

CHEMISTRY AND BIOLOGICAL ACTIVITY OF
SECONDARY METABOLITES FROM
Fusarium oxysporum

SUGATA ROY



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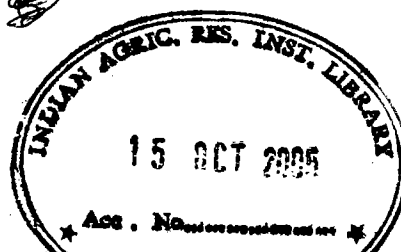


IARI

Division of Agricultural Chemicals
Indian Agricultural Research Institute
New Delhi – 110 012

2004

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**CHEMISTRY AND BIOLOGICAL ACTIVITY OF
SECONDARY METABOLITES FROM
*Fusarium oxysporum***

A Thesis

**By
SUGATA ROY**

**Submitted to the Faculty of the Post Graduate School,
Indian Agricultural Research Institute, New Delhi,
in partial fulfillment of the requirements
for the award of the degree of**

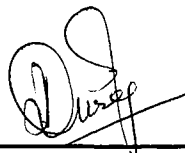
DOCTOR of PHILOSOPHY

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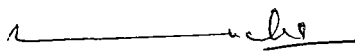
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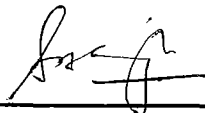
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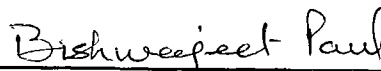
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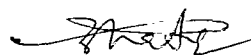
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This is to certify that the thesis entitled, "**Chemistry and biological activity of secondary metabolites from *Fusarium oxysporum***", submitted to the Faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfillment of the requirements for the award of the **degree of Doctor of Philosophy in Agricultural chemicals**, is a faithful record of *bona fide* research work carried out by **Mr. Sugata Roy** under my guidance and supervision and that no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of these investigations have been duly acknowledged by him.

Date: November 18th, 2004

Place: New Delhi



(Prem Dureja)
Chairperson,
Advisory Committee

DEDICATED

To Maa and Baba....

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Abstract

Biological control of insect pests has been a successful approach for decades. The biological control of plant diseases and weeds is not yet as well established in commercial agriculture, but is a rapidly developing area of research. The general mechanisms of biological control of plant diseases are competition, antibiosis, predation/parasitism and induced resistance. All known BCA's utilize one or more of these general mechanisms. Among the bio-control agents *F.oxysporum* has been reported to have high potentiality in controlling a number of soil borne diseases. The present study on chemistry and biological activity of the cultural filtrate of *F.oxysporum* was undertaken to isolate, identify and study antagonistic effect of the secondary metabolites against plant pathogens.

Based on this study it was found that the best media for mass culturing of non-pathogenic strain of *F. oxysporum*, production of secondary metabolites and inhibition against pathogenic fungi are ALA-II, PDA and SYP. Maximum fungicidal activity was exhibited by incubating *F.oxysporum* up to 15 days. *F.oxysporum* was raised on potato dextrose broth at $25 \pm 1^{\circ}\text{C}$ for 15 days. Culture filtrate separated from mycelia was extracted with dichloromethane and ethyl acetate. Similarly mycelia was dried and extracted with hexane, dichloromethane and methanol. After removal of solvents the residues were found active against pathogenic fungi namely *P.aphanidermatum* (ED_{50} 21.1 ppm) and *P.debaryanum* (29.9 ppm) and showed 99.8 percent mortality even after 48 and 72 hours of incubation against *R. reniformis* and *M. incognita*. Extracts also inhibited the germination of *Parthenium hysterphorus* seeds. Secondary metabolites such as dibutyl phthalate, bis(2-ethylhexyl)phthalate, sporol, sporotrichiol and sambucoid were identified from dichloromethane extract of culture filtrate and 8-hydroxytrichothecene, JM-47, nivalenol, fusarenon X and 5-butylpicolinic acid from ethyl acetate extract of culture filtrate were isolated and characterized on the basis on the spectroscopy. A number of hydrocarbons namely nonadecane, 1-tetradecanol, methyl hexadecanoate, 2-methyl pentadecane, and 4-hydroxy-4-methyl-2-pentane were identified by GC-MS in the hexane extract of mycelia along with decane, dodecane, tetradecane and nonadecane. Cyclobutane and carbonitrile, sporol, 6-butyl- α -pyrone, sambucoid, fufurigerin and moniliformin from dichloromethane and zearalenone, resorcylic acid lactone, T-2 toxin, diacetoxyscirpenol, visoltricin, 8-hydroxy-isotrichodermol, 8 β -hydroxy sambucoid, ergosterol, digitoxin, 2-dimethyl-5-amino-6-(2'-ene-4'-hydroxybutyl) -4-chromone, 8 α -hydroxysambucoid, enniatin, 2,2-dimethyl-5-amino-6-(4'-hydroxybutyl)-4-chromene and a small quantity of IAA have been identified from methanol extract of mycelia identified.

Maximum fungicidal activity was found with moniliformin against *P. aphanidermatum* (ED₅₀ 29.25, ppm), and *P. debaryanum* (ED₅₀ 78.00 ppm) followed by 6 -Butyl- α pyrone (ED₅₀ 37.2 and 34.9 ppm, respectively) and sporotrichiol (ED₅₀ 66.54 ppm against *P. aphanidermatum*) Fusaric acid and indole acetic acid showed plant growth regulating activity at lower concentrations, whereas isotrichodermin and nivalenol did not show growth promoting activity. Sambucoid was found most active with ED₅₀ 15.25 and 15.29 ppm after 24 h against *R. reniformis* and *M. incognita*. *F. oxysporum* and some of secondary metabolites were found compatible with captan, tridemorph, metalaxyl and thiophanate-methyl, but sensitive to mancozeb, sulphur dust and borax Dried mycelia based solid (dust) and pellet formulations significantly prevented tomato damping off. It allowed a stand comparable to that in the non-infested control. Derivatives of Fusaric and palmitic acid were synthesized and found active against *P. aphanidermatum* followed by *P. debaryanum*, *R. bataticola* and *S. rolfsii*

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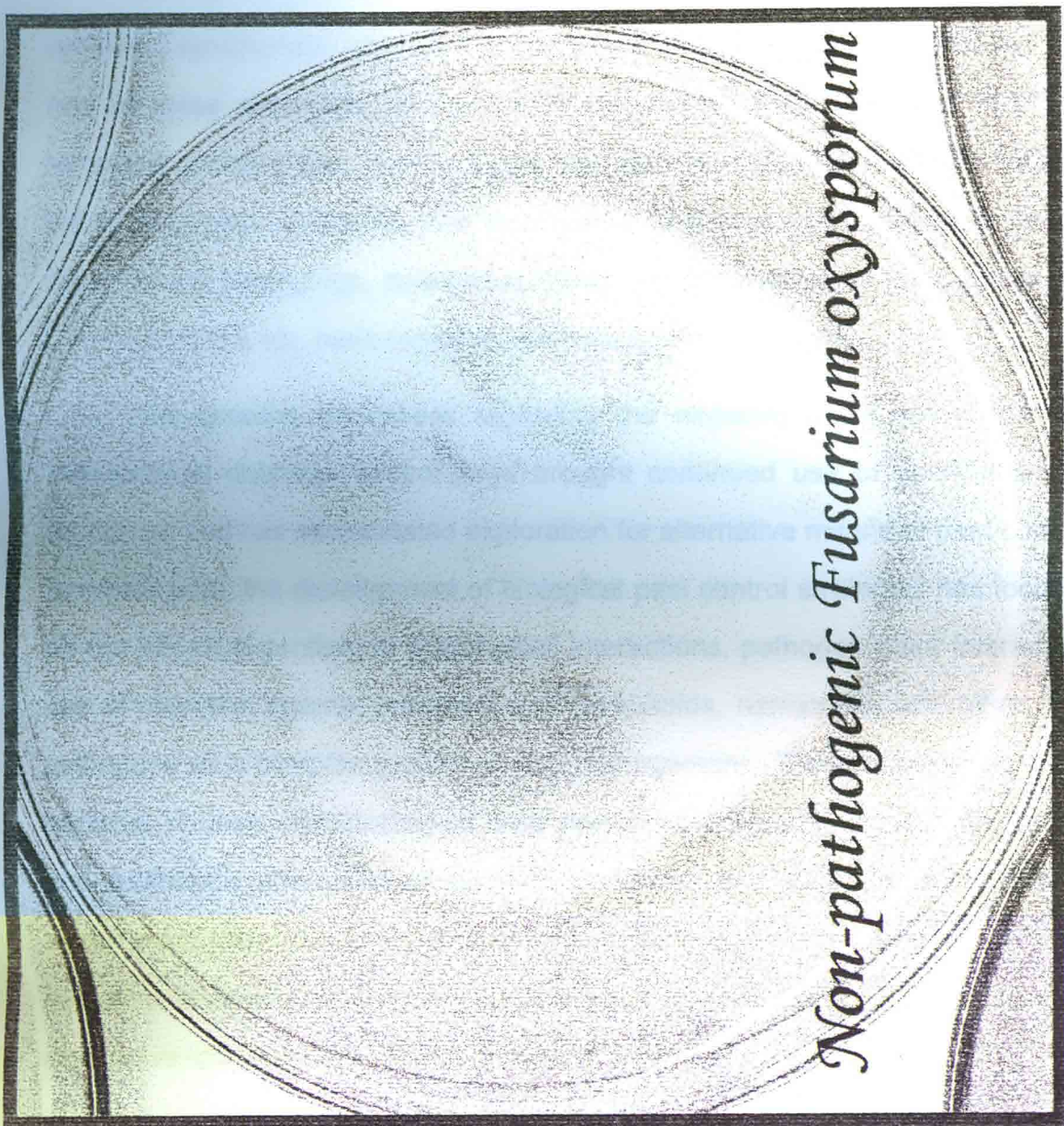
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A circular microscopic image showing a spore with several concentric rings. The outermost ring is dark and well-defined. Inside it, there are several lighter, more diffuse rings. The center of the spore is bright and appears to have some internal structure, though it is less distinct than the rings. The overall image has a grainy, high-magnification appearance typical of light microscopy.

