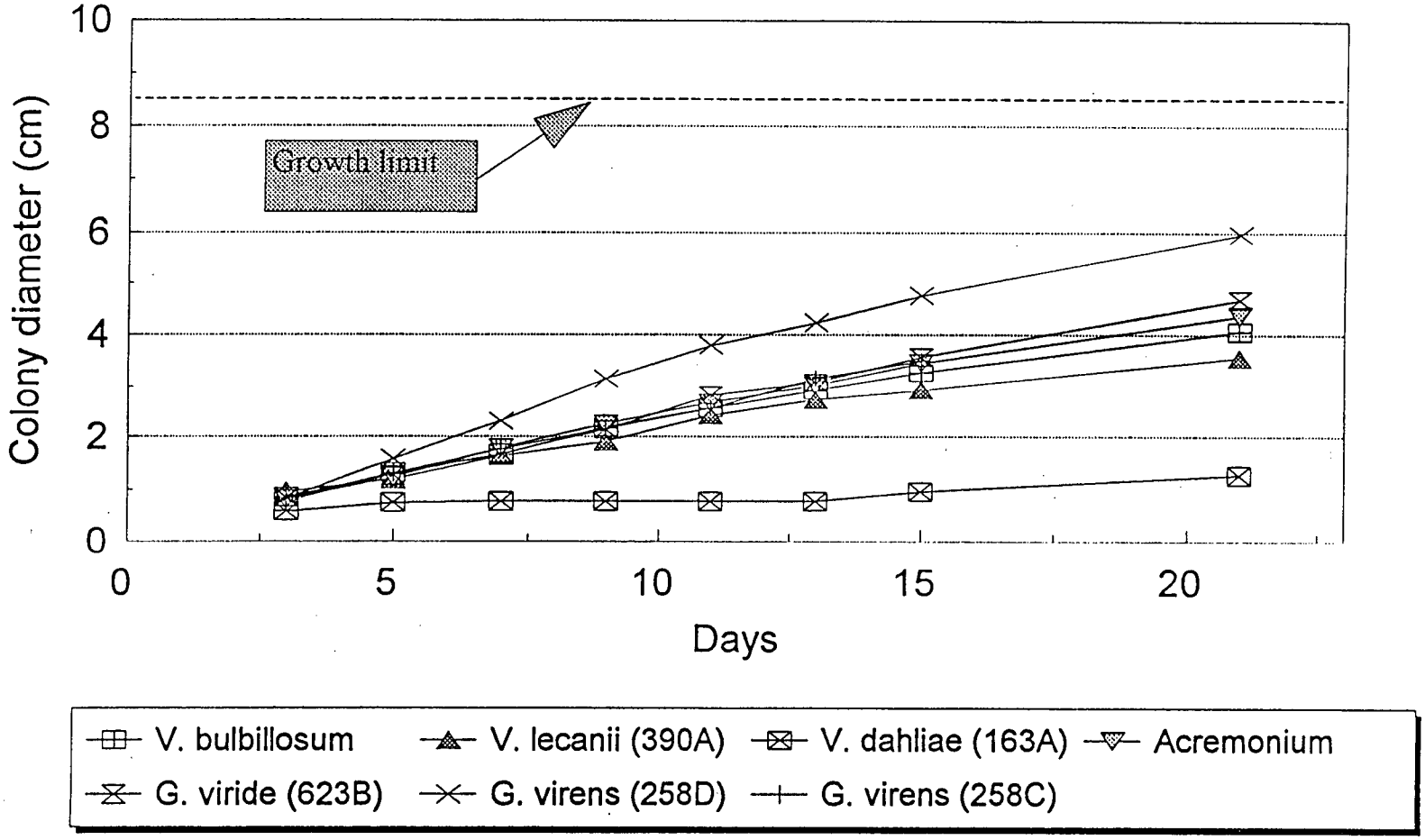


Fig. 5.3.1.6 Fungal growth on malt agar containing 500 ppm DDAC
(Standard deviations ≤ 0.6 cm)

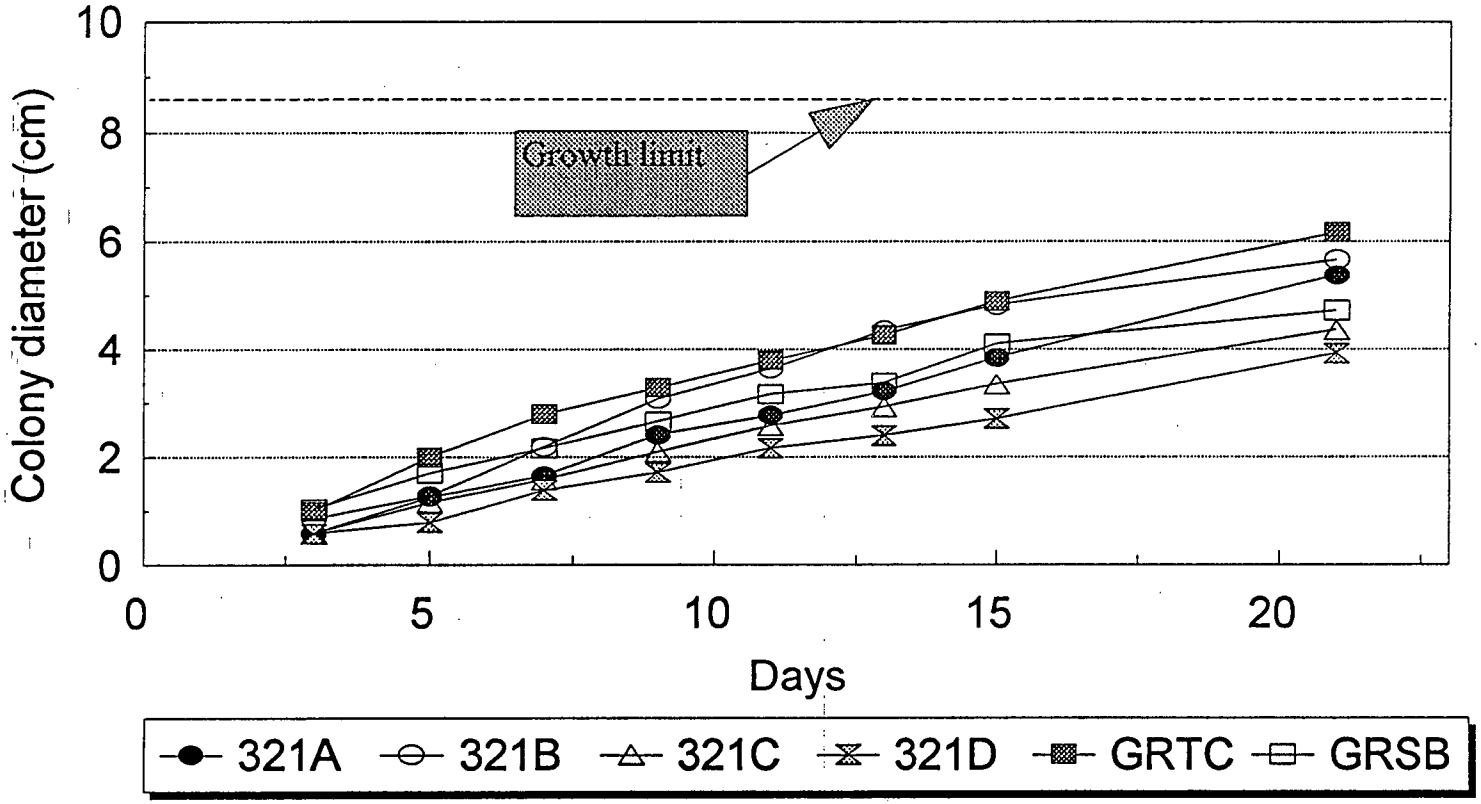


Fig. 5.3.1.7 Growth of *G. roseum* on malt agar containing 500 ppm DDAC
(Standard deviations ≤ 0.6 cm)

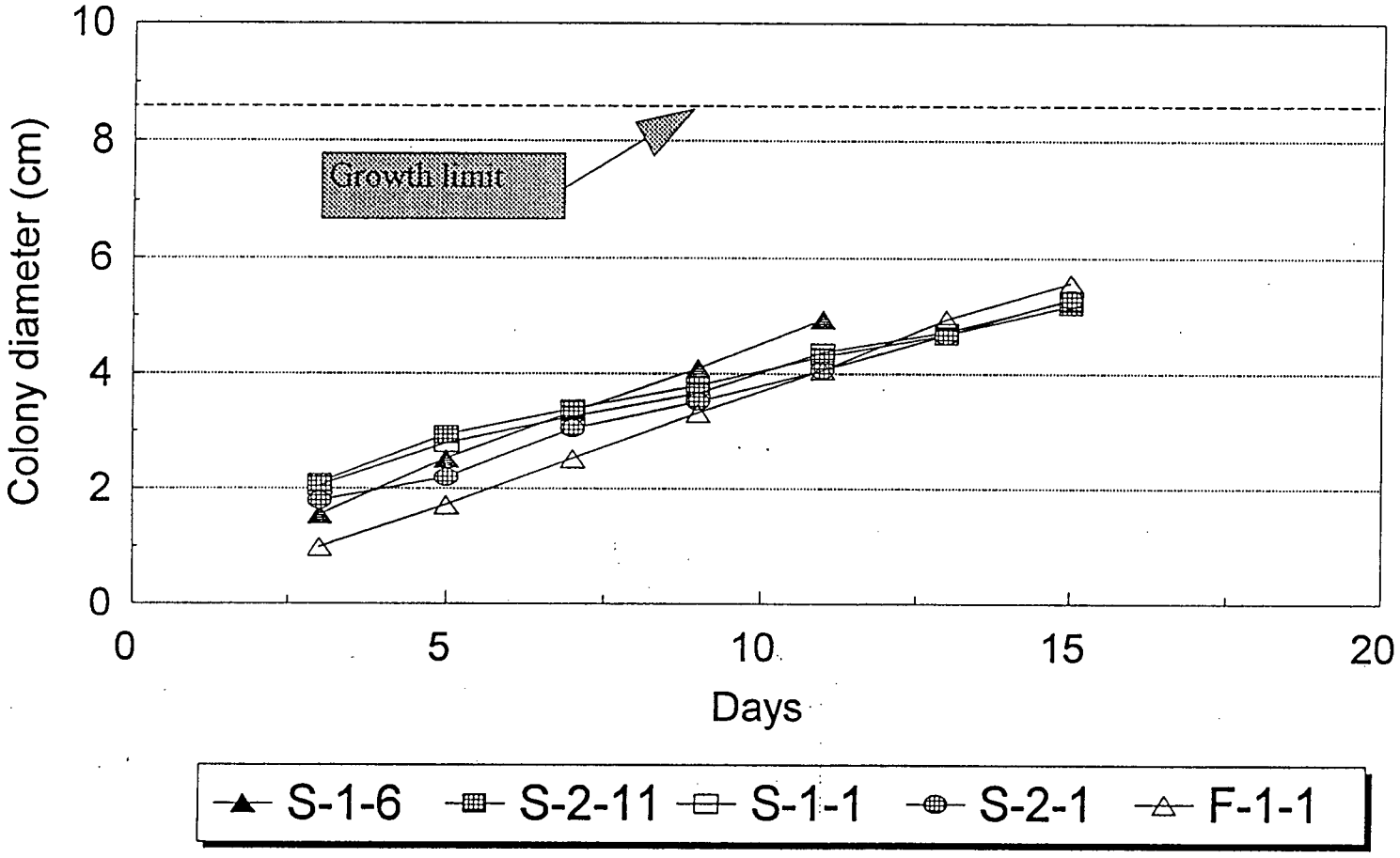


Fig. 5.3.1.8 Fungal growth on malt agar containing 750 ppm DDAC (S-1-6 is *Z. molleri;* S-2-11, S-1-1 and S-2-1 are *F. avenaceum;* F-1-1 is *T. virens*) (Standard deviations ≤ 0.6 cm)

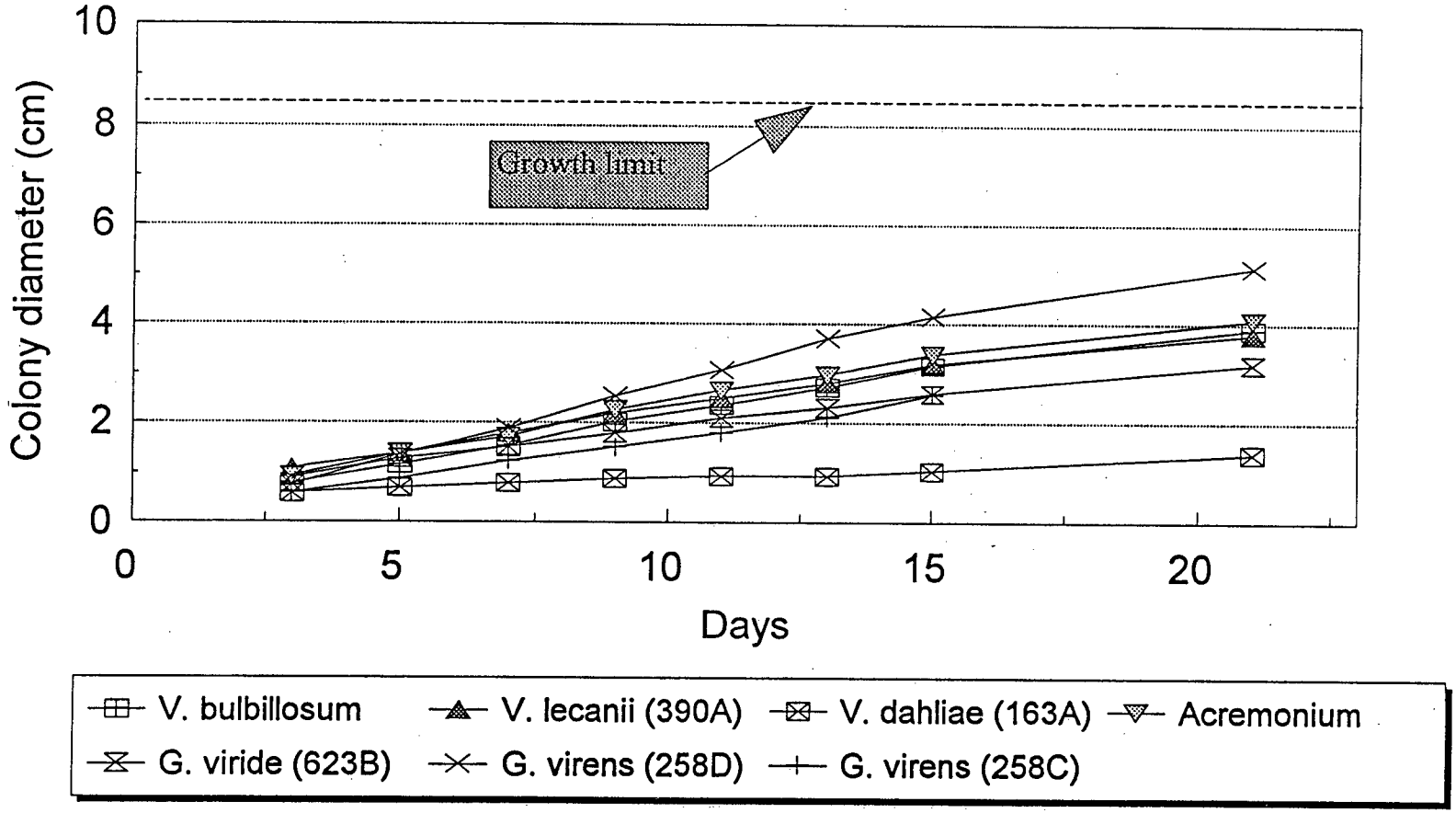


Fig. 5.3.1.9 Fungal growth on malt agar containing 750 ppm DDAC
(Standard deviations ≤ 0.6 cm)