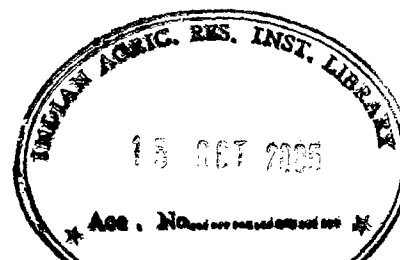
Non-pathogenic Fusarium oxysporum

1. Introduction

During the past five decades or so, the use of synthetic pesticides has remained synonymous with plant protection in agriculture and allied fields. The role of these chemicals as saviors of mammalian food, feed and fiber has remained unchallenged, even though, the various undesirable effects such as induction of pest resistance, pest resurgence, environmental pollution, hazard to man and his belongings, deleterious effects on the non-target flora, fauna and other organisms etc. have remained associated with their use.

The growing awareness regarding the negative traits associated with conventional chemical control have brought continued use of pesticides to a crossroad and has necessitated exploration for alternative means of pest control. In recent past, the development of biological pest control strategies has focused on the chemical profiles of insect plant interactions, pathogen plant interaction, use of microbial agents, predators and parasitoids, nematodes and other plant pathogens as a complex system of pest management. These agents (i.e. fungi, bacteria, viruses, nematodes) in crop protection programmes can offer a safe and ecofriendly alternative to synthetic pesticides and also offer an alternative where pests and diseases have developed resistance to these pesticides. Thus, this also implies that use of bio-pesticides is very promising due to their cumulative effect and ecofriendly nature.

Among the natural enemies of plant pathogens, antagonistic fungi have received the maximum attention. Several biofungicides and bionematicides,



containing fungi as the active agents are already in use in the United States and Europe, primarily against damping-off diseases of vegetables and ornamentals.

To date about 35 microbial products for biocontrol of plant pathogens are commercially available. Most of these products are for the control of soil borne diseases but other products aim at controlling pruning wounds, foliar diseases, post harvest diseases or diseases of woody plants.

Fusarium oxysporum is a fungus that lives in the soil. Most of the *F. oxysporum* in soil lives on dead organic matter (saprophytic strains). Some of the *F. oxysporum* in soil can cause disease (pathogenic strains), especially wilt disease in plants. Other *F. oxysporum* (biocontrol strains) can prevent disease caused by pathogenic strains. Saprophytic, pathogenic and biocontrol strains all look the same. The only way to tell them apart is to see how they behave. The only chemicals that can reduce losses due to *Fusarium* wilt disease are soil fumigants. These fumigants damage the environment. Thus, research is being conducted to see if biocontrol strains can be used to control *Fusarium* wilt. Therefore nonpathogenic strains are developed as biocontrol agents. The nonpathogenic *F. oxysporum* strains show several modes of action contributing to their biocontrol capacity. They are able to compete for nutrients in the soil, affecting the rate of chlamydospore germination of the pathogen. They can also compete for infection sites on the root, and can trigger plant defence reactions, inducing systemic resistance. These mechanisms are more or less important depending on the strain. The nonpathogenic *F. oxysporum* are easy to mass-produce and formulate, but application conditions for biocontrol efficacy under field conditions have still to be determined.

In recent years a lot of progress has been done in understanding the mechanisms involved in the antagonistic effect of these microorganisms, which can be due to any

combination of the four factors, competition for nutrition and space, parasitism, antibiosis and induced resistance. Recently new modes of action for antagonistic strains have also been discovered, including biosurfactant production and interference with enzyme production of pathogen.

Studies on biocontrol agents have revealed the presence of a diverse range of antifungal metabolites derived from amino acid, polyketones and isoprenoid biosynthetic pathways, each of which has distinctive regulatory points and is subjected to different stimuli within organism.

Among the biocontrol agents *Fusarium oxysporum* has been reported to have high potentiality in controlling a number of soil borne diseases and pathogenic strain of *F. oxysporum*. Some reports are also available about its nematocidal, insecticidal, herbicidal and plant growth regulatory potential. The exact mechanism of action of *Fusarium* spp. against plant pathogens is not well established. Most of the earlier reports on mode of action was related to, competition with the pathogen for nutrients and space, induced resistance and suppression of hydrolytic enzymes of pathogen as the main mechanisms along with others such as myco parasitism and antibiosis.

Few secondary metabolites have already been isolated and characterised from *Fusarium* spp., but not much work has been carried out on their pesticidal activity. In order to establish the role of secondary metabolites as biocontrol agents and to find novel chemical entities, which can serve as leads for chemical synthesis, the present study on "Chemistry and biological activity of secondary metabolites from *Fusarium oxysporum*." was undertaken with the following objectives:

- I. Isolation and identification of secondary metabolites from *Fusarium oxysporum*.
- II. Study of bioactivity of extracts and isolated secondary metabolites.

2. *Review of literature*

Biological control of plant disease is defined as "the involvement of the use of beneficial microorganisms, such as specialized fungi and bacteria, to attack and control plant pathogens and the diseases they cause. These "specialized" fungi and bacteria are microorganisms that normally inhabit most soils. In their native habitat they compete with other microorganisms for space and food and in some cases they produce toxic substances that parasitize and/or kill other soil-inhabiting microorganisms such as *Pythium* sp., *Phytophthora* sp., *Rhizoctonia* sp., and other plant pathogens.

Among the natural enemies, antagonistic fungi have received the maximum attention. Several antagonistic fungi have been established as useful biocontrol agent for the management of crop diseases.

About 35 different biocontrol products are available worldwide. These are based on *Trichoderma* sp. to protect against pruning wounds, soil borne diseases or leaf pathogens such as *Botrytis cinerea* Pers, *Bacillus subtilis* to control root or seed pathogens, *Burkholderia cepacia*, *Gliocladium virens* Miller and non-pathogenic *Fusarium* spp to control damping off diseases, *Agrobacterium tumefaciens* (using *Agrobacterium radiobacter* strains), *Pseudomonas tolaasi* and *Erwinia amylovora* and *Ralstonia solanacearum* (using non pathogenic *R. solanacearum* strains) to control bacterial plant pathogens, *Ampelomyces quisqualis*, *Pseudomonas*

Ampelomyces quisqualis, *Pseudomonas syringae* Van Hall and *Candida oleophila* to control post harvest diseases (Table 1).

2.1 Mechanism of Action of Biocontrol agents

There are four different mechanisms by which biocontrol agents control other microorganisms. Most biocontrol agents apply only one of these four mechanisms; however, some may employ more than one (Handelsman and Stabb, 1996; Thomashow and Weller, 1996; Goldman *et al.*, 1994).

2.1.1. Direct Competition with the Target Organism

The biocontrol agent out compete the target organisms for nutrients and space.

2.1.2. Antibiosis.

With antibiosis, the biocontrol agent produces a chemical compound such as an antibiotic or some type of toxin that kills or has some sort of detrimental effect on the target organism.

2.1. 3. Predation or Parasitism of the Target Organism.

The biocontrol agent can attack and feed directly on the target organism or the biocontrol agent can produce some sort of toxin that kills the target organism and then the biocontrol agent feeds on the dead target.

2.1. 4. Induced Resistance of the Host Plant.

It has been known for decades that once a plant is infected with a pathogen, that infection triggers some sort of reaction in the infected host plant that helps keep it from being infected with other pathogens. The infected plant becomes more "resistant" to other infections.

Additional mechanisms such as production of biosurfactants, interaction with pathogen related enzymes and undoubtedly a number of still unknown mechanisms, may complete the microbial arsenal.

2.1.5 Production of biosurfactants

Change of the leaf surface wettability by surface-active microorganisms is a common natural phenomenon. Such changes can affect leaf wetness duration, the distribution of nutrients and microorganisms and their attachment on leaves. Thus the duration of contact of pathogen on the plant surface is reduced and the attack is reduced.

2.1.6 Inference with enzyme production of pathogen

In this process, the presence of biocontrol agents inhibits the production of cutinolytic, pectinolytic and cellulolytic enzymes by pathogens, which are necessary for penetration through the cuticle and into the host tissue.

2.1.7 Primary and secondary metabolites

Secondary metabolites are compounds produced by organisms, which are not directly needed for their normal functioning. There are many theories why

The secondary metabolites can also be defined as those compounds that accumulate in the organism and to a large extent are metabolically inactive towards the organism that produces them (end-products). Strains able to make these compounds may lose, because of mutations, the capacity to synthesize them without any apparent immediate effect to the organism.

The discovery of penicillin, a secondary metabolite of *Penicillium notatum* West, in 1929 marked a milestone in the development of antibiotics. After this mycologists and microbiologists searched for further sources of antibiotics by investigating microbial antagonism and antibiosis.

In this review major emphasis will be given on the secondary metabolites isolated from *Fusarium* species and their bioactivity

2.2 *Fusarium oxysporum*

Fusarium oxysporum is a fungus that lives in the soil. Most of the *F. oxysporum* in soil lives on dead organic matter (saprophytic strains). Some of the *F. oxysporum* in soil can cause disease (pathogenic strains), especially wilt disease in plants. Other *F. oxysporum* (biocontrol strains) can prevent disease caused by pathogenic strains. Saprophytic, pathogenic and biocontrol strains all look the same. The only way to tell them apart is to see how they behave. The only chemicals that can reduce losses due to *Fusarium* wilt disease are soil fumigants. These fumigants damage the environment. Thus, research is being conducted to see if biocontrol strains can be used to control *Fusarium* wilt. Therefore nonpathogenic strains are developed as biocontrol agents. The

nonpathogenic *F. oxysporum* strains show several modes of action contributing to their biocontrol capacity. They are able to compete for nutrients in the soil, affecting the rate of chlamydospore germination of the pathogen. They can also compete for infection sites on the root, and can trigger plant defence reactions, inducing systemic resistance. These mechanisms are more or less important depending on the strain. The nonpathogenic *F. oxysporum* are easy to mass-produce and formulate, but application conditions for biocontrol efficacy under field conditions have still to be determined.