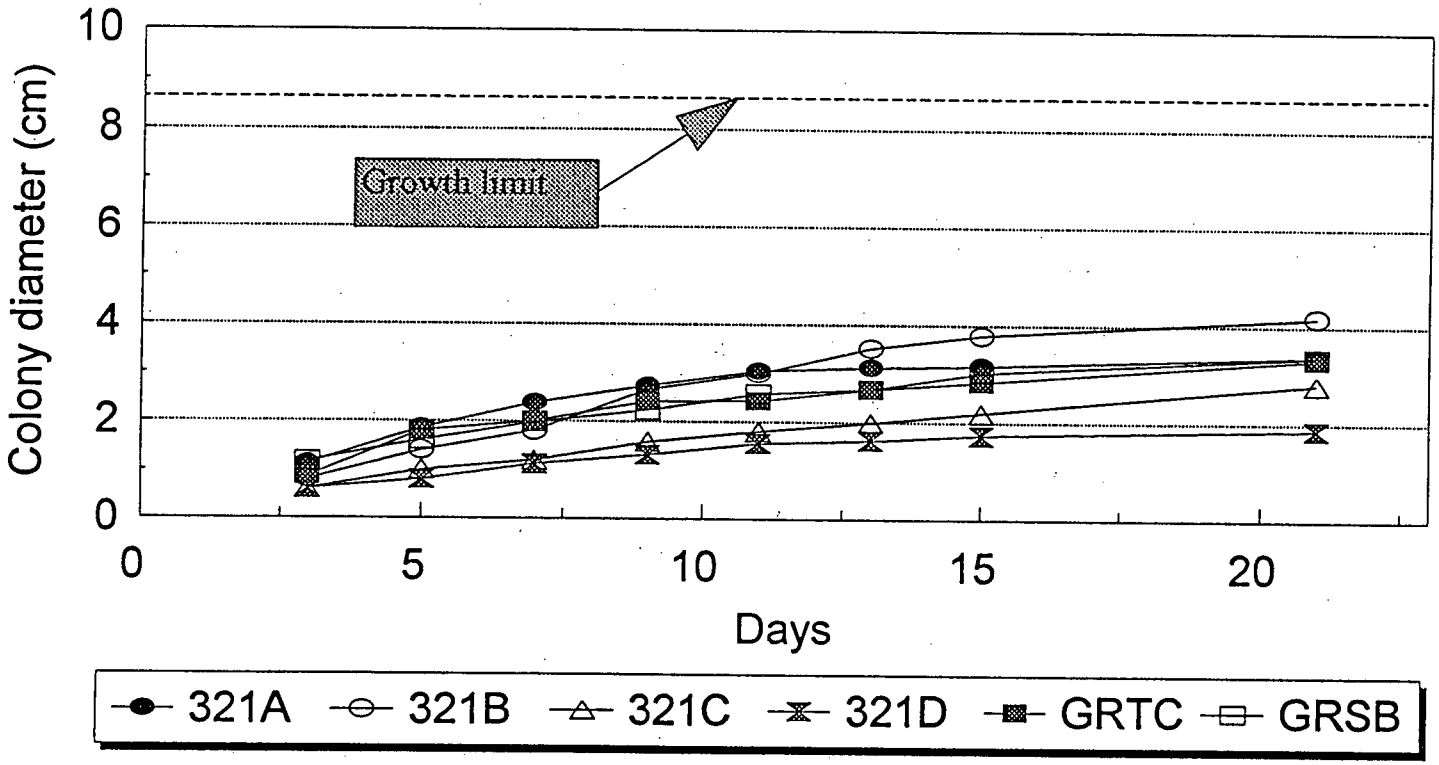


Fig. 5.3.1.10 Growth of *G. roseum* on malt agar containing 750 ppm DDAC
(Standard deviations ≤ 0.6 cm)

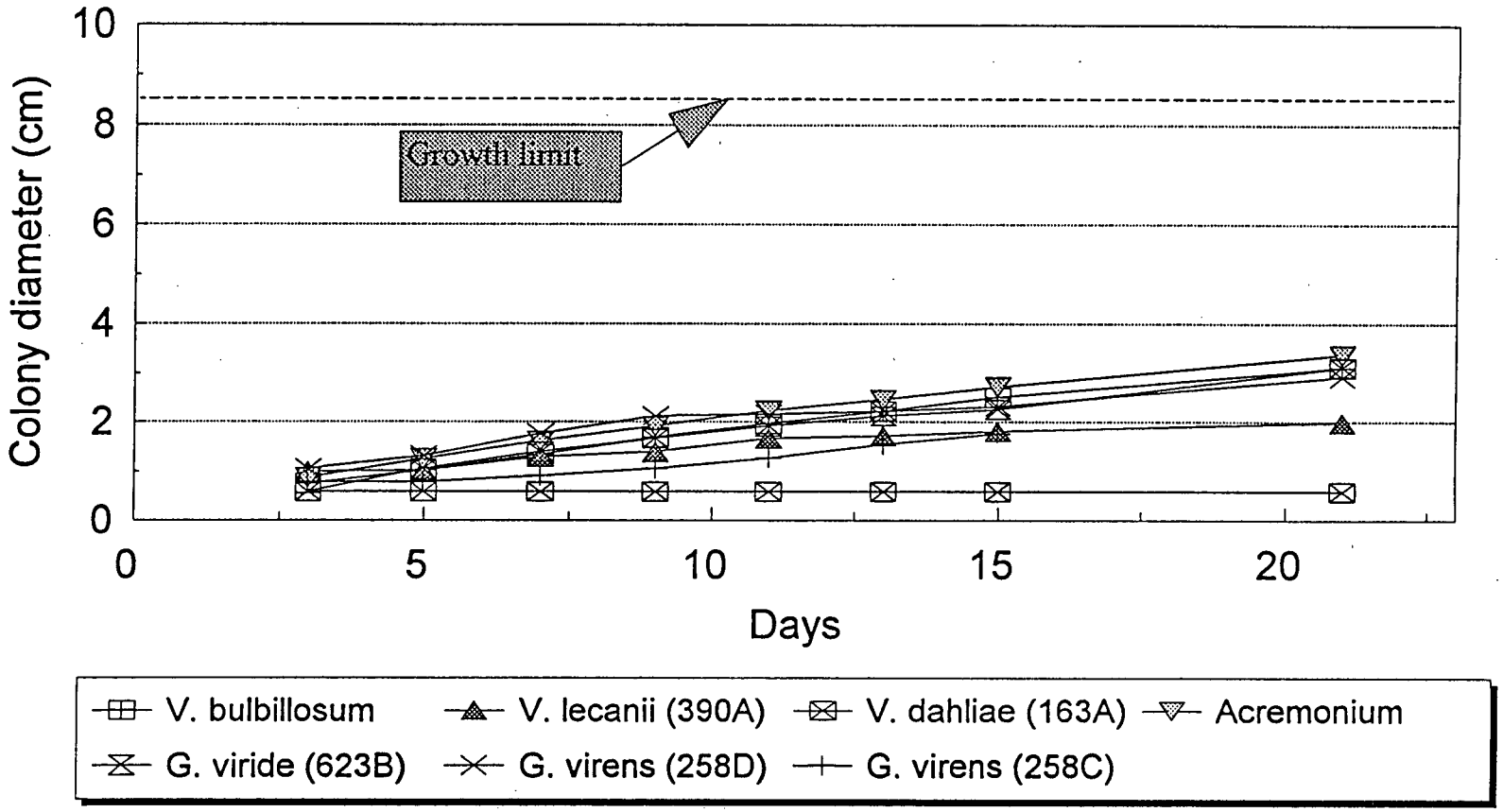


Fig. 5.3.1.11 Fungal growth on malt agar containing 1000 ppm DDAC
(Standard deviations ≤ 0.6 cm)

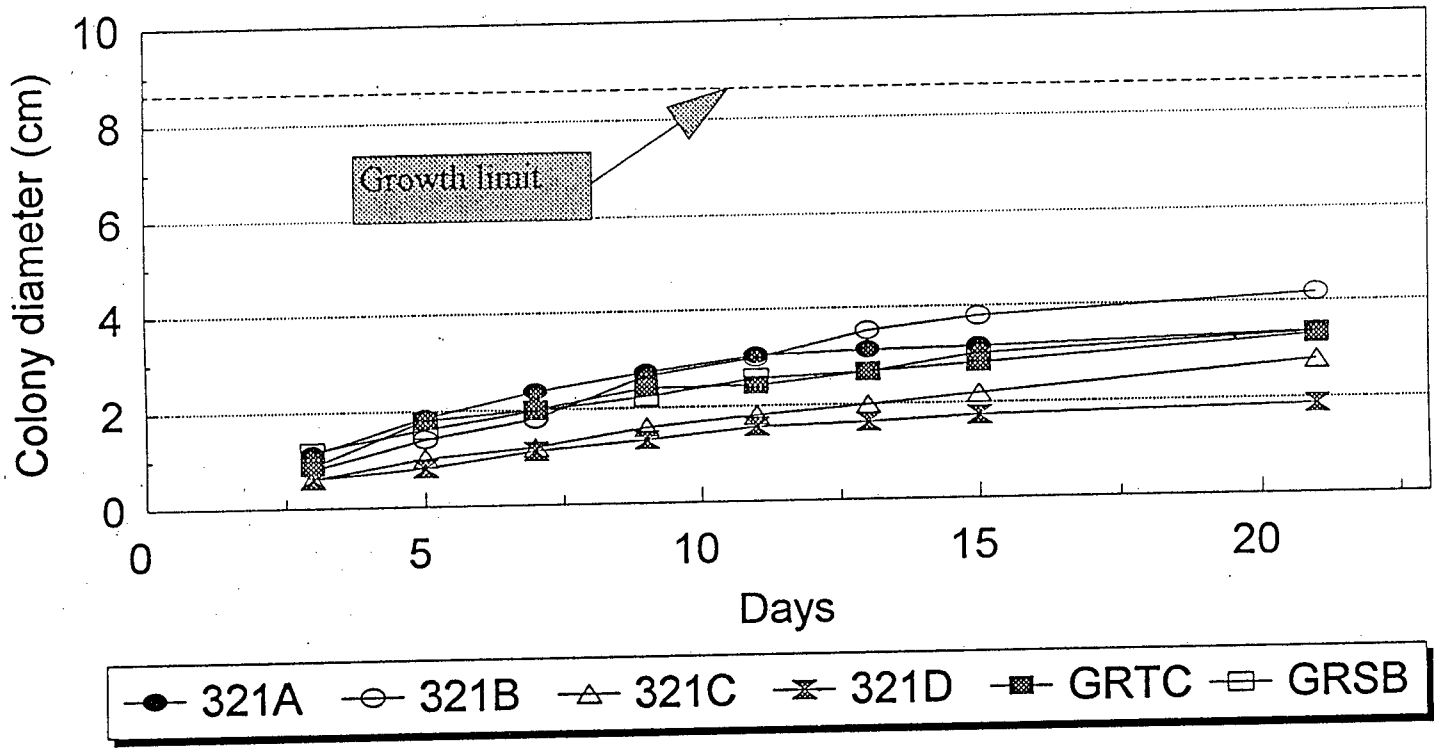


Fig. 5.3.1.12 Growth of *G. roseum* on malt agar containing 1000 ppm DDAC
(Standard deviations ≤ 0.6 cm)

During the first fifteen day of incubation, there was a linear relationship between the diameter (cm) of the mycelial mat and the incubation period (days). The slope of this plot can represent the initial growth of these fungi. The intercept can be used to show whether or not the fungal growth is subject to a lag phase. From the data which are summarized in Tables 5.3.1.1 and 5.3.1.2, it was clear that there was no relationship between the fungal growth rate for any particular fungus on malt agar alone, and the growth rate on malt agar containing DDAC. For example, of all *G. roseum* strains, the highest growth rate on malt agar was exhibited by strain 321C. However, this strain did not have the highest growth rate in malt agar containing DDAC. *G. viride* (623B) had the highest growth rate of all strains of fungi tested on malt agar alone, but it did not grow fastest on malt agar containing DDAC. Comparing the growth rate of various strains of *G. roseum*, it was interesting to note that strain 321A grew rapidly at 250 ppm of DDAC, but as the concentration increased it was clear that strain 321B was more tolerant. From the data of intercepts, it seems that none of the fungi have a lag phase during growth on malt agar or malt agar containing DDAC.

With increasing concentrations of DDAC, the appearance and growth characteristics of some fungi changed. Particularly noticeable was the production of increasing amounts of aerial hyphae. Some fungi released pigment into the malt agar causing strong yellow or red colorations (Figure 5.3.1.13). All the changes are considered to arise due to a reaction to the DDAC toxicity and its surfactant properties.

Table 5.3.1.1 Slope and intercept of linear regression of fungal growth (hyphal diameter in cm) over the first fifteen days of the incubation period

Fungal species		*V. bulbillosum*	390A	163A	*Acremonium*	623B	258D	258C	S-1-6	S-2-11	S-1-1	S-2-1	F-1-1
Slope	Control	0.32	0.24	0.34	0.29	2.75	1.53	0.69	0.46	0.46	0.45	0.43	0.08
	500 ppm	0.18	0.15	0.03	0.20	0.22	0.29	0.22	0.52	0.34	0.28	0.33	0.50
	750 ppm	0.18	0.16	0.04	0.18	0.13	0.25	0.16	0.42	0.24	0.26	0.29	0.39
	1000 ppm	0.13	0.06	0	0.14	0.13	0.10	0.13					
Intercept	control	0.44	0.43	0.28	0.63	-5.25	-1.48	5.05	1.36	1.36	2.37	1.90	7.61
	500 ppm	0.41	0.57	0.52	0.35	0.23	0.29	0.18	0.73	1.01	1.13	0.86	-0.57
	750 ppm	0.35	0.72	0.49	0.55	0.65	0.20	0.11	0.36	1.59	1.39	0.87	-0.18
	1000 ppm	0.43	0.85	0.6	0.61	0.38	0.97	0.38					

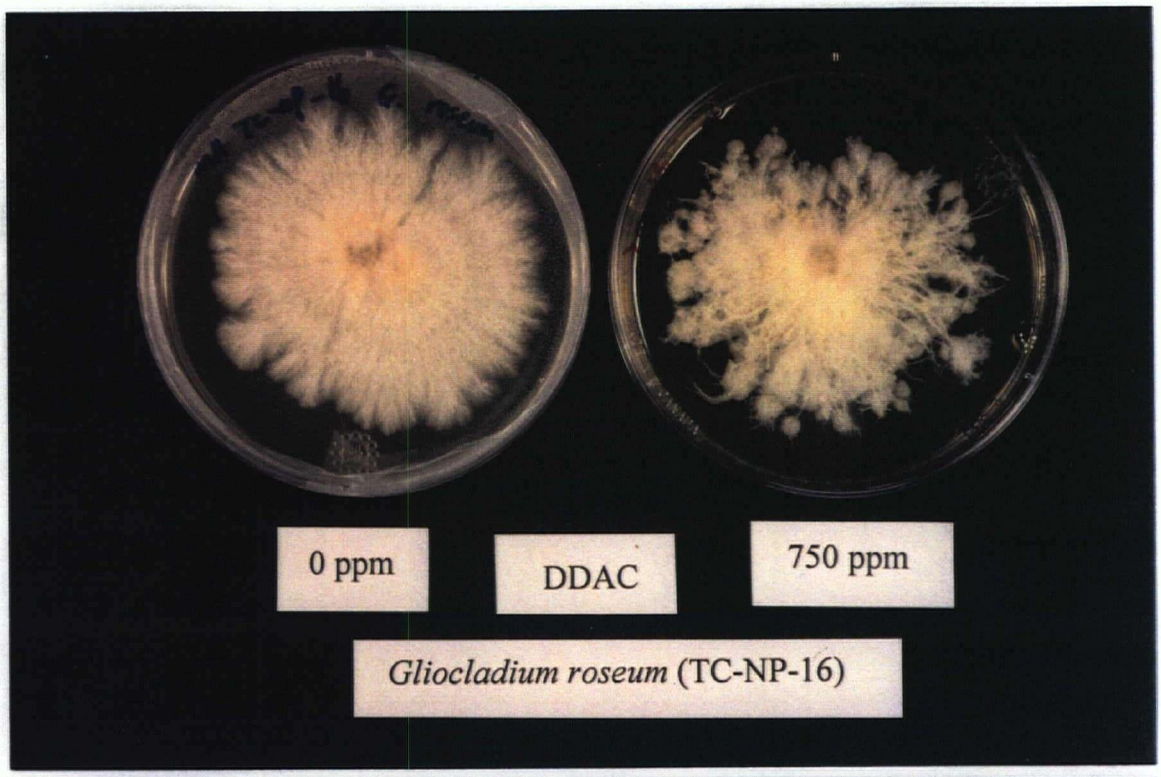
Note: 390A is *V. lecanii*, 163A is *V. dahliae*, 623B is *G. viride*, 258D and 258C is *G. virens*, S-1-6 is *Z. molleri*, S-2-11, S-1-1 and S-2-1 are *F. avenaceum*, F-1-1 is *T. virens*

Table 5.3.1.2 Slope and intercept of linear regression of growth of *G. roseum* (hyphal diameter in cm) over the first fifteen days of the incubation period (days)

		321A	321B	321C	321D	GRTC	GRSB
Slope	Control	0.45	0.44	0.59	0.45	0.45	0.34
	250 ppm	0.36	0.33	0.25	0.29	0.30	0.23
	500 ppm	0.26	0.29	0.21	0.19	0.28	0.21
	750 ppm	0.19	0.24	0.14	0.14	0.20	0.20
	1000 ppm	0.12	0.20	0.12	0.07	0.12	0.12
Intercept	control	0.56	0.89	-0.13	0.28	0.75	1.15
	250 ppm	0.17	0.04	0.31	-0.18	-0.28	0.64
	500 ppm	0	0.24	0.14	0.02	0.61	0.70
	750 ppm	0.94	0.74	0.75	0.46	0.79	0.79
	1000 ppm	1.35	0.56	0.39	0.56	1.04	1.05

Note: 390A is *V. lecanii*, 163A is *V. dahliae*, 623B is *G. viride*, 258D and 258C is *G. virens*, S-1-6 is *Z. molleri*, S-2-11, S-1-1 and S-2-1 are *F. avenaceum*, F-1-1 is *T. virens*

A



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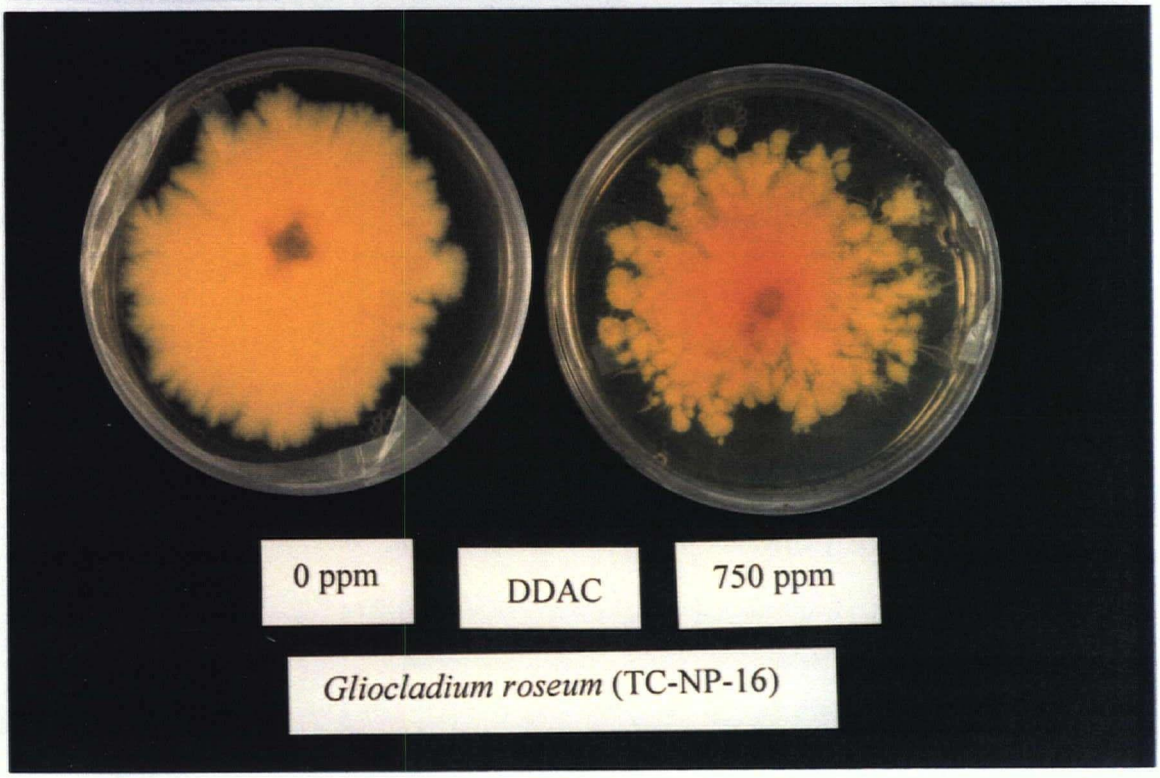


Fig. 5.3.1.13 *G. roseum* (TC-NP-16) grown on malt agar containing 0 ppm and 750 ppm DDAC

A: Face up
B: Face down

5.3.2 Liquid media

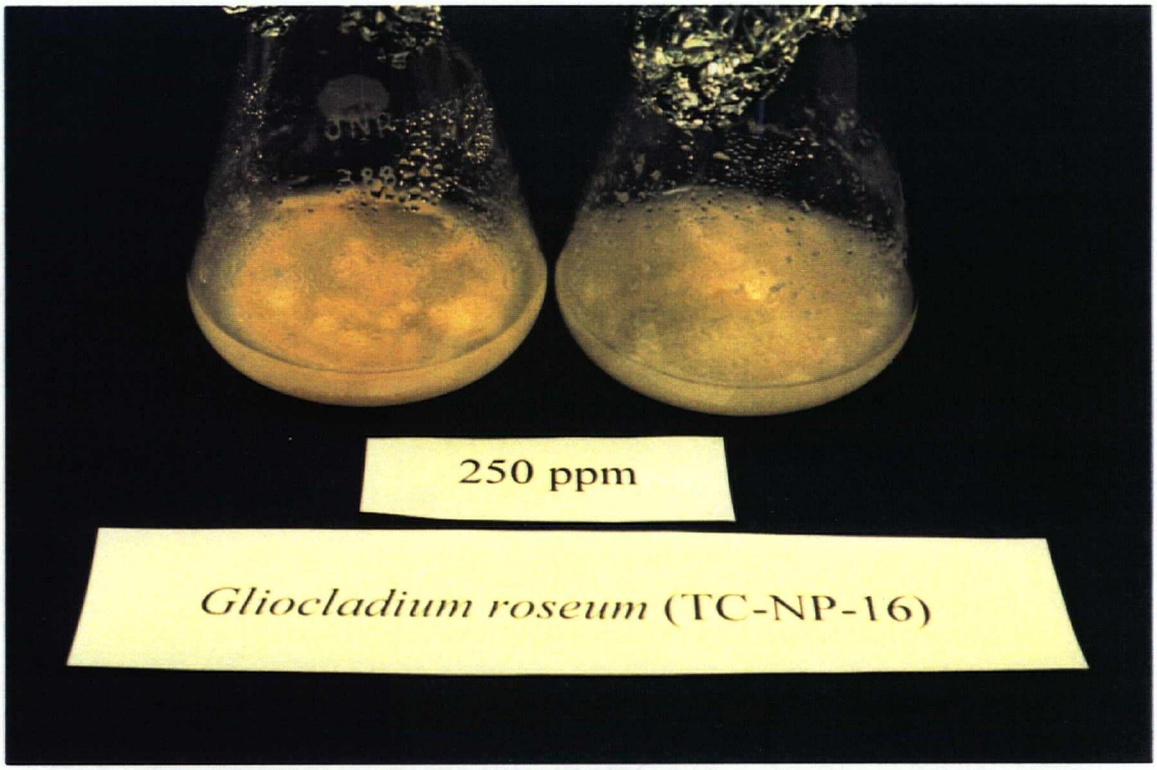
The fungi *V. bulbillosum* and *G. roseum,* were inoculated in all three liquid media with and without DDAC. After two weeks, both fungi grew well in Vogel and Czapek liquid media containing no DDAC but they did grow well in 2% malt extract. When the three liquid media contained DDAC, fungi grew only in the Vogel liquid media. Consequently this was chosen for the liquid culture experiment.

The tolerant fungi were chosen for growth in liquid media with DDAC, based on the growth rate of fungi on solid media containing DDAC. The biomass of fungi in liquid media without DDAC and with 250 ppm DDAC is shown in Table 5.3.2.1. The fungal strains of *G. roseum* (TC-NP-16), *G. roseum* (321C), *V. bulbillosum*, *V. lecanii* (390A), *Acremonium sp.*, and *F. avenaceum* (F-1-1) grew very well in liquid media containing 250 ppm DDAC. Figure 5.3.2.1 shows the growth of *G. roseum* (TC-NP-16) and *F. avenaceum* (S-1-1) in liquid media containing 250 ppm DDAC. Since a threshold of 250 ppm DDAC in solid agar media was employed to select fungi for further work, the same concentration was also used for the determination of fungal tolerance in liquid media. Examination of the results of fungal growth in solid media and liquid media (Fig. 5.3.1.4 to 5.3.1.12 and Table 5.3.2.1), however, revealed that there was no relationship between the fungal tolerance in solid media and liquid media. Some fungi *T. virens* (F-1-1), *Z. molleri* (S-1-6), *G. roseum* (321A and 321D) grew very well in solid media containing 750 ppm or 1000 ppm DDAC, but failed to grow in liquid media containing 250 ppm DDAC.

Table 5.3.2.1 Fungal growth in the liquid media containing 250 ppm DDAC

	Biomass (mg/ml)	
Fungi	0 ppm	250 ppm
G. roseum (321A)	6.46 ± 0.30	0
G. roseum (321B)	2.84 ± 0.49	1.81 ± 0.22
G. roseum (321C)	7.15 ± 0.08	6.17 ± 0.65
G. roseum (321D)	2.21 ± 0.01	0
G. roseum (GRSB)	6.26 ± 2.39	2.99 ± 0.05
G. roseum (TC-NP-16)	5.86 ± 0.01	6.83 ± 0.34
V. bulbillosum	8.17 ± 0.13	6.25 ± 0.15
V. lecanii	7.08 ± 1.12	6.34 ± 0.05
F. avenaceum (S-2-11)	9.17 ± 0.71	1.85 ± 0.20
F. avenaceum (S-1-1)	6.64 ± 0.73	7.07 ± 0.56
G. viride (623B)	5.78 ± 0.18	3.24 ± 0.49
G. virens (258D)	7.65 ± 0.30	0
Acremonium sp.	6.83 ± 0.01	6.40 ± 0.26
T. virens (F-1-1).	7.46 ± 0.25	0
Z. moelleri (S-1-6)	6.38 ± 0.01	0