2.2.1 *Fusarium oxysporum* as a potential bioherbicide

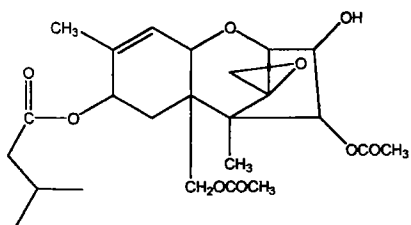
A strain of *Fusarium oxysporum* was isolated from infected sickle pod stems in Stoneville, MS, in 1989. When formulated as granules either in a fungus-infested rice preparation or encapsulated in a wheat-gluten matrix called 'Pesta', biocontrol of sickle pod, coffee senna, and hemp sesbania was achieved with pre-emergence or pre-plant-incorporated treatments. Liquid conidial applications were less effective. Plants were killed by a pre-emergence damping-off. Applications made post emergence were significantly less effective. Results of these tests suggest that this fungus has potential as a myco-herbicide for controlling sickle pod, coffee senna, and hemp sesbania (Wagner, 1993). Wagner, J. J. (1993). "Evaluation of *Fusarium oxysporum* as a potential bioherbicide for sicklepod (*Cassia obtusifolia*), coffee senna (*C. occidentalis*), and hemp sesbania (*Sesbania exaltata*).".

2.3 *Fusarium* toxins

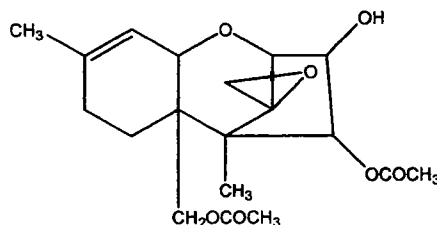
Fusarium has a wide habitat; principally as saprophyte, plant pathogen, entomopathogen, and sometimes parasite. So, as a natural defense strategy it produces a wide array of compounds.

2.3.1 Secondary metabolites from *Fusarium* species

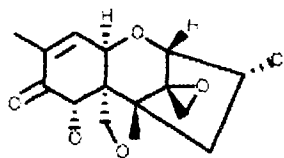
Bottalico, (1997) reported different *Fusarium* species in preharvest cereals of Europe and production of mycotoxins namely trichothecenes, including T-2 toxin (T2) (1) and its derivatives, diacetoxyscirpenol (DAS) (2), deoxynivalenol [vomitoxin] (DON) (3), fusarenone X (FUS) (4) and nivalenol (NIV) (5); zearalenone (ZEN) (6); and fumonisins (FB1) (7). Moniliformin (MON) (8), beauvericin (BEA) (9) and fusaproliferin (FUP) have been also reported from *Fusarium* infected cereal ears. Different toxigenic species of *Fusarium* identified are *F. moniliforme* [*Gibberella fujikuroi*], *F. proliferatum*, *F. subglutinans*, *F. avenaceum*, *F. graminearum* [*Gibberella zeae*], *F. culmorum*, *F. crookwellense*, *F. poae*, *F. sporotrichioides*, and *F. oxysporum*.



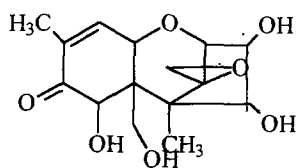
T-2 toxin (T2) (1)



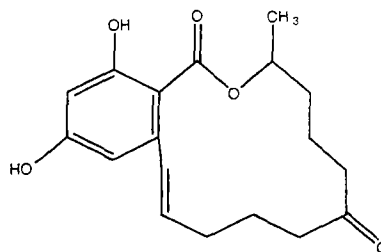
Diacetoxyscirpenol (2)



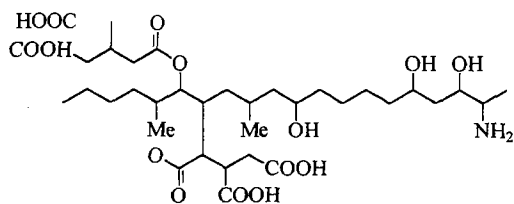
Vomitoxin (3)



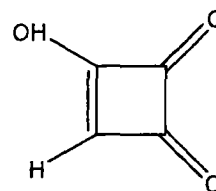
Nivalenol (5)



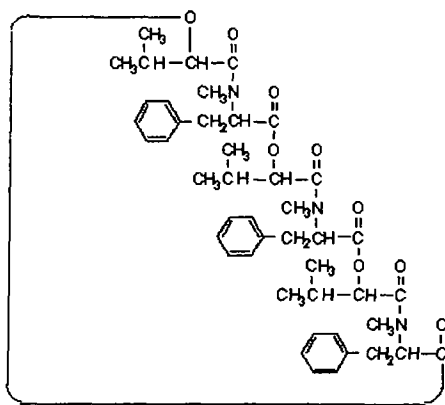
Zearalenone (6)



Fumonisin (FB1) (7)

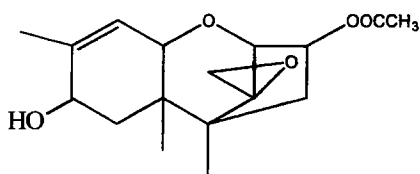


Moniliformin (MON) (8)

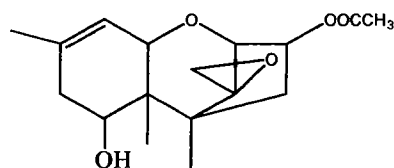


Beauvericin (BEA) (9)

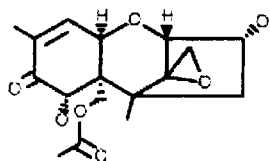
Lauren, (1988) screened a number of *Fusarium spp.* for their toxin producing potential and described isolation and taxonomy of 25 isolates of *Fusarium spp.* from a New Zealand farm pasture. The ability of these isolates to produce mycotoxins both in liquid culture and on rice grain culture has been assessed by a combination of chemical and analytical methods. The toxins reported include zearalenone, zearalenols, butenolide and various trichothecenes. An isolate of *F. culmorum* produced beta-zearalenol as the major toxin, whereas trichothecene production was greatest by an isolate of *F. crookwellense*. The major trichothecenes produced by this species are 4,15-diacetoxynivalenol followed by 8-hydroxy and 7-hydroxyisotrichodermin. Several previously unreported toxins have also been isolated. Known trichothecenes produced by other species are deoxynivalenol [vomitoxin], 15-acetoxydeoxynivalenol and 4,15-diacetoxyscirpenol.



8-hydroxy isotrichodermin



7-hydroxyisotrichodermin



15-acetoxydeoxynivalenol

A systematic study on the presence and concentration of pathogenic fungi and mycotoxins in maize has been reported by Munoz, (1990). The toxicity assays (*Artemia salina* and rabbit skin test) followed by chromatographic analysis showed the presence of zearalenone (0.7-9.9 ppm) and deoxynivalenol [vomitoxin] (43-315 ppb) in a significant number of samples. While aflatoxins, T-2 toxin and diacetoxyscirpenol have not been detected from any of the samples. Ten different fungus species were isolated from these toxic samples; eight of them were *Fusarium*, of which *F. moniliforme* [*Gibberella fujikuroi*] and *F. poae* were the most common. According to them, the storage procedure was found to influence greatly the development and nature of mycotoxins.

Hussein, *et al* (1991) isolated forty different *Fusarium* isolates from maize fields in New Zealand and screened for moniliformin production on maize kernels. Twelve isolates produced moniliformin at levels ranging from 0.4 to 64 ppm. Twenty-six isolates were also screened for production of deoxynivalenol [vomitoxin], diacetoxyscirpenol, T-2 toxin and zearalenone. Of these 22, including all 11 isolates of *F. graminearum* [*G. zeae*], produced zearalenone at levels ranging from 0.1 to 96.0 ppm, while 13 produced T-2 toxin at low levels (<1.1 ppm.). 6 isolates produced vomitoxin and diacetoxyscirpenol at low levels of <1.0 ppm. Three isolates of *G. zeae* and one isolate of *F. sambucinum* [*G. pulicaris*] produced 4 toxins simultaneously.

Greenhalgh *et al* (1990) reported production of secondary metabolites in trace amounts from large-scale fermentations of *F. sporotrichioides* DAOM 165006 namely 2 trichothecenes, 4-deacetoxy T-2 toxin and 4,8-isonesosolaniol, and 5

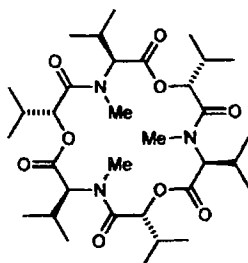
new 11-epiapotrichothecenes. 4-Deacetoxy T-2 toxin is unique from this species with oxygen function at C-4. All the 11-epiapotrichothecene toxins isolated comprise of an acetoxy moiety at C-2, and in addition some had oxygen functions at C-8 and C-15. The structures are interpreted in terms of the activity of the hydroxylases present in this fungal species. The absolute configuration of 13-hydroxy-3 α ,11-epoxyapotrichothec-9-ene was determined by X-ray crystallographic analysis.

Pettersson, (1991) reported production of a number of secondary metabolites from different *Fusarium* spp. Deoxynivalenol [vomitoxin], 3-acetyldeoxynivalenol and zearalenone were produced by most isolates of *F. graminearum* [*Gibberella zeae*] and *F. culmorum*. Whereas, zearalenone was produced by some isolates of *F. avenaceum* [*G. avenacea*], *F. poae* and *F. oxysporum*. Six of the ten isolates of *F. poae*, produced nivalenol.). The highest concentration of zearalenone (44.7 $\mu\text{g g}^{-1}$) was obtained when it was cultured on rice for 4 week at room temperature and under near UV-light. Fields infected with *F. poae* when treated with fungicides reduced the production of nivalenol concentration in the harvested grain. .

Hashmi *et al*, (1990) reported production of mycotoxins and naphthaquinone pigments from *F. moniliforme* [*Gibberella fujikuroi*], *F. [G. fujikuroi var.] subglutinans*, *F. semitectum* [*F. pallidoroseum*], *F. oxysporum*, *F. equiseti* and *F. solani* on *Capsicum*, *Coriandrum sativum* and *Trigonella foenum-graecum*. Mycotoxin namely equisetin was reported for the first time from several isolates of *F. pallidoroseum*. Secondary metabolites such as Zearalenone, zearalenol,

deoxynivalenol [vomitoxin], moniliformin, anhydrofusarubin, fusarubin and bostrycoidin were detected from *Gibberella fujikuroi*, *F. pallidroseum*, *F. equiseti* and *F. solani*. The study was based on the TLC profiles of secondary metabolites of different species and their growth response to tannin.