A



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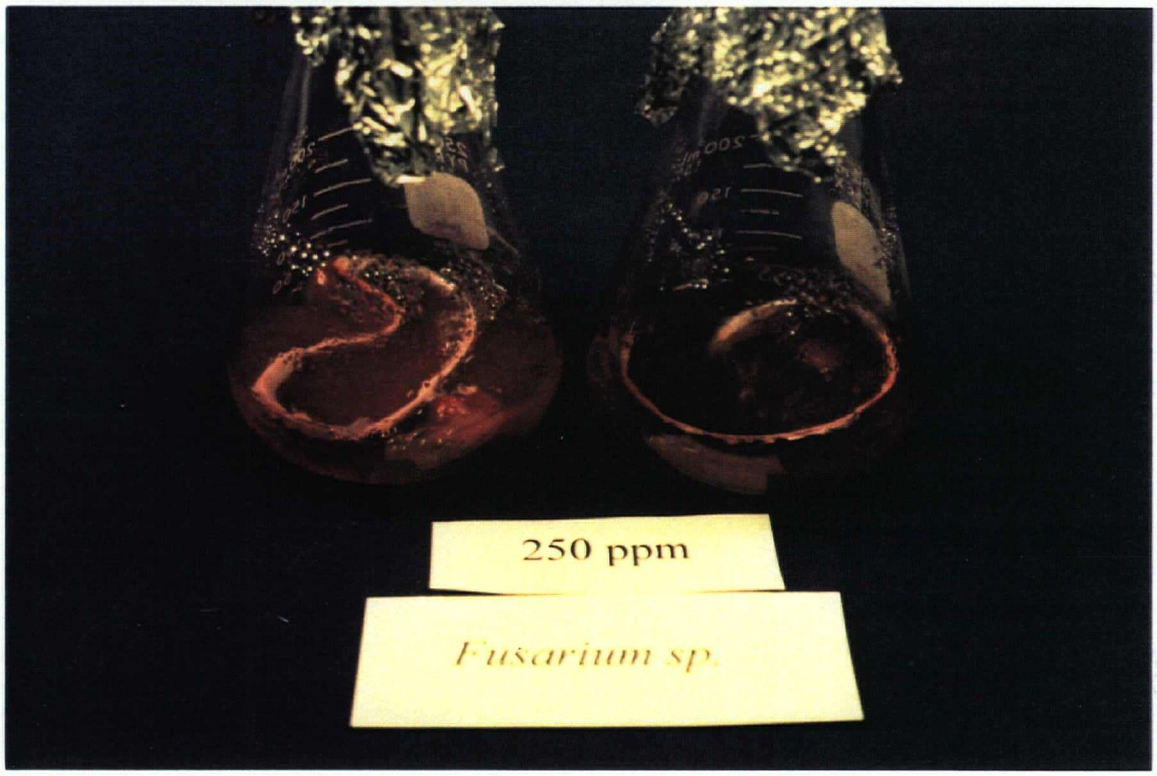

Fig. 5.3.2.1 Fungal growth in Vogel liquid media containing 250 ppm DDAC
A: *G. roseum* (TC-NP-16)
B: *F. avenaceum* (S-1-1)

5.3.3 The growth curve of *V. bulbillosum*.

The production of biomass by *V. bulbillosum* in Vogel liquid media without DDAC at different time intervals is shown in figure 5.3.3.1. Growth curves of fungal cultures normally show three stages of growth. According to the growth curve of *V. bulbillosum*, there was only a very short lag phase where the organism adjusted to the growing medium. The lack of a lag phase probably resulted from the transfer of cores containing highly active, growing mycelium, to a liquid media. Between days three to seven growth was linear. The fungal biomass reached a maximum during day seven and eight. After this, between the ninth day and eighteenth days, the biomass reduced in magnitude. During this period, degradation of DDAC may occur as the fungus seeks alternative source of carbon. However, according to the fungal growth on malt agar containing DDAC, the amount of DDAC under the edge of the colony was substantially reduced, which could indicated that degradation of the chemical must also take place during the early stages of fungal growth.

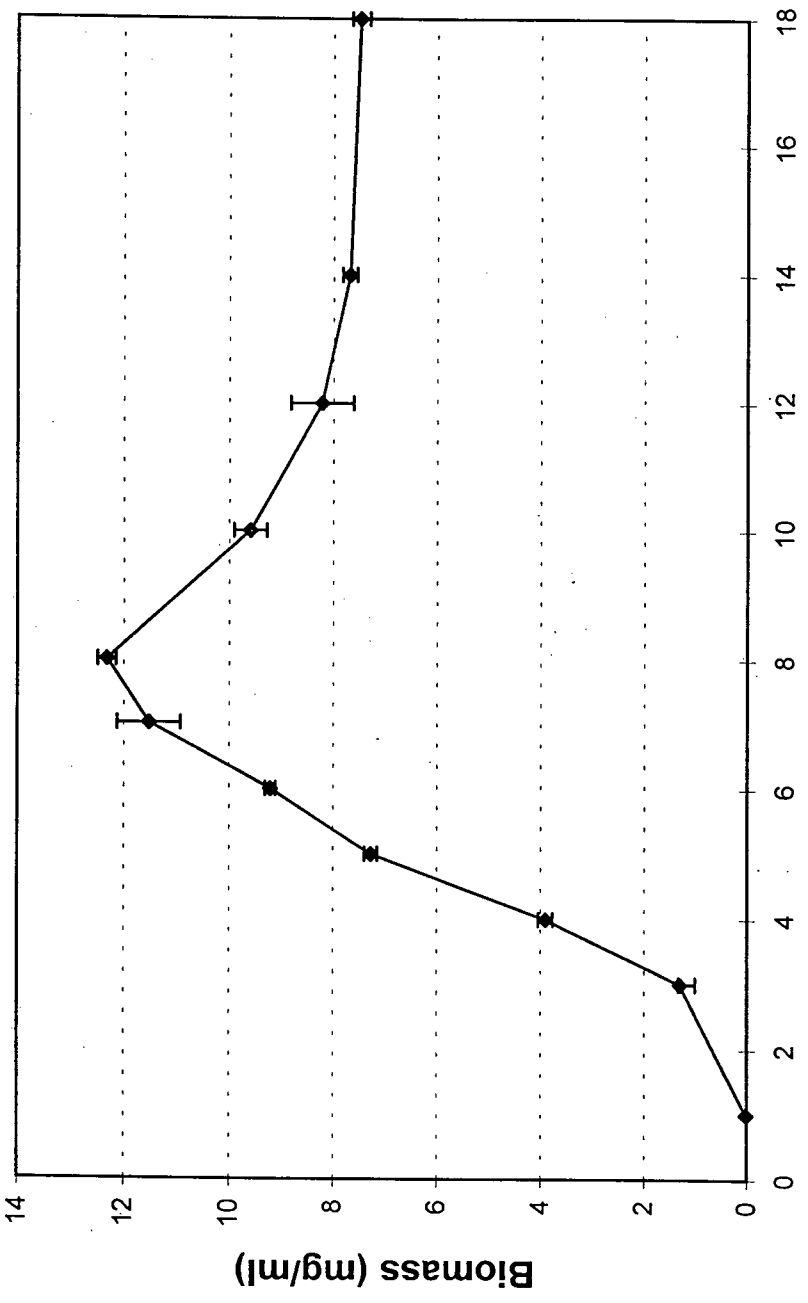

Fig. 5.3.3.1 Growth curve of *V. bulbillosum*

5.4 Fungal degradation of DDAC

Previous research with *Gliocladium roseum* and *Acremonium* sp demonstrated that fungi could modify the structure of QACs in solid agar and wood, based upon the disappearance of the peak due to DDAC in the HPLC chromatogram. In the present study, the ability of the fungi from standard cultures to degrade DDAC was compared with strains of *G. roseum, V. bulbillosum* and *Acremonium* sp. isolated from lumber by Dr. Andress Doyle. The DDAC degrading capacity of the recent isolation of *Fusarium sp.*, *Zygorrhynchus sp.* and *Trichoderma sp.* were also assessed in solid and liquid media.

5.4.1 Solid media

Table 5.4.1.1 shows the concentration of DDAC in malt agar before fungal growth and the residual DDAC concentration in selected positions on the malt agar plates following fungal overgrowth. The positions on the malt agar are shown in Figure 5.4.1.1 and correspond to: P) the perimeter of the plate where there has been no fungal growth; E) beneath the growing limit of the hyphal mat; and C) close to the center of the plate. The percent reduction of DDAC was calculated from the baseline DDAC assay determined for each plate at the perimeter. The decrease in DDAC concentration may be considered as evidence of primary degradation. Although the concentration decreased, there was no evidence to suggest that ultimate degradation (or mineralization) had occurred. Nor was there any information on the toxicity of the products formed (environmentally acceptable degradation). *V. bulbillosum*, *G. roseum* 321C and 321D seemed to have the best potential to degrade DDAC, based upon their ability to cause a major reduction in the DDAC peak when analyzed by HPLC. There did not appear to be any correlation between the decrease in

Table 5.4.1.1 DDAC degradation in solid media

Fungal species	DDAC content (ppm)			Percentage of DDAC reduction (%)	
	No growth	Growth		Growth	
		Edge	Center	Edge	Center
G. roseum (321A)	826	710	477	16	42
G. roseum (321B)	833	755	659	9	21
G. roseum (321C)	1119	11	11	99	99
G. roseum (321D)	966	170	75	82	98
GRSB	765	551	145	28	81
TC-NP-16	821	520	164	37	80
V. bulbillosum	801	94	8	88	99
Acremonium	898	707	380	21	58
F. avenaceum (S-1-1)	688	101	173	85	75
F. avenaceum (S-2-11)	793	719	295	9	63
F. avenaceum (S-2-1)	1051	955	620	9	41
Z. moelleri (S-1-6)	915	731	514	20	44
T. virens (F-1-1)	953	590	564	38	41
G. virens (258D)	859	481	383	44	55
G. virens (258C)	944	506	443	46	53
G. viride (623B)	760	190	154	75	80

Note: Standard deviations are between 2 - 50 ppm.

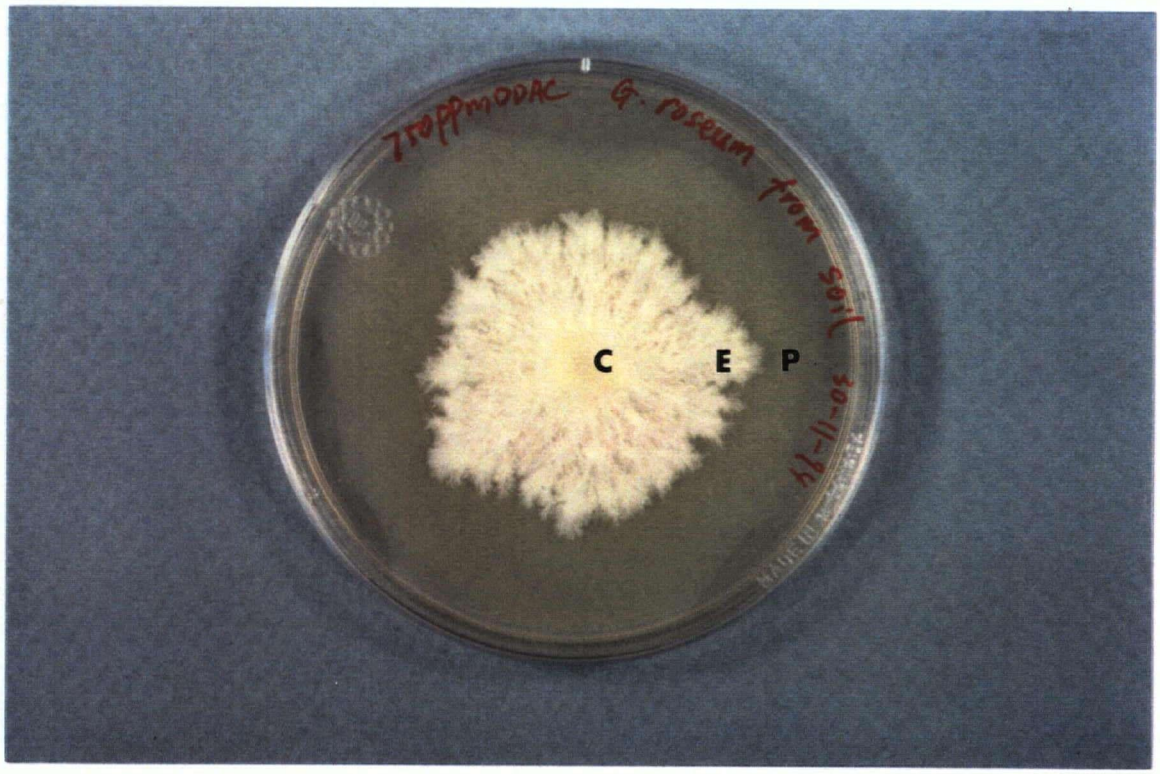

Fig. 5.4.1.1 The different locations on the malt agar plate for assessment of DDAC
C: center
E: edge
P: perimeter

DDAC concentration and the fungal growth rate. For example, *T. virens* (F-1-1) and *Z. molleri* (S-1-6) grew much faster on malt agar containing DDAC than *V. bulbillosum*, but the residual DDAC concentration was much higher than that after growth of *V. bulbillosum*.

5.4.2 Liquid media

Recovery of DDAC from the liquid media

DDAC is soluble in water and a wide range of solvents. DDAC contains two long alkyl chains, which are responsible for its hydrophobic character. However, the positive charged nitrogen atom enhances the hydrophilic property of DDAC. This combination of hydrophobic and hydrophilic character, its surfactant property and the common ion effect, makes the quantitative recovery of DDAC from solution or substrates challenging.

Table 5.4.2.1 contains the percent of DDAC recovered from liquid media, using different extraction procedures outlined in Section 4.4.2. It was found that the recovery of DDAC using partitioning in methylene chloride was not good enough. If instead, the liquid media containing DDAC was roto-evaporated and extracted in acidified methylene chloride, with an ultrasonic bath, the best recovery of DDAC (>90%) from buffer and fungal cultures was obtained. Because DDAC contains cationic ions according to the following equation

$$[DDAC] \leftrightarrow [DDA]^+ + [Cl]^-$$

($[DDA]^+$ here is the didecyldimethylamonium cation), the use of an acidified solvent can increase the recovery of DDAC solubility in water or polar solvents, because the protonic solvent can prevent exchange of the DDA onto solids such as glassware etc, as well as enhence its release from wood at solid media.

Table 5.4.2.1 The recovery of DDAC from liquid media and buffer using different extraction procedures

Extraction procedure		Original DDAC (mg)	Recovered DDAC (mg)	Recovery (%)
a	Vogel liquid media			
		9.76	4	46
		9.2	4	43
b	Buffer			
		3.8	1.9	50.6
		3.8	1.6	41.5
	Vogel liquid media			
		5.9	3	5.9
		5.9	3	5.9
c	Buffer			
		10	9	90
		10	8	80
	Vogel liquid media			
		10	1.5	14
		10	0.5	4.8
d	Buffer			
		29	27	93
		19	18	96
	Vogel liquid media			
		29	16	57
		36	33	90
e	Fungal culture			
		24	24	100
		28	25	89
		23	21	91

a = Liquid media extracted with methylene chloride
b = Liquid media roto-evaporated to dryness, extracted with methylene chloride
c = Liquid media roto-evaporated to dryness, extracted with acidified methylene chloride
d = Liquid media roto-evaporated to dryness, extracted ultrasonically with acidified methylene chloride
e = Fungal culture roto-evaporated to dryness, extracted ultrasonically with acidified methylene chloride

Residual amount of DDAC in liquid media

The fungi which showed very good growth in liquid media containing 250 ppm DDAC were chosen for these experiments to determine the amount of DDAC degraded in liquid media. Table 5.4.2.2 shows the residual DDAC after two week's fungal growth. It was found that DDAC concentrations were reduced to below the detectable limit of approximately 10 ppm in all but one case (*G. roseum* 321C). One explanation for this observation is that the DDAC had been modified or degraded into chemicals which can not be detected by the HPLC method, which was specific for cationic QACs. Figures 5.4.2.1 to 5.4.2.6 show the reduction or disappearance of the DDAC peaks at ca 9.85 minute retention time and the formation of other peaks at longer retention times. Since all the peaks are negative (ie. give a response similar to the DDAC, but with a different retention time), this suggests that these metabolites are cationic in nature. These new peaks are thought to arise from the fungal action on DDAC. They were not observed in the HPLC spectra of the control samples (Vogel liquid media containing 0 ppm DDAC after fungal growth or Vogel liquid media containing 250 ppm DDAC without any fungal growth). It was interesting to note that all the retention times of the negative peaks from the different cultures were similar, except for those in the chromatogram of *G. roseum* 321C. This suggests that the degradation of DDAC by different moulds follows the same mechanism. The longer retention time is consistent with the formation of QAC type compounds with shorter alkyl chain length. According to their retention times (C_8/C_8: 11.15 min; C_8/C_{10}: 10.05min; C_{10}/C_{10}: 10.49 min), two of the negative peaks could possibly correspond to C_8/C_8 and C_8/C_{10} dialkyldimethylammonium salts. It may also be formed by QACs containing an oxidized

Table 5.4.2.2 Residual concentration of DDAC in liquid media after fungal growth

Fungi	*Original concentration (ppm)	Residual concentration (ppm)
V. bulbillosum	296	ND
Acremonium sp.	296	ND
G. roseum (TC-NP-16)	296	ND
G. roseum (321C)	296	8.6
F. avenaceum (S-1-1)	296	ND

ND: not detected (Detection limit is 10 ppm)
* Concentrations of DDAC based on HPLC analysis

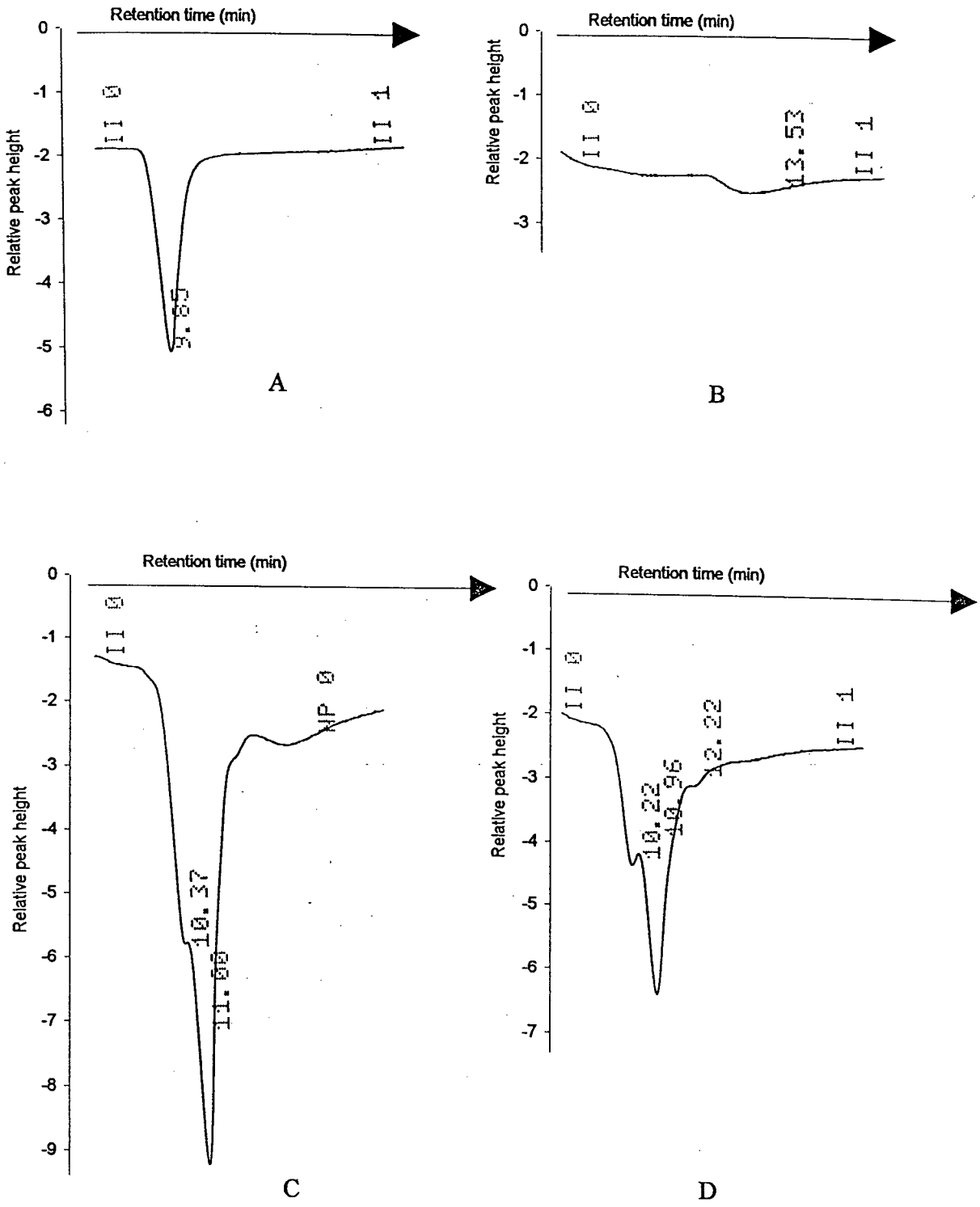

Fig.5.4.2.1 HPLC spectra of extract from liquid media containing 250 ppm DDAC after fungal growth of *V. bulbillosum*

A: Standard DDAC (776 ppm)

B: Control (concentrated extract from liquid media without DDAC)

C: Concentrated extract from Vogel liquid media with DDAC

D: Concentrated extract from Vogel liquid media with DDAC and silica gel

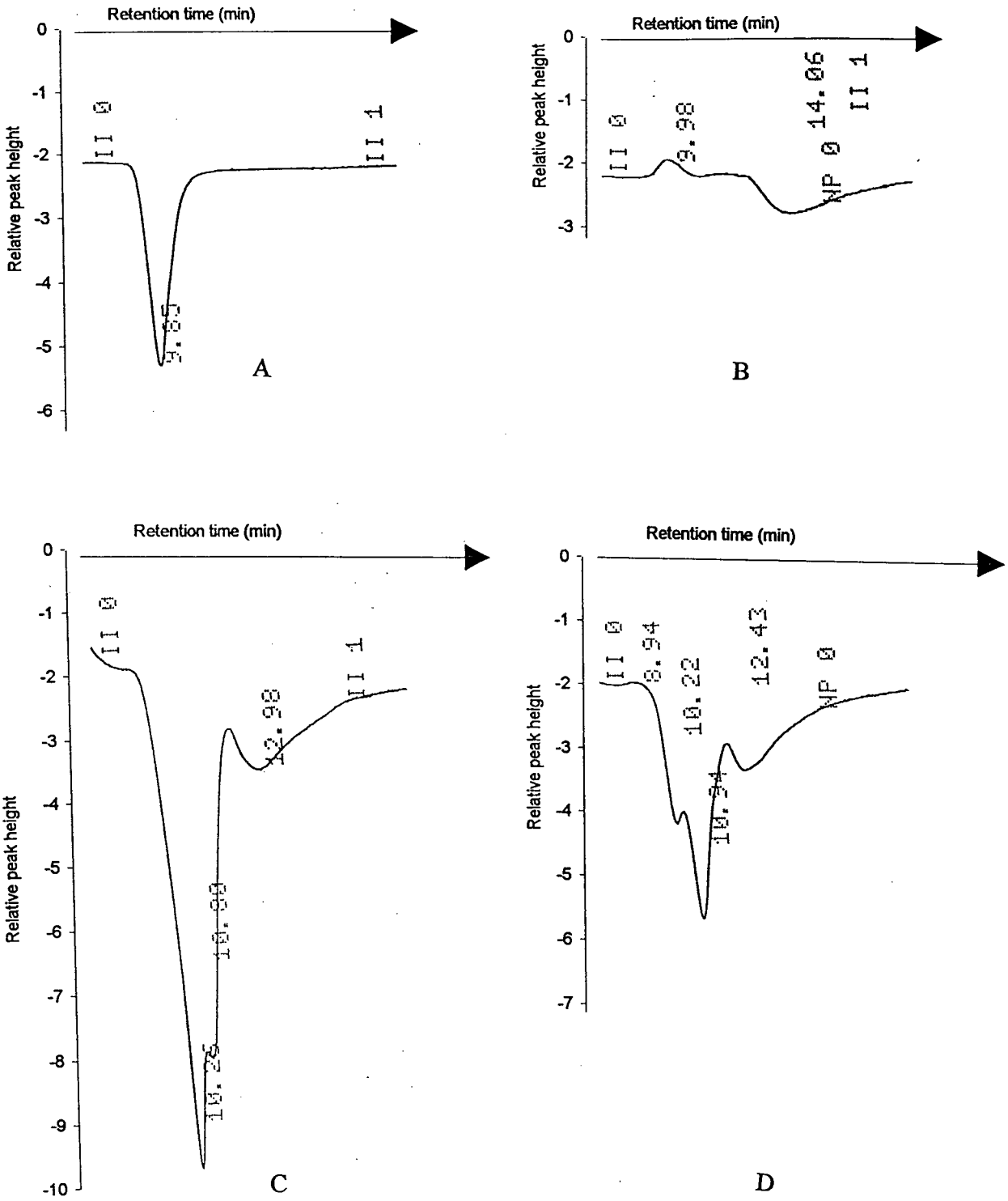

Fig. 5.4.2.2 HPLC spectra of extracts from liquid media containing 250 ppm DDAC after fungal growth of *Acremonium sp.*

A: Standard DDAC (776 ppm)

B: Control (concentrated extract from liquid media without DDAC)

C: Concentrated extract from Vogel liquid media with DDAC

D: Concentrated extract from Vogel liquid media with DDAC and silica gel

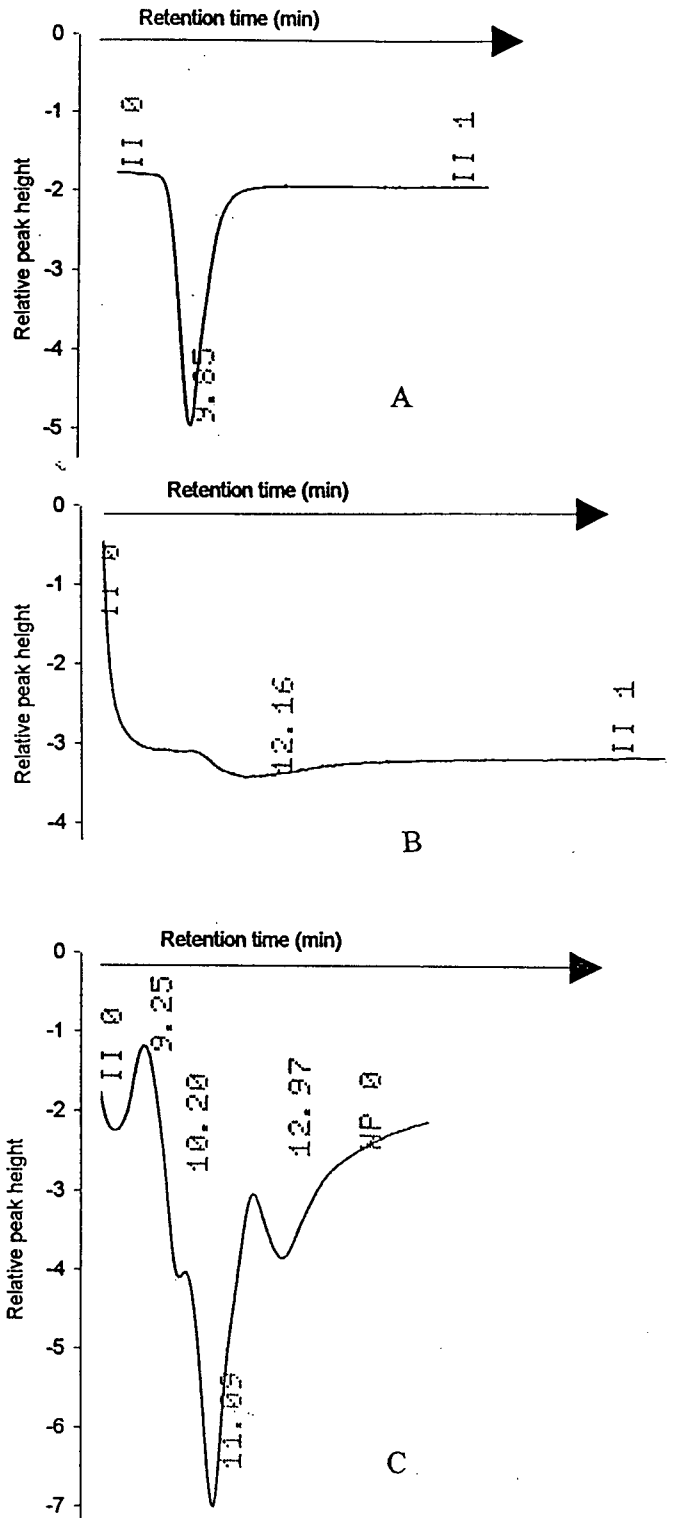

Fig. 5.4.2.3 HPLC spectra of extracts from liquid media containing 250 ppm DDAC after fungal growth of *G. roseum* (TC-NP-16)
A: Standard DDAC (776 ppm)
B: Control (concentrated extract from liquid media without DDAC)
C: Concentrated extract from Vogel liquid media with DDAC