Fusarium spp. namely *F. anthophilum* [*Gibberella fujikuroi* var. *subglutinans*], *F. avenaceum* [*G. avenacea*], *F. crookwellense*, *F. culmorum*, *F. graminearum* [*G. zeae*], *F. nivale* [*Monographella nivalis*], *F. sambucinum* [*G. pulicaris*], *F. oxysporum*, *F. semitectum* [*F. pallidroseum*] and *F. tricinctum* were isolated and identified from New Zealand (Bosch *et al*, 1989) isolated. These isolates when grown on autoclaved rice and tested for toxicity to rats in feeding tests, total of 82% of the isolates showed toxicity, of which 24% were severely toxic and caused haemorrhages of stomach and intestine, haematuria and finally death. Cultures of the most toxic isolates contained toxins namely deoxynivalenol [vomitoxin] (0.1-104 ppm) and 15- and 3-acetyldeoxynivalenol (0.7 and 7 ppm respectively), fusarenon-X (0.2-4 ppm), zearalenone(11-1021 ppm), haemorrhagic factor [wortmannin] (40-272 ppm), moniliformin(2100-7200 ppm), cytotoxic factor [HM-8] (565 ppm) and enniatin in substantial concentration.



Enniatin (13)

Abbas *et al*, (1989) studied the production of trichothecene and non-trichothecene mycotoxins from *Fusarium* spp., isolated from infected maize samples. Eighty-two cultures of *Fusarium* spp. isolated from maize and were cultured on rice for 4 weeks and found to produce following mycotoxins deoxynivalenol [vomitoxin] (DON, 4-225 $\mu\text{g g}^{-1}$), 3-acetyldeoxynivalenol (3-ADON, 2-4 $\mu\text{g g}^{-1}$), 15-acetyldeoxynivalenol (15-ADON, 1-35 $\mu\text{g g}^{-1}$) and zearalenone (ZEA, 5-4350 $\mu\text{g g}^{-1}$), fusarin C (detectable amounts to 1000 $\mu\text{g g}^{-1}$) and moniliformin.

Park *et al*, (1991) reported the presence of the nivalenol, deoxynivalenol and T-2 toxin from Korean cereals infected with *Fusarium* spp. Samples of rice, barley, millet, maize and Indian millet were found to be contaminated with nivalenol (NIV), deoxynivalenol (DON) [vomitoxin] and T-2 toxin. Six samples were found to be contaminated with NIV and DON in the ranges of 189-624 and 168-506 $\mu\text{g kg}^{-1}$, respectively.

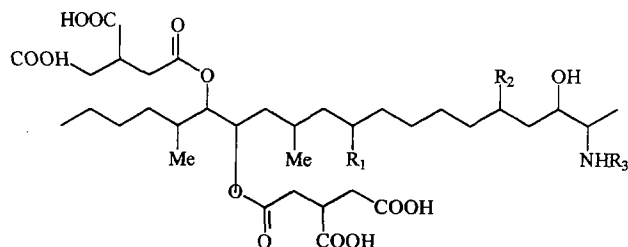
Muller *et al* (1993) reported the presence of *Fusarium* toxins namely, zearalenone (ZON), alpha- and beta-zearalenol (alpha,beta-ZOL), deoxynivalenol [vomitoxin] (DON), 3-acetyldeoxynivalenol (3-AcDON), nivalenol (NIV), T-2 toxin (T-2), HT-2 toxin (HT-2) and diacetoxyscirpenol (DAS) in wheat.

Srobarova *et al* (1994) reported the presence of fatty acids such as palmitic, stearic, oleic, linoleic and linolenic acids in wheat roots infected by *F. oxysporum*. Along with different fatty acids, other *Fusarium* spp produced toxins like zearalenone, deoxynivalenol [vomitoxin], diacetoxyscirpenol and T-2 toxin, both

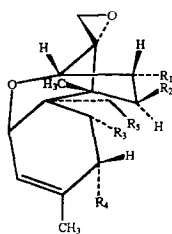
in vitro and *in vivo*, and mycotoxin production *in vivo* was reported more than *in vitro*.

The trichothecenes fraction produced by liquid culture of *F. crookwellense* DAOM 193611 was 4, 15- diacetoxynivelenol. Other secondary metabolites isolated were 7 and 8-hydroxy derivatives of isotrichodermin, 7,8-dihydroxyisotrichodermin, 8-keto isotrichodermin and 4,15-diacetoxy-7-deoxynivaenol. (Denis *et al*, 1987)

Table 2. Some Common *Fusarium* Fuminosins



FUMINOSIN	R ₁	R ₂	R ₃
Fuminosin B ₁	OH	OH	H
Fuminosin B ₂	H	OH	H
Fuminosin B ₃	OH	H	H
Fuminosin A ₁	OH	OH	COCH ₃
Fuminosin A ₂	H	OH	COCH ₃
Fuminosin C ₁	C ₁ - H replaces CH ₃		

Table 3. Some Common *Fusarium* Trichothecenes

NAME	R ₁	R ₂	R ₃	R ₄	R ₅
Scirpene	H	H	H	H	H
Trichodermol	H	OH	H	H	H
Trichodermin	H	OAc	H	H	H
4-Acetoxyscirpendiol	OH	OAc	H	H	H
15-Acetoxyscirpenediol	OH	H	H	H	OAc
Diacetoxyscirpenol	OH	OAc	H	H	OAc
Neosolaniol	OH	OAc	H	OH	OAc
HT-2 Toxin	OH	OH	H	Isovaleroxy	OAc
Nivalenol	OH	OH	OH	Oxo	OH
Fusarenon-X	OH	OAc	OH	Oxo	OH
Diacetyl nivalenol	OH	OAc	OH	Oxo	OAc
Triacetoxyscirpendiol	OH	OAc	OH	OAc	OAc
Verrucarol	H	OH	H	H	OH
Vomitoxin	OH	H	Oxo	OH	OH

Table 4. Some Common *Fusarium* Mycotoxins and the Species that Produce Them

Mycotoxin	Organism
Acetoxyscirpenediol	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
Acetyldeoxynivalenol	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>

Mycotoxin	Organism
Acetylneosalaniol	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> , <i>F. roseum</i> , and <i>F. nivale</i>
Acetyl T-2 toxin	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
Avenacein	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
Beauvericin	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> , <i>F. roseum</i> , and <i>F. nivale</i>
Butenolide	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
Calonectrin	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
Deacetylcalonectrin	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
Deoxynivalenol diacetate	<i>Fusarium moniliforme</i> and <i>F. nivale</i>
Deoxynivalenol monoacetate	<i>Fusarium moniliforme</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
Diacetoxyscirpenol	<i>Fusarium moniliforme</i> , <i>F. equiseti</i>
Enniatins	<i>Fusarium moniliforme</i> , <i>F. avenaceum</i> , <i>F. roseum</i> , <i>F. solani</i> and <i>F. nivale</i>
Fructigenin	<i>Fusarium moniliforme</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> and <i>F.roseum</i>
Fumonisin B ₁	<i>Fusarium moniliforme</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> and <i>F.nivale</i>
Fusaric acid	<i>Fusarium moniliforme</i>
Fusarin	<i>Fusarium moniliforme</i>

Mycotoxin	Organism
HT-2 toxin	<i>Fusarium moniliforme</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> and <i>F. nivale</i>
Ipomeanine	<i>Fusarium moniliforme</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> and <i>F. nivale</i>
Lateritin	<i>Fusarium moniliforme</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> and <i>F. nivale</i>
Lycomarasmin	<i>Fusarium moniliforme</i>
Moniliformin	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
Monoacetoxyscirpenol	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
Neosolaniol	<i>Fusarium moniliforme</i> , <i>F. solani</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> and <i>F. roseum</i>
Nivalenol	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
NT-1 toxin	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
NT-2 toxin	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. solani</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
Sambucynin	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. solani</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
Scirpentriol	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. oxysporum</i> , <i>F. culmorum</i> , <i>F. solani</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
T-1 toxin	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. culmorum</i> , <i>F. solani</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
T-2 toxin	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. culmorum</i> , <i>F. solani</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>

Mycotoxin	Organism
Triacetoxyscirpendiol	<i>Fusarium moniliforme</i> , <i>F. equiseti</i> , <i>F. avenaceum</i> , <i>F. roseum</i> and <i>F. nivale</i>
Yavanicin	<i>Fusarium culmorum</i> , <i>F. graminearum</i> , <i>F. oxysporum</i> , <i>F. roseum</i> , <i>F. moniliforme</i> , <i>F. avenaceum</i> , <i>F. equiseti</i> and <i>F. nivale</i>
Zearalenone	<i>Fusarium culmorum</i> , <i>F. graminearum</i> , <i>F. oxysporum</i> , <i>F. roseum</i> , <i>F. moniliforme</i> , <i>F. avenaceum</i> , <i>F. equiseti</i> and <i>F. nivale</i>

2.4 Effect of media and days of incubation on production of secondary metabolites by different *Fusarium* species

Morozumi *et al* (1988), evaluated a number of synthetic media for production of mycotoxins namely trichothecene by *Fusarium* spp. *F. sporotrichioides* str. F-135 when inoculated in a synthetic medium (SSA) containing: sucrose 200 g, asparagine 10 g, Ca-panthothenate 0.01 g, NaNO₃ 1 g, MgSO₄.7H₂O 1 g, KH₂PO₄ 0.75 g, ZnSO₄.7H₂O 0.1 g, FeSO₄.7H₂O 0.01 g, MnCl₂.4H₂O 0.001 g, CuSO₄.5H₂O 0.001 g, Co(NO₃)₂.6H₂O 0.0001 g, NaMoO₄.2H₂O 0.001 g, NiCl₂.6H₂O 0.001 g, Na₂B₄O₇ 0.001 g and distilled water 1000 ml, after 14 d of incubation at 25°C, produced diacetoxyscirpenol (420 µg ml⁻¹) and T-2 toxin (2265 µg ml⁻¹) along with a large amount of fusarenon-X, nivalenol, neosolaniol, butenolide and moniliformin. It was concluded that the SSA medium was found most suitable for production of trichothecenes and other mycotoxins by *F. spp.*

Lu *et al* (1990), standardized a liquid media for *F. graminearum* for production of deoxynivalenol. *F. graminearum* was grown at different

temperature, pH and for different time period and production of metabolite was monitored by HPLC. The optimum conditions standardized for liquid static and shake cultures were: temperature, $21.92 \pm 0.92^{\circ}\text{C}$ and $28.98 \pm 0.96^{\circ}\text{C}$ ($P=0.05$), pH, 7.50 ± 0.17 and 5.83 ± 0.17 ($P=0.05$), respectively. The study concluded that, carbon source affected growth and toxigenicity but light played no prominent role in growth and metabolite production profile.