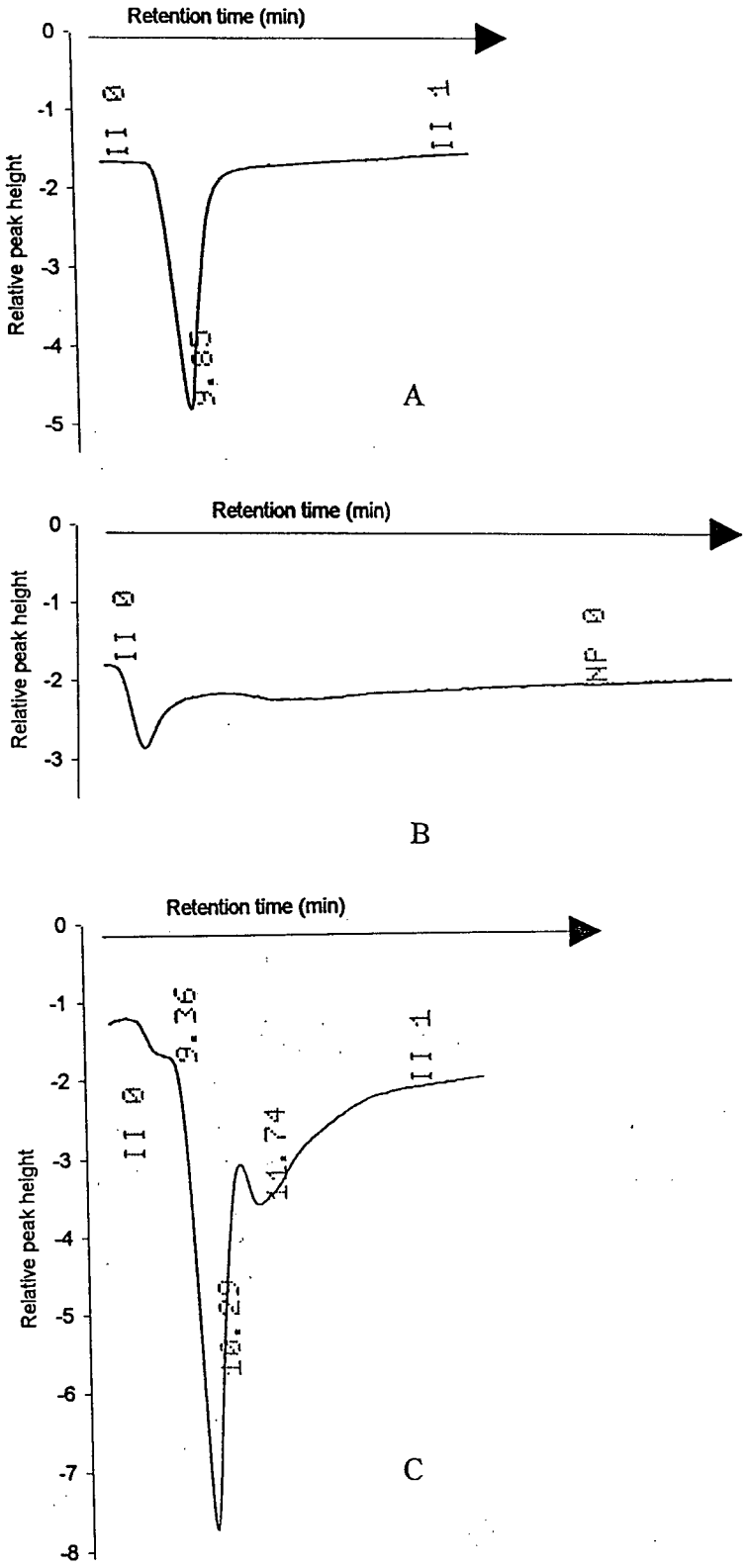

Fig. 5.4.2.4 HPLC spectra of extracts from liquid media containing 250 ppm DDAC after fungal growth of *F. avenaceum* (S-1-1)
A: Standard DDAC (776 ppm)
B: Control (concentrated extract from liquid media without DDAC)
C: Concentrated extract from Vogel liquid media with DDAC

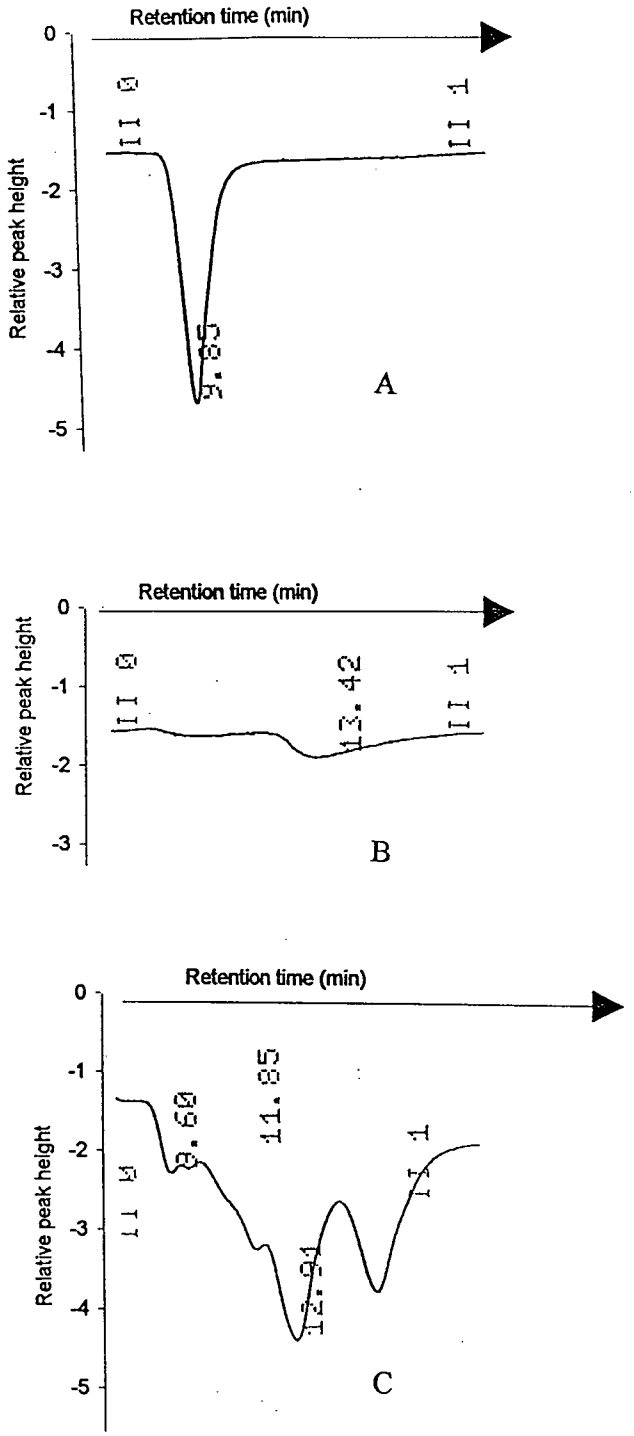

Fig. 5.4.2.5 HPLC spectra of extracts from liquid media containing 250 ppm DDAC after fungal growth of *G. roseum* (321C)
A: Standard DDAC (776 ppm)
B: Control (concentrated extract from liquid media without DDAC)
C: Concentrated extract from Vogel liquid media with DDAC

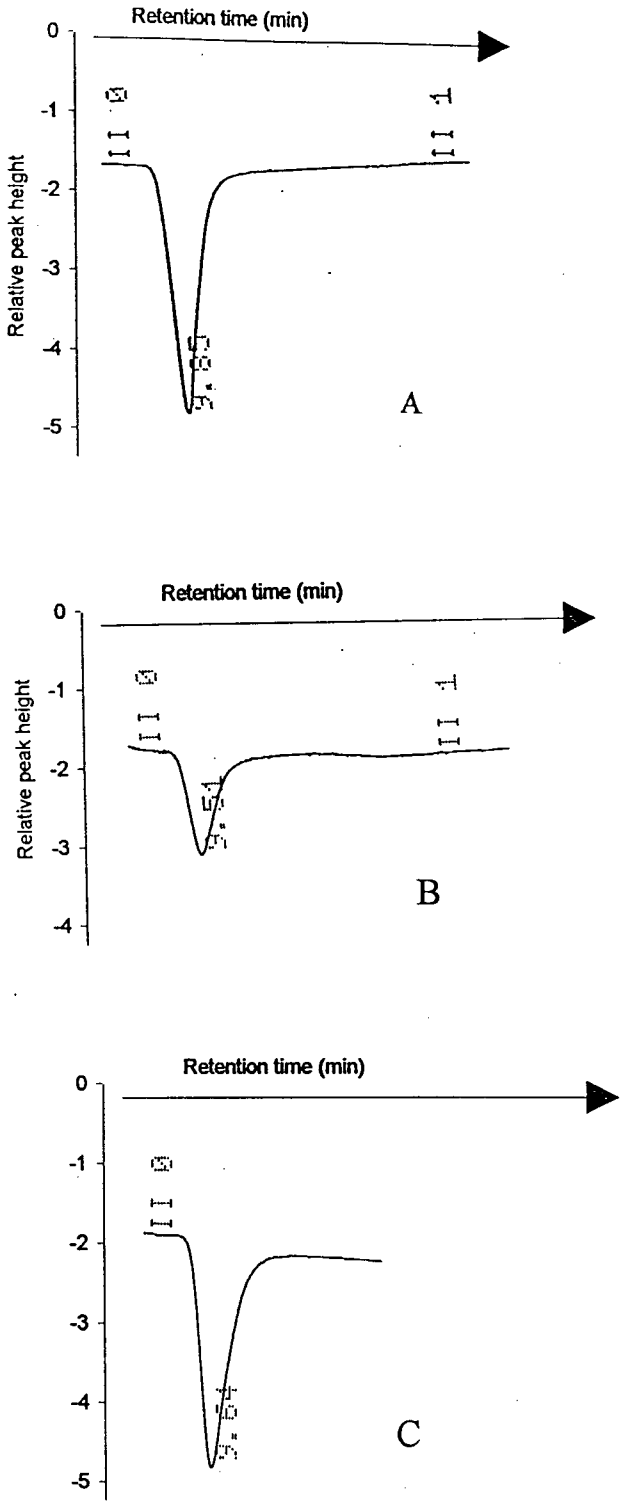

Fig. 5.4.2.6 HPLC spectra of extract from liquid media containing DDAC without any fungal growth

A. Standard DDAC (776 ppm)

B. Extract from Vogel liquid media with 250 ppm DDAC

C. Extract from Vogel liquid media with 800 ppm DDAC and siica gel

terminal carbon atom. During the extraction, the chloride of DDAC may be replaced by the formate anion which would affect the retention time of HPLC peak. However, this should not pose any the problem since the standards and reference chromatograms of DDAC were all prepared using the same procedure. Based on the study of the tolerance and DDAC degrading ability of all of the fungi from the culture collection and those isolated from treated stakelets exposed in the field test, it did not seem that the fungi isolated from the field area were specially tolerant to DDAC. Nor did they have a better ability to degrade DDAC. Instead it seemed that the tolerance and the ability to degrade DDAC were common properties of these moulds.

5.5 Identification of DDAC metabolites and implications for fungal degradation process

This research and the former study by Doyle (1995) have both shown that the concentrations of DDAC were reduced in solid and liquid media by fungal growth. But where did the DDAC go? The final products of biodegradation were not established in the previous study. In these experiments, an attempt was made to identify the major metabolites present in liquid media and thereby provide some evidence for the biodegadation pathway and the degree to which this degradation takes place.

5.5.1 Decanoic acid

Based on the literature review, one plausible biodegradation route for DDAC would involve the initial removal of one decyl chain. The resulting metabolites formed would be a ten carbon chain organic chemical and a tertiary amine. Both of these are likely to be soluble in methylene chloride and should be detectable with GC-MS.

When methylene chloride extract of the *V. bulbillosum* and *G. roseum* (TC-NP-16) cultures containing 250 ppm DDAC were subjected to GC-MS analysis, decanoic acid was identified as one of the compounds present (Figures 5.5.1.1 to 5.5.1.2). Analysis of liquid cultures of *Acremonium sp.*, resulted in a closely related product, decanediol, being identified (Figure 5.5.1.3). The GC-MS analysis of the extracts of corresponding cultures containing 0 ppm DDAC or Vogel liquid media containing 250 ppm DDAC without fungal growth, did not identify the presence of either decanoic acid or decanediol. The DDAC metabolites produced by different fungi after the same incubation period may differ, because their growth rate may be different. Two metabolites contain a ten carbon chain (decanoic acid and decanediol). This would suggest that during degradation of DDAC, breakage of the ten

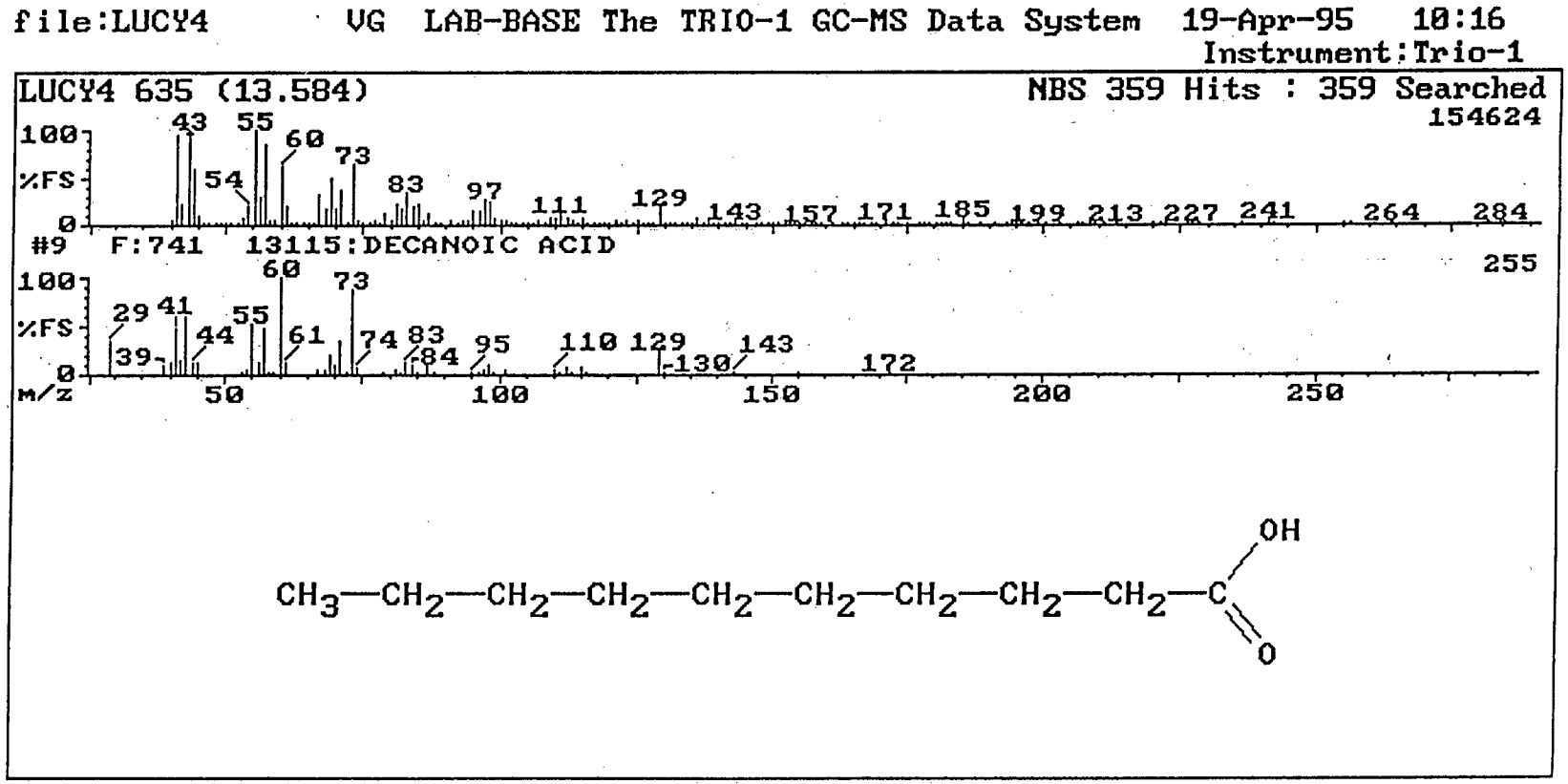

Fig. 5.5.1.1 GC-MS spectra of a component (retention time 13.584 min) in the extract from a liquid culture of *V. bulbillosum*