Thrane *et al* (1988), screened yeast extract sucrose agar containing yeast extracts from 6 different manufacturers for the support of mycotoxin production. T-2 toxin and fusarin C production by *F. sporotrichioides*, zearalenone and fusarin C by *F. culmorum* and antibiotic Y by *F. avenaceum* [*Gibberella avenacea*] was tested. It was found that mycotoxin production was dependent on the yeast extract used.

Effect of media on the production of zearalenone, from *F. oxysporum* was studied by Paster (1991). He reported an increase in the concentration of sucrose and xylose in Czapek's medium resulted in increased toxin production, while no toxin was produced when lactose was used in the medium. Methionine (10^{-2} and 10^{-3} M) and cystine (10^{-3} M) added to Czapek's medium inhibited zearalenone production. When amino acids or nitrogen salts such as alanine, tryptophan and NH_4Cl were added as the sole source of nitrogen, zearalenone production was completely inhibited. Its production was also inhibited in high-moisture maize grains (27%) kept under conditions enriched with high CO_2 levels (60, 40 or 20%) with either 20% or 5% O_2 .

Optimal conditions for production of deoxynivalenol in liquid medium by isolates of *F. graminearum* were standardized by Lu *et al* (1991). Production of deoxynivalenol was affected by carbon source. Maximum growth occurred at pH 7.5, while toxigenicity was greatest at pH 5.8. Optimum incubation times for growth of *F. graminearum* were found to be 23 and 7 d for static and shake cultures, respectively, and 25 and 5 d for toxin production. Effect of light or dark condition no effect on growth or toxigenicity.

Production of zearalenone, moniliformin and trichothecenes in intact sugar beets under laboratory conditions were reported by Bosch *et al* (1992) for the first time. Five toxigenic isolates of *Fusarium* spp. (*F. moniliforme* var. *subglutinans* [*Gibberella fujikuroi* var. *subglutinans*], *F. poae*, *F. sporotrichioides*, *F. equiseti*, *F. graminearum* [*G. zeae*]) were tested for the production of zearalenone, moniliformin and trichothecenes (deoxynivalenol [vomitoxin], 15-acetyldeoxynivalenol, T-2 toxin, HT-2 toxin and neosolaniol) when grown on solid sugar beet slices in the laboratory for 30 days.

In a similar experiment, Badawey *et al* (1992), reported the influence of substrate (corn, wheat and rice) on zearalenone production by different *Fusarium* species. After 21 days of incubation, culture filtrate was extracted with ethyl acetate and zearalenone concentration was analysed by HPLC. *F. zeae*, *F. roseum* and *F. culmorum* all yielded the highest quantities of toxin on maize (220, 178 and 98 mg kg⁻¹, respectively), followed by rice (95, 83 and 72 mg kg⁻¹, respectively) and wheat (70, 35 and 14 mg kg⁻¹, respectively). The study

concluded maize as a substrate far better than rice or wheat grain for the production of zearalenone by all the three *Fusarium spp* studied.

2.5 Bioactivity of *Fusarium* toxins

2.5.1 Insecticidal activity

Insecticidal activity of secondary metabolites isolated from *F. semitectum* and *F. moniliforme* var. *subglutinans* have been reported by Gupta *et al* (1991). Methylene chloride extracts of mycelia of both fungi were toxic to *Leptinotarsa decemlineata*. Insecticidal activity was found to be due to presence of cyclodepsipeptide beauvericin. This was the first report of the isolation of beauvericin from fungi of the genus *Fusarium*.

The chronic and acute toxicity of fumonisin B1 (FB1), a mycotoxin from *F. moniliforme* [*Gibberella fujikuroi*], to the larvae of *Tenebrio molitor* was assessed by Abado *et al* (1998) by giving via the diet or injected directly into the larvae. Young *T. molitor* larvae when fed on a diet containing 450 µg FB₁ g⁻¹ exhibited reduced growth, rate of carbon dioxide production, food consumption and protein metabolism. The concentrations of FB1 in the diet did not increase mortality, even when tested at the highest dose of 450 µg FB1. Injection of 25 ng FB1 per larva decreased the CO₂ production after 11 days of the injection and was reversible with time. A parallel analysis of the retention of FB1 by the larvae indicated that about 40% of the ingested FB₁ was excreted in the faeces.

2.5.2 Nematicidal activity

Secondary metabolites produced by *Fusarium spp.* and other soil fungi were evaluated against plant parasitic nematodes by Ciancio (1995). Nematotoxicity was evaluated *in vitro* using at 0.02, 0.2, 2, 20, and 200 ppm concentrations in 4% methanol. T2-toxin, moniliformin, verrucarins A and B and cytochalasin B significantly reduced the viability of *Meloidogyne javanica* juveniles at 2 ppm or higher concentrations after 72 h of exposure at 22.5°C. Enniatin B showed a significant increase in *M. javanica* mortality at 20 ppm. T2-toxin also showed a nematicidal effect on *M. hapla* and *Pratylenchus neglectus* at 0.2 and 2 ppm, respectively. When tested in soil against *M. javanica* parasitizing tomato plants, T2-toxin and moniliformin did not affect nematode density. In the treated pots a larger number of galls per g of root was also observed. Data suggest that mycotoxins can interact with nematodes through antagonistic or synergistic effects related to the concentration levels present in the plant rhizosphere.

2.5.2 Plant growth regulatory activity

Lui (1991) reported the growth hormone-like action of deoxynivalenol toxin on induction and differentiation of wheat calluses. 10^{-6} - 10^{-3} M pure or crude deoxynivalenol antagonized the callus-inducing effects of 2,4-D and promoted root differentiation. The lowest concentration promoted plantlet regeneration but

a concentration of 10^{-2} M inhibited callus differentiation and growth of regenerated plantlets.

Effect of T-2 toxin on seed germination and seedling growth of mung (*Vigna radiata* L) was reported by Bilgrami *et al* (1991). It was applied at eight different concentrations ranging from 500–4000ppb and its effect on seed germination and seedling growth (root and shoot lengths) was observed. Seed germination was inhibited at all tested concentrations of toxin, whereas seedling growth was significantly reduced ($P < 0.02$) at > 1000 ppb.

Root growth of maize embryos was reduced by moniliformin Shi *et al* (1994). The study proved Richard's medium as the best of 3 medium tested for mycelium and moniliformin production. No differences were observed either in mycelium and toxin production, or in the capacity to reduce root growth, among 5 isolates of the pathogen tested. Mycelial dry weight increased with temperature over the range 15–30°C, but toxin production was optimum at 20°. There was no positive correlation between dry wt and amount of toxin. All the five isolates of moniliformin producers produced the same amount of toxin and affected the root growth at as low as 250 ppm concentration.

The effects of dihydrofusarubin produced by *F. solani* on tobacco leaves and on spinach chloroplasts was reported by Albrecht *et al* (1998). The effect of dihydrofusarubin was compared with herbicide methyl viologen. Toxin treatment of tobacco leaves and leaf discs resulted in a light dependent chlorophyll degradation and simultaneous ethane production. Experiments with spinach chloroplasts showed that dihydrofusarubin interacted with the electron transport

chain, thus forming reactive oxygen species. The effects of the dihydrofusarubin on chloroplasts were found to be comparable with the effects of methyl viologen.

Ravikumar *et al* (2002), evaluated the effect of fusaric acid on *in vitro* pollen germination and tube growth in chickpea. A series of pollen germination medium (PGM) containing 0, 50, 100, 150 and 200 μg fusaric acid ml^{-1} were prepared and tested on resistant chickpea genotypes, WR 315 and K 850. In the absence of fusaric acid in PGM, there was 80% pollen germination of both genotypes with prominent pollen tube, whereas decrease in pollen germination and tube growth was observed in presence of fusaric acid in PGM. No drastic reduction in germination with 50 μg fusaric acid ml^{-1} was observed but increase in fusaric acid concentration, drastically reduced pollen germination.

2.5.4 Fungicidal activity

reported the Antifungal effect of fumonisin B1, a secondary metabolite isolated from a non-pathogenic strain of *F. poae* on different fungi like *F. verticillioides*, *F. proliferatum*, *F. globosum*, *F. subglutinans* [*Gibberella fujikuroi* var. *subglutinans*], *F. graminearum* [*G. zeae*], *Penicillium expansum*, *Aspergillus flavus*, *Alternaria alternata* and *Botrytis cinerea*, at 40-0.05 mM (Keyser *et al* 1999). Fumonisin B1 inhibited mycelial growth of 5 of the 9 fungi tested. The minimum inhibitory concentration of FB1 ranged from 0.25-0.5 mM for *A. alternata*, 1-5 mM for *P. expansum* and *B. cinerea*, and 5-10 mM for *G. zeae*, whereas the other fungi tested showed no activity. A small inhibition zone was observed with *F. proliferatum*, a FB1-producing species, at 40 mM. The mycelial

growth of the 2 other FB1-producing species, *F. verticillioides* and *F. globosum* was not affected by the toxin.

2.5.5 Herbicidal activity

The Herbicidal potential of *F. moniliforme* and fumonisin under laboratory condition has been evaluated by Abbas *et al* (1991). The study revealed that application of aqueous suspensions of spores or mycelia ($>1 \times 10^7$ propagules ml^{-1}) of *F. moniliforme* [*Gibberella fujikuroi*] str. JW 1, grown on potato-dextrose agar, to the potted soil in which 2- to 4-week-old jimsonweed plants (*Datura stramonium*) were grown caused local lesions or mosaic-like patterns on the leaves and inhibited its growth. Application of lower concentration or dose to the leaves produced no visual effects. When the fungus was grown on autoclaved rice medium and the mixture was applied to jimsonweed, the plants wilted within 24 h of application and died after 48 h. Crude and cell-free filtrates of the fungal-rice culture also killed 1- to 4-week-old plants after 24-72 h. The toxin responsible for herbicidal activity was identified as fumonisin B1, isolated from fermented rice at $400 \mu\text{g g}^{-1}$. Fumonisin B1, applied in water at $2.5 \mu\text{g } 100\mu\text{l}^{-1}$ to excised jimsonweed leaves, caused similar symptoms as the cell-free extract and the crude culture filtrates, i.e. soft rot that diffused along leaf veins within 24 h. Similar damage occurred to intact plants treated with crude or cell-free filtrates or purified aqueous fumonisin B1 solutions.

reported the Herbicidal property of secondary metabolites produced by a strain of *F. oxysporum* against *Striga hermonthica* was reported by Savard *et al*

(1997). The extracts showed a promising herbicidal potential. Culturing of the fungus under conditions similar to those that would be used in the field produced no detectable toxins of concern for human health. It was observed that fusaric acid and its dehydro analogue have promising herbicidal activity along with satisfactory safety.

Similar report on biocontrol potential of *Fusarium* toxin as mycoherbicide was reported by Zonno *et al* (1999). They assayed fourteen fungal toxins *in vitro* to evaluate their effects on *S. hermonthica* seed germination. The T-2 toxin from *Fusarium spp.* was found to be most active, inhibiting 100% seed germination at 10^{-5} M concentration. Deoxynivalenol [vomitoxin], produced by *Fusarium sp.*, was also very active, causing 100% and 69% reduction in germination when assayed at 10^{-4} and 10^{-5} M, respectively. The high activity shown by these fungal toxins suggest that they may have potential as more natural and safe herbicides to suppress *S. hermonthica* seed germination.

Sugimoto *et al* (2002) reported some of the trichothecene produced by *F. solani* inhibitors of *Striga hermonthica* germination. The active principles were identified as trichothecenes acuminatin, neosolaniol, 8-acetylneosolaniol, and tetraacetoxyl T-2 tetraol (neosolaniol diacetate) on the basis of their chromatographic behavior and nuclear magnetic resonance and mass spectra. Inhibitory activity of the four trichothecenes against germination of *S. hermonthica* increased with acetylation of the hydroxyl moieties. 8-acetylneosolaniol produced by *F. solani* completely inhibited germination of *S. hermonthica* at a concentration of 24 μ M. The toxin did not affect the germination

of sorghum, a host crop, but retarded root and shoot elongation of the seedlings by 60 and 30%, respectively, at the same concentration. Thus, the study clearly indicated that, tricothecenes isolated from *F. spp.* can selectively control *Striga* without affecting the crop plant.

Oleskevich *et al* (1998) evaluated *F. avenaceum* for the control of invasive *Rubus species*. Fungus was isolated from naturally infected *R. strigosus*, *R. parviflorus* and *R. spectabilis* plants. Three endemic fungi, *F. avenaceum* [*Gibberella avenacea*], *Colletotrichum dematium* and a *Phomopsis sp.* were selected for further study after they were found to induce >50% leaf area necrosis when inoculated onto detached *Rubus* leaves using *in vitro* pathogenicity tests. Foliar infection was increased significantly when inoculum of *F. avenacea* was grown on a rice-grain substrate and applied in combination with an organosilicone surfactant (0.4% Silwet L-77 R) to *R. strigosus* and *R. parviflorus* plants. Extraction and analysis of infected rice filtrates for metabolite production showed that a single toxin, moniliformin, was present at levels of 3300 ppm.

Inhibition of germination of *Orobancha ramosa* seeds by *Fusarium* toxins was reported by Zonno, M. C. *et al*(2002). Eighteen toxins produced by *Fusarium* species were tested at different concentrations on *O. ramosa* seeds to evaluate their effectiveness in inhibiting seed germination. Many of the toxins were active at the highest concentration used. Seven of them: fusarenon-X, nivalenol, deoxynivalenol, T-2 toxin, HT-2 toxin, diacetoxyscirpenol and neosolaniol, were highly active at 10 μ M, causing 100% inhibition of germination. Many of them

were still active when assayed at a concentration ten times lower, with T-2, HT-2, nivalenol, neosolaniol and diacetoxyscirpenol still able to cause total inhibition. Diacetoxyscirpenol was very active also at 0.1 μ M, causing more than 90% inhibition. The results show that the use of toxic secondary metabolites could represent a useful alternative strategy in the management of parasitic weeds, by interfering with the induced germination process, and that fungal culture extracts could be an interesting source of new compounds acting as natural and original herbicides.

2.5.6 Antiprotozoan activity

Nishie *et al* (1989) studied the inhibitory effects of secondary metabolites of *Fusarium*, *Trichothecium*, *Myrothecium* and *Penicillium* on the protozoan *T. pyriformis*. The order of toxicity according to the ID₅₀ values reported were: T-2 toxin > trichothecin > 4,15-diacetylverrucarol > patulin > trichothecolone > verrucarol > zearalenone > PR-toxin > 3 α -acetyldiacetoxyscirpenol > zearalenol > griseofulvin > acetyl T-2 > iso T-2 > T-2 triol > scirpentriol > rubratoxin B > T-2 tetraol > moniliformin. In analogous pairs of trichothecenes their toxicities depended upon the substituents of the molecules. Among trichothecenes (without ester groups) with H and OH substituents, the toxicity was inversely related to the number of OH groups in the molecule, verrucarol (2 OHs) > scirpentriol (3 OHs) > T-2 tetraol (4 OHs). Zearalenone showed approx. 3-fold more toxicity than its analogue zearalenol.

Table 1. Commercial Biocontrol products and their Uses

Product/ Biocontrol organism	Target pathogen/ Disease	Crop	Formulation	Application Method	Manufacturer
AQ10 Biofungicide <i>Amelomyces quisqualis</i> isolate M-10	Powdery mildew	apples, cucurbits, grapes, ornamentals strawberries, tomatoes	water-dispersible granule	spray	Ecogen, Inc.
Bio-Fungus <i>Trichoderma</i> spp.	<i>Sclerotium, Phytophthora, Rhizoctonia solani, Pythium</i> spp., <i>Fusarium, Verticillium</i>	flowers, strawberries trees, vegetables	granular, wettable powder; sticks and crumbles	applied after fumi- gation; incorporated in soil; sprayed or injected	Grondortsmettingen De Cuester n.v.
Aspire <i>Candida oleophila</i> I-182	<i>Botrytis</i> spp. <i>Penicillium</i> spp.	citrus, pome fruit	wettable powder	post harvest application to fruit as drench, drip or spray	Ecogen, Inc.
Binab T <i>Trichoderma harzianum</i> (ATCC 20476) and <i>Trichoderma polysporum</i> (ATCC 20475)	Pathogenic fungi that cause wilt, take-all, root rot, and internal decay of wood products and decay in tree wounds	flowers, fruits, ornamentals, turf and vegetables	wettable powder and pellets	spray, mixing with potting substrate, mixing with water and painting on tree wounds, inserting pellets in holes drilled in wood	Bio-Innovation AB
Biofox C <i>Fusarium oxysporum</i> (nonpathogenic)	<i>Fusarium oxysporum</i> <i>Fusarium moniliforme</i>	basil, carnation, cyclamen, tomato	dust or alginate granule	seed treatment or soil incorporation	S.I.A.P.A.
Bio-save100, Bio-save1000 <i>Pseudomonas syringae</i> ESC-10	<i>Botrytis cinerea, Penicillium</i> spp. <i>Mucor pyroformis</i> <i>Geotrichum candidum</i>	pome fruit (Bio-save 100) and citrus (Bio- save 1000)	frozen cell concentrated pellets	pellets added to water to produce liquid suspension, post-harvet application	Eco Science Corp.

Bio-sve 110 <i>Pseudomonas syringae</i> ESC-11	<i>Botrytis cinerea</i> , <i>Penicillium</i> spp., <i>Mucor Pyroformis</i> <i>Geotrichum candidum</i>	pome fruit	frozen cell concentrated pellets	to fruit as drench dip or spray
Blight Ban A 506 <i>Pseudomonas fluorescens</i> A506	Frost damage, <i>Erwinia amylovora</i> , and russet-inducing bacteria	almond, apple, apricot, blueberry, cherry, peach pear, potato, strawberry tomato	wettable powder	pellets added to water to produce liquid suspension, post-harvest application to fruit as drench dip or spray
Cedomon <i>Pseudomonas chlororaphis</i>	leaf stripe, net blotch spot blotch, <i>Fusarium</i> sp.	barley, oat, wheat	seed treatment	bloom time spray of the flower and fruit
Companion <i>Bacillus subtilis</i> GB03	<i>Rhizoctonia</i> , <i>Pythium</i> , <i>Fusarium</i> , <i>Phytophthora</i>	horticultural crops, turf	liquid	Plant Health Technologies
Conquer <i>Pseudomonas fluorescens</i> Contans <i>Coniothyrium</i> Biologischer <i>minitans</i> GmbH	<i>Pseudomonas tolaassii</i> <i>Sclerotium sclerotiorum</i> and <i>S. minor</i>	mushrooms Canola, sunflower, peanut, soybean and vegetables (lettuce, bean, tomato)	wettable powder water dispersible granule	seed dressing drenching, spray
Deny/Blue Circle <i>Burkholderia cepacia</i> Products (<i>Pseudomonas cepacia</i>) type Wisconsin	<i>Rhizoctonia</i> , <i>Pythium</i> <i>Fusarium</i> and disease caused by lesion, spiral lance, and sting nematodes	alfalfa, barley, beans clover, cotton, peas, grain sorghum, vegetable crops and	peat-based dried biomass from solid fermentation; aqueous suspension	Microbial Stine applied to seeds with a sticking agent in planter box (aqueous suspension formulation)
				Prophyta Pflanzenschutz
				Mauri Foods

Epic <i>Bacillus subtilis</i>	<i>Rhizoctonia solani</i> , <i>Fusarium</i> spp. <i>Alternaria</i> spp. and <i>Aspergillus</i> spp. that attack roots	cotton, legumes	dry powder	added to a slurry; mix with a chemical fungicide for commercial seed treatment	Gustafson, Inc.	is for use in drip irrigation or as a seedling drench)	wheat
Fusalclean <i>Fusarium oxysporum</i> Protection (nonpathogenic)	<i>Fusarium oxysporum</i>	asparagus, basil, carnation, cyclamen, gerbera, tomato	spores, microgranule	in drip to rock wool; incorporate in potting mix; in row	Natural		
Galltrol-A <i>Agrobacterium radiobacter</i> Strain 84	Crown gall disease caused by <i>Agrobacterium tumefaciens</i>	fruit, nut, and ornamental nursery stock	petri plates with pure culture grown on agar	bacterial mass from one plate transferred to one gallon of nonchlorinated water suspension applied to seeds, seedlings, cuttings roots, stems, and as soil drench	Ag Bio Chem, Inc.		
Intercept <i>Pseudomonas</i> Corp <i>cepacia</i>	<i>Rhizoctonia solai</i> , <i>Fusarium</i> spp., <i>Pythium</i> sp.	maize, vegetables, cotton	powder	applied to seed with sticker, soil mixing	Soil Technologies		
Kalisena <i>Aspergillus niger</i> , strain AN-27	soil borne diseases	vegetable, cereals etc.	powder	Dusting, soil mixing	Cadilla		
Kodiak, Kodiak HB Kodiak AT <i>Bacillus subtilis</i>	<i>Rhizoctonia solani</i> <i>Fusarium</i> spp. <i>Alternaria</i> spp. and <i>Aspergillus</i> spp.	Cotton, legumes	dry powder, usually applied with chemical fungicides	added to a slurry mix for seed treatment; hopper box treatment	Gustafson, Inc.		