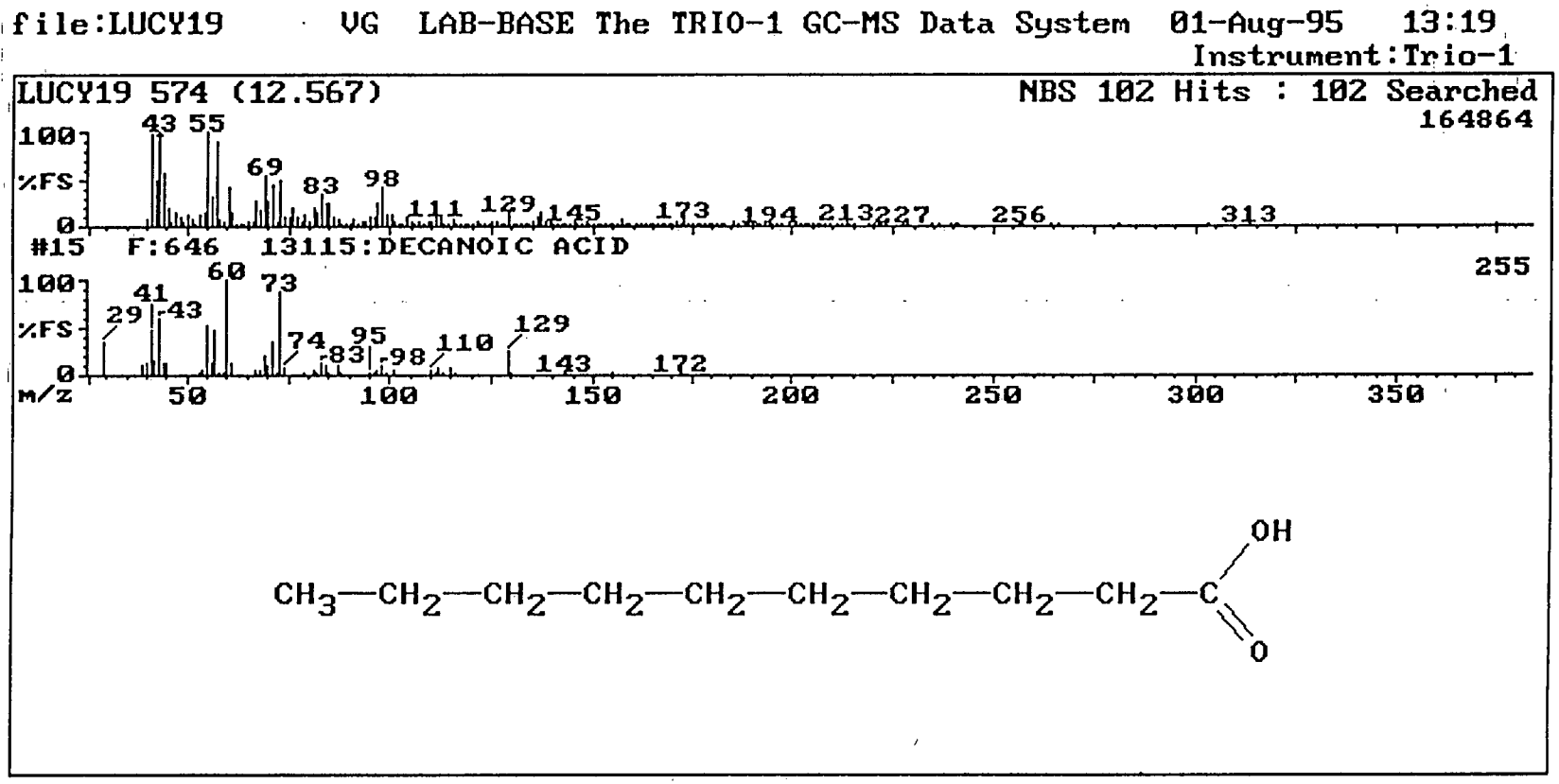

Fig. 5.5.1.2 GC-MS spectra of a component (retention time 12.567 min) in the extract from a liquid culture of *G. roseum* (TC-NP-16)

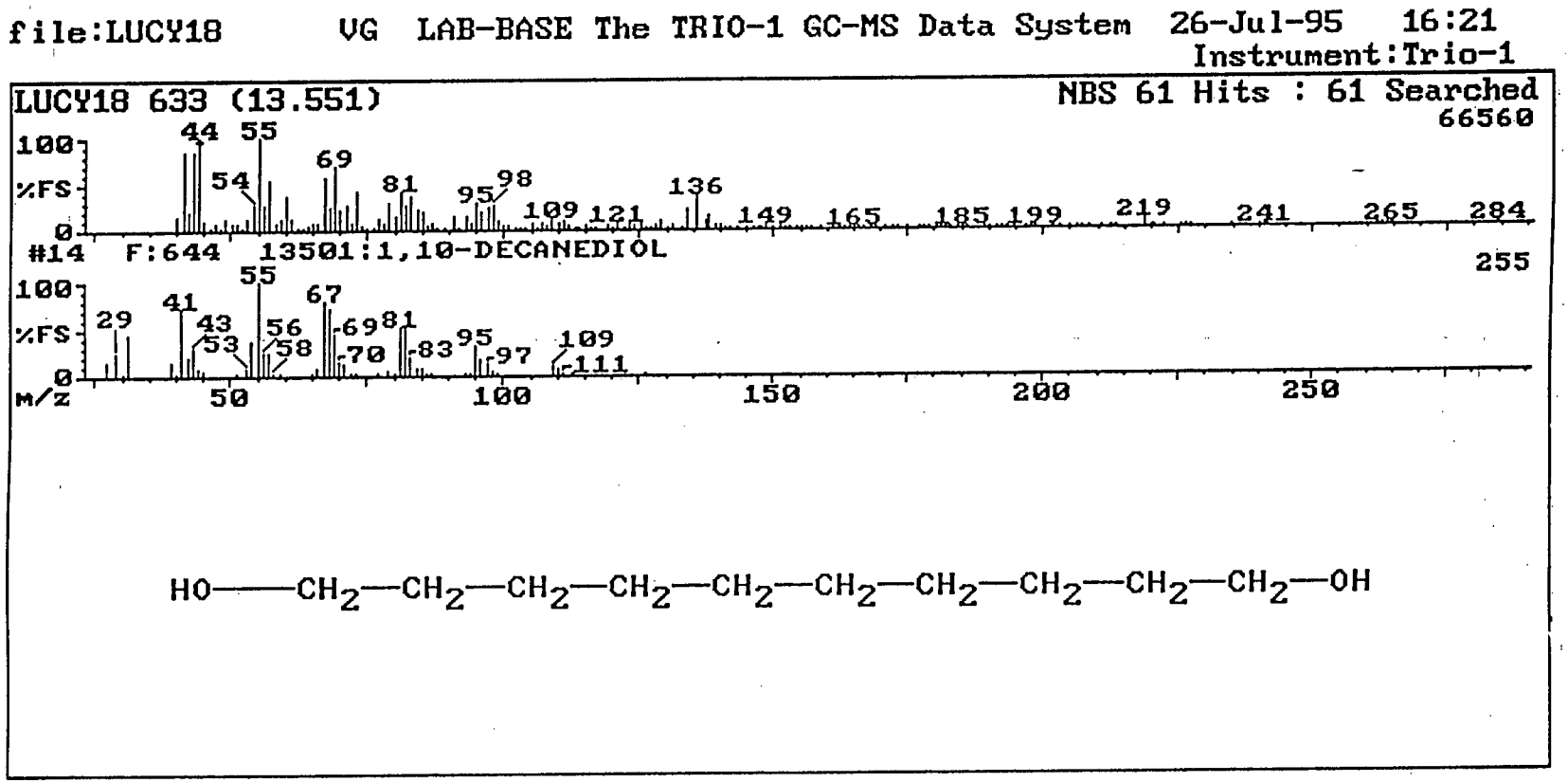

Fig. 5.5.1.3 GC-MS spectra of a component (retention time 13.551 min) in the extract from a liquid culture of *Acremonium sp.*

carbon alkyl chain from the nitrogen is the initial step. It has been reported (Cain, 1976) that during the bacterial degradation of QACs, initial rupture of the long alkyl chain occured yielding a tertiary amine and an alkan-1-al. The alkan-1-al was then further oxidized and degraded by β-oxidation. If this mechnism is correct for moulds, a tertiary amine should be formed. Attempts to detect a tertiary amine (eg. decyldimethylamine) were unsuccessful. Studies conducted using an authenticated sample of decyldimethylamine, found that it was removed during the pre-treatment with the cationic ion-exchange resin column designed to remove the QACs present. An alternative strategy was therefore attempted to separate the tertiary amine and the residual DDAC. Although the computer fitting of the GCMS spectra suggested the presence of decanoic acid and decanediol, some characteristic peaks were missing from the mass spectra, notably those at approximately 29 m/c and while others were observed at higher mass than the molecular weight. This might suggested that impurities were present, or that recombination of ion fragments has taken place. Since the spectra are dependent to some extent on the GCMS conditions of operation, the spectra of authenticated samples of decanoic acid and decanediol should be run.

5.5.2 Tertiary amine

Cationic surfactants can form insoluble compounds with anionic surfactants. Advantage was taken of this to form an insoluble ionic compound between the anionic surfactant sodium dodecyl sulphate and DDAC. This compound was insoluble in water and methylene chloride. Sodium dodecyl sulphate was also insoluble in methylene chloride. Based on this procedure, the DDAC would complex with the sodium dodecyl sulphate leaving the tertiary amine in solution. It could then be separated and analyzed by GC-MS.

GC-MS analysis of the extract recovered from the liquid of culture of *V. bulbillosum.* and *Acremonium sp.* which contained 250 ppm DDAC, showed that after two weeks' growth, decyldimethylamine could be detected. This spectrum was identified by comparing it with the authenticated decyldimethylamine run on GC-MS. Although this may have originated from the degradation process, it was also found in the extract of liquid media containing 250 ppm DDAC but without fungal growth. Since decyldimethylamine is not a major inpurity in DDAC, either the amount is small as it must have arisen frim another source (such as the GCMS analysis of DDAC which had not been removed during the complex reaction with the anionic surfactant, or which had dissolved in the methylene chloride).

5.5.3 Possible process in biodegradation of QACs by moulds

Based on the available information on biodegradation mechanism of QACs, it is possible to consider two possible pathways for degradation of DDAC by moulds. In the first mechanism, the fungus initiates attack (ω-oxidation) of the decyl chains, after which further degradation (β-oxidation) of this chain gradually removes additional atoms. The presence of such a process is supported by the HPLC analysis (Fig. 5.4.2.1 to Fig. 5.4.2.5) which showed additional peaks at a longer retention time. Such peaks could well indicate QACs with shorter alkyl chains.

However, in the liquid culture experiments, in two cases decanoic acid was identified by GCMS. This would support the second pathway which involves the initial breaking of the long decyl group. After cleavage of the decyl group, it is possbly further biodegraded by β-oxidation leading ultimately to mineralization.

5.6 The growth of fungi in liquid media containing decyldimethylamine

One proposed mechanism for the degradation of QACs by bacteria and fungi involves carbon-nitrogen fission as the first step, which would result in the formation of decyldimethylamine. This experiment was carried out to confirm whether or not, decyldimethylamine could support fungal growth.

V. bulbillosum failed to grow in normal Vogel liquid media containing 1000 ppm of decyldimethylamine as either the sole nitrogen- or sole carbon-source.

In a second experiment, the mould *V. bulbillosum*, the brown rot fungus *C. puteana*, and the white rot fungus *T. versicolor* all failed to grow in normal Vogel liquid media containing 1000 ppm decyldimethylamine (Table 5.6.1). When the concentration of decyldimethylamine was decreased to 100 ppm, four moulds *V. bulbillosum, Acremonium sp. G. roseum* (TC-NP-16), and *F. avenaceum* (S-1-1) were able to grow in normal Vogel liquid media. The resulting biomass is recorded in Table 5.6.2. This experiment was carried out at room temperature (20 °C), and the shaker speed was approximate by 143 rpm. Besides these incubation conditions, the source of inoculum may be responsible for the low biomass produced by these fungi compared to that measured in the previous experiments (cf Table 5.6.1). While the low concentration of decyldimethylamine did not inhibit the growth of the moulds, it did not function as carbon source to increase the biomass produced.

Table 5.6.1 Fungal growth in the normal Vogel liquid media containing 1000 ppm decyldimethylamine

	Biomass (mg/ml)	
	decyldimethylamine	
	0 ppm	1000 ppm
V. bulbillosum	5.68 ± 0.05	0
C. puteana	5.68 ± 0.01	0
T. versicolor	8.72 ± 0.01	0

Table 5.6.2 Fungal growth in the normal Vogel liquid media containing 100 ppm decyldimethylamine

	Biomass (mg/ml)	
	decyldimethylamine	
	0 ppm	100 ppm
Acremonium sp.	2.20 ± 0.03	1.95 ± 0.00
V. bulbillosum	2.91 ± 0.25	2.72 ± 0.12
G. roseum (TC-NP-16)	2.22 ± 0.04	1.97 ± 0.26
F. avenaceum (S-1-1)	1.76 ± 0.01	2.31 ± 0.28

5.7 The distribution of DDAC in starch, silica gel and aqueous solution

During the latter stages of research, difficulties were encountered when repeating some of the earlier experiments. Analysis of the experimental procedure revealed the observation that the presence of a small amount of suspended starch in the liquid media, had a profound impact on the amount of DDAC dissolved in the solution. To better understanding this problem, the relative amounts of DDAC adsorbed onto the suspended starch and dissolved in the liquid media, were determined. To further investigate the phenomenon, reference experiments were made using silica gel, which is used as an absorbing surface in liquid culture studies involving micro-organisms. Liquid media containing 50 ppm of DDAC, made using soluble starch purchased from two sources did not support good growth of *Acremonium sp., Verticillium sp. G. roseum, or Fusarium avenaceum* (Fr.) Sacc. Previous experiments with a two year old bottle of soluble starch from a third source supported fungal growth when amended with 500 ppm DDAC.