that attack roots						
Koni <i>Coniothyrium minitans</i>	<i>Sclerotium sclerotiorum</i> and <i>S. minor</i>	greenhouse crops	granule	granules incorporated into soil or soilless mix	Bioved, Ltd.	
Mycostop <i>Streptomyces griseoviridis</i> strain K61	<i>Fusarium</i> spp., <i>Alternaria brassicola</i> , <i>Phomopsis</i> spp. <i>Botrytis</i> spp. <i>Pythium</i> spp. <i>Phytophthora</i> spp. that cause seed, root and stem rot and wilt disease	ornamental and vegetable crops	powder	drench, spray or through irrigation system	Kemira Agro Oy.	
Nogall, Diegall <i>Agrobacterium radiobacter</i>	<i>Agrobacterium tumefaciens</i>	trees	washed plates; culture suspensions	root dips	Bio-Care Technology Pvt. Ltd.	
Norbac 84C <i>Agrobacterium</i> Inc.	Crown gall disease caused by <i>Agrobacterium tumefaciens</i>	fruit, nut and ornamental nursery stock	aqueous suspension	root stem, cutting	New Bio Products	
<i>radiobacter</i> strain K84			containing bacterial cells, methyl cellulose and phosphate buffer (refrigerate)	dip or spray		
Paecil <i>Paecilomyces lilacinus</i>	Various nematode spp.	banana, tomato, sugarcane, pineapple, citrus, wheat etc.	dry spore concentrate	seedling or soil drench	Technological Innovation Corpn. Pvt. Ltd.	
Protus WG <i>Talaromyces flavus</i> V117b	<i>Rhizoctonia solani</i> <i>Verticillium</i> sp.	tomato, cucumber strawberry, rape oil seed	water dispersable powder containing ascospores	seed treatment, soil drench	Prophyta Biologischer Pflanzenschutz Gmb. H.	
Phagus <i>Bacteriophage</i> Protection	<i>Pseudomonas tolaasii</i>	<i>Agaricus</i> spp. <i>Pleurotus</i> spp.	bacterial suspension	root dips, spray	Natural	Plant

Polygandron <i>Pythium oligandrum</i> product-	<i>Pythium ulimum</i>	sugarbeet	granule or powder	seed treatment or soil incorporation	Vyskumny ustav rastinnej [platn tion Institute], Bratislavsk cesta
Pre Stop, Primastop <i>Gliocladium cetenulatum</i>	<i>Pythium</i> spp., <i>Rhizoctonia solani</i> spp. <i>Botrytis</i> spp. <i>Didymella</i> spp.	greenhouse crops	wettable powder	Spray	Kemira Agro Oy.
PSSOL <i>Pseudomonas Protection solanacearum (nonpathogenic)</i>	<i>Pseudomonas solanacearum</i>	Vegetables	powder	seed treatment spray	Natural Plant
Rhizo-Plus, Rhizo-Plus Konz <i>Bacillus subtilis</i> FZB24	<i>Rhizoctonia solani</i> , <i>Fusarium spp.</i> , <i>Alernafia</i> spp. <i>Sclerotium</i> , <i>Verticillium</i> , <i>Streptomyces scabies</i>	Field (potatoes corn) vegetables, and ornamental plants	water dispersible granule	as suspension for seed treatment, soil drench, dip and addition to nutrient solutions	KFZB Biotechnik GmbH
Root Shield/ Bio-Trek T-22G <i>Trichoderma harzianum</i> Rifai strain KRL-AG2 (T-22)	<i>Pythium</i> spp., <i>Rhizoctonia solani</i> , <i>Fusarium</i> spp.	trees, shrubs, transplants, all ornamentals, cabbage tomato, cucumber	granules or wettable powder	granules mixed with soil or potting medium powder mixed with water and added as a soil drench	Bioworks, Inc.
Rotstop, P-g Suspension <i>Phlebia gigantea</i>	<i>Heterobasidium annosum</i>	trees, forest plants	spores in inert powder	spray, chain saw oil	Kemira Agro Oy
Serenade					

<i>Bacillus subtilis</i>	Powder mildew, downy mildew, <i>Cercospora</i> leaf spot, early blight, late blight, brown rot, fire blight, and others	cucurbits, grapes hops, vegetables. peanuts pome fruits, stone fruits and others	wettable powder	spray, root dip.	Agra Quest, Inc.
Soi Gard/ Glio Gard <i>Gliocladium virens</i> GL-21	Damping off and root rot pathogens especially <i>Rhizoctonia solani</i> and <i>Pythium</i> spp.	ornamental and food crop plants grown in greenhouses nurseries homes, and interior scapes	granules	granules are incorporated in soil or soilless growing media prior to seedling	Thermo Trilogy
Spot-less <i>Pseudomonas aureofaciens</i> strain Tx-1	Dollar spot Anthracnose Pink snow mold	Turfgrass	liquid	overhead irrigation	Eco Soil Systems Inc.
Supersivit <i>Trichoderma</i> Reitzel	Various fungi <i>harzianum</i>	vegetable, fruit and ornamental crops	Wettable powder pellets	Spray, root dip, soil and drench	Borregaard and
System 3 <i>Bacillus subtilis</i> GB03 and chemical pesticides	Seedling pathogens	barley, beans, cotton peanut, pea, rice, soybean	dust	seed treatment in planter box	Helena Chemical Co.
T22G, T-22 Planter BoxT/Bio-Trek <i>Trichoderma harzianum</i> Rifai strain KRL-AG2	<i>Pythium</i> spp. <i>Rhizoctonia solani</i> , <i>Fusarium</i> spp. and <i>Sclerotinia homeocarpa</i>	bean, cabbage, corn, cotton, cucumber, peanut, potato, sorghum, soybean, sugarbeet, tomato turf, and greenhouse ornamentals	granules or dry powder	granules added in furrow with granular applicator by broadcast application to turf, mixed with greenhouse soil, or by mixing powder with seeds in planter box, or in commercial seed treatment slurry	Bioworks Inc.

Trichodex <i>Trichoderma</i> Chemical <i>harzianum</i>	Primary <i>Botrytis cinerea</i> also <i>Collectotrichum</i> spp. <i>Fulvia fulva</i> , <i>Monilia laxa</i> <i>Plasmopara viticola</i> , <i>Pseudoperonospora</i> <i>cubensis</i> , <i>Rhizopus stolonifer</i> <i>Sclerotinia sclerotiorum</i>	cucumber, grape, nectarine, soybean strawberry, sunflower tomato	wettable powder	spray	Makhteshim works Ltd.
Trichopel, Trichosject Trichodowels, Trichoseal <i>Trichoderma</i> Technologies <i>harzianum</i> and <i>T. viride</i>	<i>Armillaria</i> , <i>Botryosphaeria</i> <i>Chondrosternum</i> , <i>Fusarium</i> <i>Nectria</i> , <i>Phytophthora</i> , <i>Pythium</i> , <i>Rhizoctonia</i> sp.	Cucumber, cotton, corn, peanut, potato, sorghum, soybean etc.	dry powder	spray, dusting	Agrimm Ltd.
Trichoderma 2000 <i>Trichoderma</i> sp.	<i>Rhizoctonia solani</i> <i>Sclerotium rolfsii</i> , <i>Pythium</i> spp. <i>Fusarium</i> spp.	nursery and field crops	granules	incorporated into soil or potting medium	Mycontrol Ltd.
Trieco <i>Trichoderma viride</i>	<i>Rhizoctonia</i> spp. <i>Pythium</i> spp. <i>Fusarium</i> spp.	Chillies, tomato, sugarcane, citrus, grape, vegetables	Powder	seed dressing, soil drench	Ecosense Labs (I) Pvt. Ltd.
Victus <i>Pseudomonas</i> NCIB 12089	<i>Pseudomonas tolaasii</i> <i>fluorescens</i> strain blotch disease	mushroom that causes bacterial	aqueous suspension	spray of fermentor biomass	Sylvan Spawn Laboratory

3. *Materials and Methods*

3.1 **Materials**

3.1.1 **Solvents and Chemicals**

Laboratory grade reagents were locally procured and purified before use. The solvents used were of analytical grade (AR), dried and distilled before use.

Chemicals such as alkyl bromides, succinimide, aluminium chloride, triethyl amine, malonic acid, urea, sodium methoxide, isoprene, different alkyl chloride/bromides, potassium tertiary butoxide, piperidine were procured locally. Triethyl amine was purified by storing overnight over sodium hydroxide pellets prior to distillation.

3.1.2 **Biological agents**

Non pathogenic strain of *Fusarium oxysporum* previously isolated from mustard aphid (*Lipaphis erisimi*) and confirmed to be non pathogenic and plant pathogenic fungi such as *Sclerotium rolfsii* Saccardo, *Rhizoctonia bataticola* (Taub.) Butler, *Pythium aphanidermatum* (Edson) Fitz. *Macrophomina phaseolina*, *Pythium debaryanum* and *Rhizoctonia solani* Nees were collected from Indian Type Culture, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi, India.

Root knot nematodes *Meloidogyne incognita* and cyst nematode *Rotylenchulus reniformis* were collected from infested tomato roots from the experimental fields of Indian Agricultural Research Institute, New Delhi.

Wheat seeds (var. HW 2009) from Division of Genetics, seeds of *Parthenium hysterophorus* and *Phalaris minor* from Division of Agronomy, Indian Agricultural Research Institute, New Delhi, were collected for different studies.