5.7.1 The recovery of DDAC from liquid media without starch but with silica gel

In the initial experiment, the recovery of DDAC was tested by adding known amount to: a) Vogel liquid media without starch but with silica gel, and b) liquid culture containing silica gel in which the fungus *V. bulbillosum* had grown for two weeks. The recoveries (shown in Table 5.7.1.1) were high. The acidified methylene chloride allowed the recovery of 85% - 96% of the DDAC.

Table 5.7.1.1 The recovery of DDAC from liquid media

Original DDAC (mg)	Recovered DDAC (mg)	Recovery (%)
Vogel liquid media without starch but with silica gel		
41.2	38	92
41.2	35	85
Vogel liquid media after growth of *V. bulbillosum*		
41.2	38	92
41.8	40	96

5.7.2 The distribution of DDAC

The second experiment focused on the distribution of DDAC when added to media containing silica gel. Table 5.7.2.1 shows the percentage distribution of DDAC distributed in solutions containing starch, silica gel, or starch and silica gel. The results confirmed that when particles of starch remained in the Vogel liquid media, they absorbed a very high proportion (88.5%) of the DDAC, leaving less than 11.5% in the solution.

This phenomenon can be explained by the highly sorptive nature of QACs. They are very strongly bound on to surfaces such as glass, soil, sewage, humus and proteins (Lawrence, 1970, Swisher, 1987, MacQuillen, 1950, Barbaro and Hunter, 1965). The adsorption of organic cationic surfactants such as QACs to starch and silica gel, occurs mainly by electrostatic attraction, although Van der Waals forces also exert an influence for QACs that have long alkyl chains (Theng, 1974). With the removal of most of the QAC from the media by the starch particles, the fungi are able to metabolize the small amount of DDAC remaining in the aqueous solution. Consequently the fungi could grow in liquid media containing large amounts (e.g. 250 ppm) of DDAC.

Different sources of starch may have quite different solubility in water. This will lead to wide variations in the amount of DDAC present in the solution. When the solubility of starch is high, (as in the latter experiment), the DDAC concentration available to fungi was also high. This may inhibit fungal growth. The simulation of this effect by the addition of silica gel (which has large surface area) allowed *V. bulbillosum* and *Acremonium sp.* to grow very well. The resulting biomass is shown in Table 5.7.2.2.

Table 5.7.2.1 Distribution of DDAC in starch, silica gel, starch and silica gel and aqueous solution

Media	Relative percent of DDAC on soilds and in solution (%)				Percent recovery based up upon the amount added (%)
	Starch	Silica gel	Starch and silica gel	Aqueous solution	
Vogel liquid media	88.5 ± 0.5			11.5 ± 0.0	74.5 ± 0.5
Vogel liquid media without starch but with silica gel		94 ± 2.1		6 ± 2.1	96.0 ± 5.0
Vogel liquid media with silica gel			97 ± 1.0	3 ± 1.0	87.5 ± 0.5

Table 5.7.2.2 The biomass of fungi grown in liquid media containing 250 ppm DDAC and silica gel

	Biomass (mg/ml)	
Fungi	Control	250 ppm
V. bulbillosum	7.6 ± 0.2	6.4 ± 0.2
Acremonium sp.	7.3 ± 0.2	7.0 ± 0.9

5.7.3 Residual amount of DDAC after fungal growth in liquid media with silica gel

Figures 5.4.3.1 and 5.4.3.2 show that DDAC disappeared after two week growth of *V. bulbillosum* and *Acremonium sp.* based on the HPLC analysis. From the chromatographs, the metabolites of DDAC appeared to be similar to those formed in the liquid media containing DDAC without silica gel. It can be concluded that the silica gel did not change the mechanism of fungal degradation of DDAC in liquid media. The results were consistent with the degradation of the DDAC with the formation of QACs having shorter alkyl chain QACs. It is also possible that oxidation of the terminal carbon occurred producing either alcohol, aldehyde or carboxylic acid.

5.8 Attempts to fractionate the protein produced by fungi growing in liquid media with and without DDAC

Experiments involving fungal growth on/in amended media have demonstrated a loss of DDAC based upon the reduction in the peak intensity in the HPLC spectra. The presence of several metabolites has also been suggested from the number of peaks in the chromatogram. To better understand the mechanism of fungal action, an attempt was made to fractionate the protein responsible for the degradation process.

5.8.1 Extracellular protein pattern

Fig. 5.8.1.1 shows on SDS-PAGE profile of the extracellular protein produced by *V. bulbillosum* and *Acremonium sp.* derived from liquid media containing 0 ppm and 250 ppm DDAC. The protein pattern for the control differed from that of the liquid media containing DDAC. The latter contained two extra protein bands. The molecular weight of one band is at approximately 20-30 kDa and while that of the other band is slightly higher than 94 kDa. The corresponding IEF gel for the extracellular protein from media containing DDAC (Fig. 5.8.1.2) exhibited a few extra protein bands. The pI (isoelectric point) of the extra bands was between 4.55 to 5.20. It is possible that these proteins are the enzymes responsible for the degradation of DDAC. Another possibility is that they may come from the fungal cell wall or cell contents. It has been reported that the surfactant nature of DDAC leads to leakage of the cell contents due to alteration of the cell membrane structure.

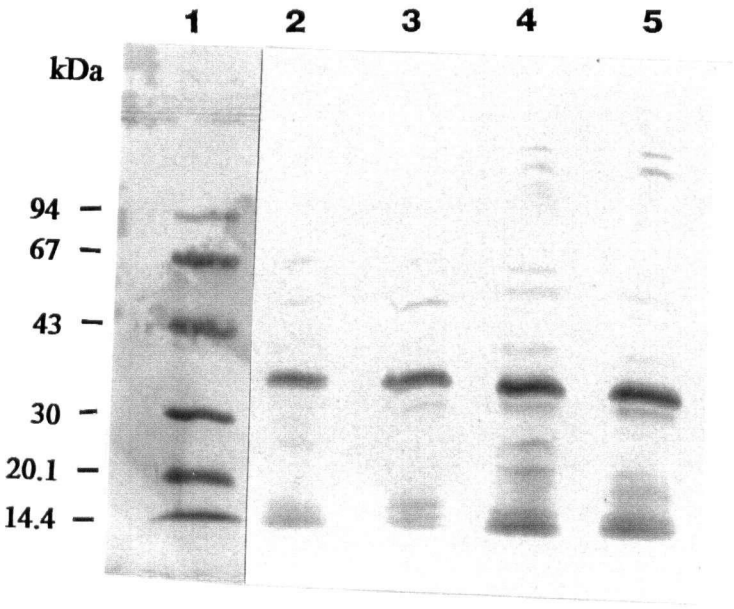

Fig.5.8.1.1 Silver stained SDS-PAGE phast gel (homogenious 12.5%) of extracellular proteins from culture of *V. bulbillosum* and *Acremonium sp.* containing 250 ppm DDAC and 0 ppm DDAC

lane 1: low molecular weight standard

lane 2: 2 µg protein from *Acremonium sp* containing 250 ppm DDAC

lane 3: 2 µg protein from *Acremonium sp* containing 0 ppm DDAC

lane 4: 2 µg protein from *V. bulbillosum* containing 250 ppm DDAC

lane 5: 2 µg protein from *V. bulbillosum* containing 0 ppm DDAC

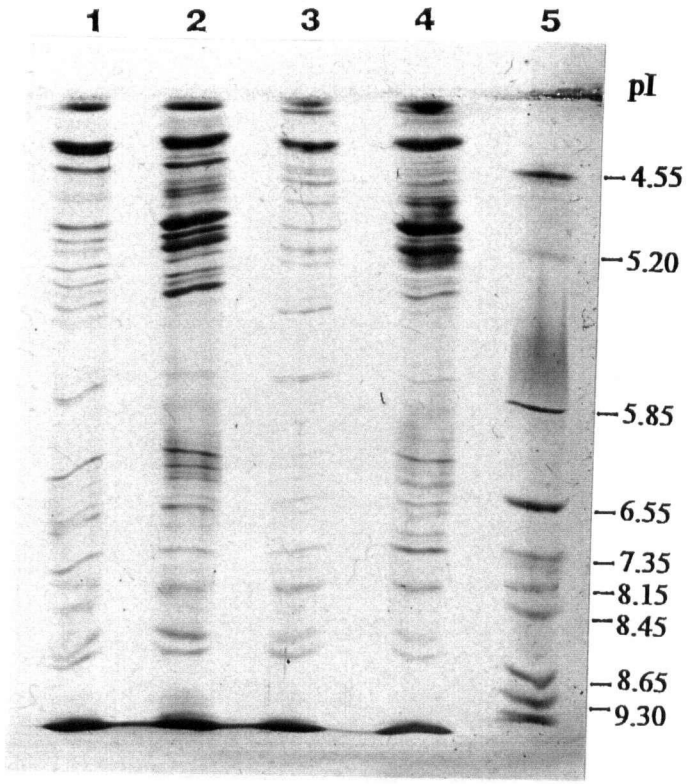

Fig. 5.8.1.2 Silver staining IEF gel (pI 3-9) of extracellular protein from culture supernatants of *V. bulbillosum* and *Acremonium sp.* containing 0 ppm DDAC and 250 ppm DDAC. Pharmacia IEF standards are shown in lane 5. Protein were loaded at approximately 2 μg per lane.
lane 1: protein from culture of. *V. bulbillosum* containing 0 ppm DDAC
lane 2: protein from culture of. *V. bulbillosum* containing 250 ppm DDAC
lane 3: protein from culture of. *Acremonium sp* containing 0 ppm DDAC
lane 4: protein from culture of *Acremonium sp.* containing 250 ppm DDAC

5.8.2 Intracellular protein pattern

From the SDS-PAGE of the intracellular protein shown in figure 5.8.2.1, there did not appear to be any difference in the protein patterns derived from the control media and the liquid media containing DDAC. This would suggest that the DDAC degradation possibly took place outside the fungal cell wall.

5.8.3 Decreased amount of DDAC after treatment with intracellular extracts and supernatants from cultures of *V. bulbillosum* containing 0 ppm and 250 ppm DDAC

The data in Table 5.8.3.1 shows that the amount of DDAC decreased after treatment with the supernatant from a liquid culture of *V. bulbillosum* containing 250 ppm DDAC, while the amount of DDAC was only slightly decreased after treatment with supernatant from culture of *V. bulbillosum* containing 0 ppm DDAC. Intracellular extracts from mycelia of *V. bulbillosum* which were grown in liquid media containing 0 ppm and 250 ppm DDAC also failed to reduce the DDAC content during incubation. This confirmed that DDAC degradation took place outside the fungal cell wall. However, it was not certain that the degradation of DDAC results from additional proteins shown on the SDS and IEF gels.

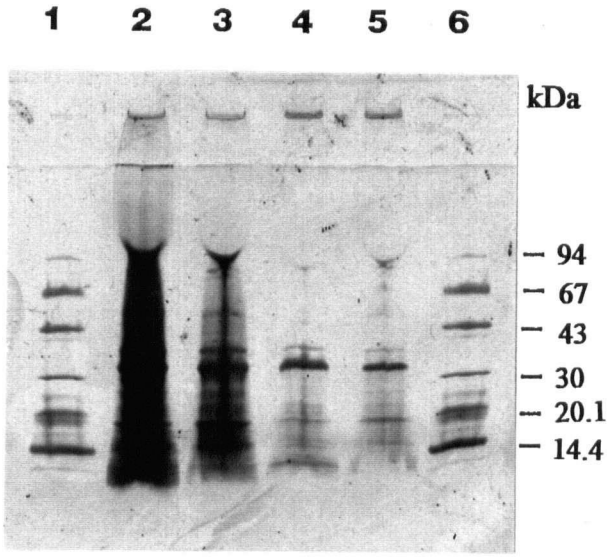

Fig.5.8.2.1 Silver stained SDS-PAGE phast gel (8-25%) of intracellular protein from culture of *V. bulbillosum* with 500 ppm DDAC and without DDAC
lane 1 and 6: low molecular weight standard
lane 2: 2μg protein from *V. bulbillosum* with DDAC
lane 3: 2μg protein from *V. bulbillosum* without DDAC
lane 4: 1μg protein from *V. bulbillosum* with DDAC
lane 5: 1μg protein from *V. bulbillosum* without DDAC

Table 5.8.3.1 Amount of DDAC in buffer after treatment with extracellular and intracellular supernatants from cultures containing 0 ppm DDAC and 250 ppm DDAC

Supernatant	Amount of DDAC (mg) (before treatment with supernatant)	Amount of DDAC (mg) (after treatment with supernatant)
a. Extracellular		
Containing 0 ppm DDAC	2.83	2.27 ± 0.03
Containing 250 ppm DDAC	2.83	0.84 ± 0.10
b.Intracellular		
Containing 0 ppm DDAC	0.18	0.22 ± 0.08
Containing 250 ppm DDAC	0.18	0.29 ± 0.06

5. CONCLUSIONS

Based upon all of the observation in this study, the following conclusions may be made.

(1) It was not feasible to use bromophenol blue or methylene orange colored complexes to monitor the changes in the DDAC concentration in liquid media.

(2) The tolerance and degrading capacity of the moulds examined in this study did not appear to indicate a special resistance in those strains isolated from DDAC treated wood. Further, it appeared to be a general property of this group of fungi.

(3) There was no relationship between the fungal growth on solid malt agar and corresponding growth on malt agar containing DDAC.

(4) There was no relationship between the tolerance of fungi to DDAC in solid media and that observed in liquid media.

(5) Only the fungi which degraded DDAC on solid media were able to grow in liquid media containing DDAC.

(6) Fungi produced additional extracellular protein when grown in liquid culture containing DDAC. Based on the examination of the extracellular and intracellular supernatant solutions, the DDAC degradation occurred outside the cell wall. GC-MS analysis of the DDAC containing liquid media following fungal growth, provided evidence for both C-N fission, and ω- and β- oxidation of a long alkyl chain, during fungal degradation. This may have arisen because of the presence of DDAC both adsorbed onto starch particles and dissolved in the liquid media.

6. RECOMMENDATIONS

(1) The role of other fungi such as Zygomycetes and Actinomycetes in limiting the performance of QACs as wood preservatives should be studied.

(3) It was found that the turbidity of the liquid media which caused by the variable solubility of the starch, affected the DDAC content in the solution. This may lead to the different mechanisms of the DDAC degradation. Further work should employ a carbon source which is totally soluble in water.

(4) Additional research is necessary to find out whether the tertiary amine exists in the media after fungal growth, to confirm the pathway of degradation of DDAC.

(5) It appears that the degradation of DDAC in solid media may be different from that in liquid media. Since the study focused on how the fungi reduced the ability of QACs to protect wood, more research need to be conducted on identifying the metabolites produced in DDAC amended solid media.

(6) A more intensive examination of the intracellular protein and extracellular protein is recommended. The current experiments suggested that intracellular protein can not degrade DDAC. The new proteins shown on the SDS and IEF gels should be purified and their ability to degrade DDAC should be confirmed.

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