Insects such as *Spodoptera litura* and *Corcyra cephalonica* were collected from the field of Indian Agricultural Research Institute; and NCIPM, New Delhi respectively.

3.1.3 Culture media

A wide range of media such as Ala-2, Nutrient Agra, Yems media, Luria Agar, Malt Extract Agar, Rose Bengal, Sucrose yeast Peptone and Potato Dextrose Agar were purchased from Hi Media for growing fungi. The appropriate quantity of media was suspended in distilled water (1L) and heated to boiling till media was completely dissolved. A pinch of streptomycin was added to it. These media were sterilized in an autoclave at 15psi at 121°C autoclaved

3.1.4 Potato Dextrose Broth

(PDB) was prepared using the literature method (Johnston and Booth, 1983) with potato (200 g) and dextrose (20 g).

Potatoes (200 g) were peeled, cut into small pieces and boiled with water (600 ml), till the potato pieces became soft (20-30 min). The potato extract was strained through a clean muslin cloth and kept in a container. Dextrose (20 g)

was added to the filtrate and volume was made up to 1000 mL. The filtrate was then heated with stirring till uniform media was obtained. A pinch of streptomycin was added to it. The media (250 mL) was transferred to each one litre conical flasks. The flasks were plugged with non-absorbent surgical cotton and sterilized in an autoclave at 15psi at 121°C for 15 min.

3.1.5 Culturing

Collected cultures of *F. oxysporum* (non pathogenic strain) and pathogenic fungi were maintained on PDA (Potato Dextrose Agar) slants at 28°C and were sub cultured on Potato Dextrose Agar plates in Petri plates for 5-6 days at 28°C. Cultures were stored at 4°C and transferred weekly.

3.1.6 Growth of *F. oxysporum* in different media

Non-pathogenic strain of *F. oxysporum* was inoculated at the center of Petri plates containing different media (section 3.1.3) by placing an eight mm actively growing culture disc of the isolate cut from a seven-day-old culture by means of a cork borer. The inoculated Petri plates were incubated in a BOD incubator at 28±2°C for 7 days. Three replicates were maintained for each culture medium. The observations were recorded after 7 days by measuring the radial growth of *F. oxysporum*.

3.1.7 Standardization of incubation period in PDA media

F. oxysporum was inoculated at the center of Petri plates containing PDA media by placing an eight mm actively growing culture disc of the isolate cut from a seven-day-old culture by means of a cork borer. The inoculated Petri plates

were incubated in a BOD incubator at $28 \pm 2^{\circ}\text{C}$. The Petri plates were drawn at different time intervals (5, 7, 9, 11, 15 and 20 days), and fungal mycelia mat was scraped by a spatula and extracted by chloroform and methanol. Three replicates were maintained for each set of experiment. Solvent was evaporated on a rotary vacuum evaporator and analyzed by GLC and HPLC.

3.1.8 Growth of *F. oxysporum* in liquid culture

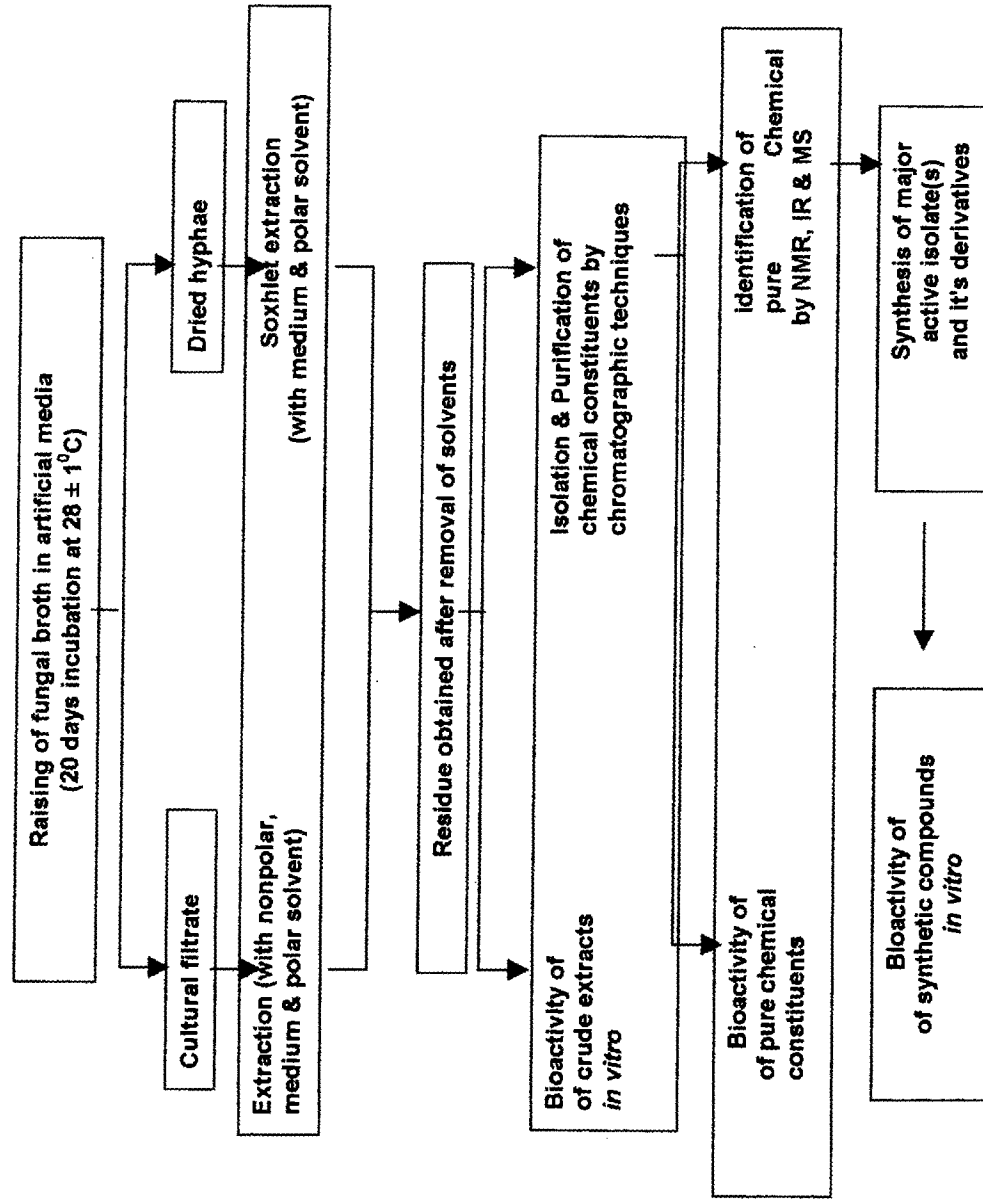
Potato dextrose broth (250 mL) was inoculated with three mycelia discs (1.2 cm diam) obtained from 5-7 day old culture of fungus grown on PDA. A number of such flasks were prepared. The inoculated medium was incubated at 28°C in a BOD incubator for 20 days. During incubation, the flasks were exposed to light for 5-6 hours for enhancing sporulation, after 5 days. At the end of the incubation period the culture was harvested by filtering through two layers of muslin cloth. The culture filtrate was stored at 0°C until use. The mycelia mass was dried either by lyophilization or by drying in a fume cupboard.

3.1.9 Extraction of culture filtrate

The cultural filtrate (1000 mL) was extracted first with hexane (500 mL) followed by dichloromethane (1L) and ethyl acetate (500 mL). The solvents were dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure. The residues thus obtained were used for bioactivity and separation of secondary metabolites.

3.1. 10 Extraction of mycelium

Scheme 1. Flowchart of the overall experimental procedure



The powdered lyophilized mycelia mass was extracted first with hexane followed by dichloromethane and methanol by Soxhlet extraction for 20 hours. Solvents were dried over anhydrous sodium sulphate and removed by distillation under reduced pressure. The residues thus obtained were used for bioactivity and separation of secondary metabolites.

3.1.11 Equipments

Special equipments like autoclave (for sterilization), Laminar flow (for culturing the biological agents in aseptic condition), lyophilizer (for freeze drying of hyphae) and biological oxygen demand (BOD) incubator for growing the biological agents at particular temperature were used. All melting points were recorded with electrical melting point apparatus using open capillary method

3.2 Chromatography

3.2.1 Thin layer chromatography (TLC)

Silica-gel TLC plates were prepared by spreading a slurry of silica gel (G) (60 - 120 Mesh) containing 10 per cent binder (gypsum) in distilled water on 20 x 20 cm and 20 x 5 cm glass plates uniformly by using a TLC applicator. The thickness of silica-gel layer on plate was maintained as 250 μm . These silica-gel coated plates were activated at 100 – 120°C for 2-3 h before use in an oven. The sample solutions were spotted on activated TLC plates using capillary tubes. The TLC plates were developed in a number of different solvent systems. Iodine vapour was used as a visualizing agent. The spots were marked on the TLC plates and their R_f values were determined by using following formula

$$R_f = \frac{\text{Distance moved by the compound}}{\text{Distance moved by the solvent front}}$$

3.2.2 Column chromatography

Secondary metabolites were separated by column chromatography using a glass column (1cm i.d., 50 cm long) packed with slurry of silica gel (60-120 mesh, pre-activated at 120⁰C for 4 h) in hexane. The column was successively eluted with hexane, hexane-dichloromethane, dichloromethane, and dichloromethane: ethyl acetate (up to 10%), collecting 25 ml fractions. These fractions were distilled on water bath and monitored by TLC and GLC. Fraction containing similar compound(s) were mixed together and further purified either by preparative TLC or by crystallization from appropriate solvents.

3.2.3 Gas liquid chromatography

Crude extracts and column fractions were analyzed on a Hewlett Packard model 5890 A gas liquid chromatograph coupled with a Hewlett Packard 3390 A integrator and equipped with a flame ionization detector (FID). The unstable and thermo labile metabolites were analyzed by suitably converting to silyl derivative by silylation with trimethyl silyl chloride. The GLC operating conditions were as follows:

Detector - Flame Ionization Detector (FID)

Column	- HP-1 (methyl silicon gum, capillary column, 10 m, 0.53 mm i.d., 2.65 mm film thickness)
Injector temperature	- 250°C
Detector temperature	- 300°C
Column temperature	- 70°C to 270°C @ 5°C min ⁻¹
Carrier gas	- Nitrogen
Flow rate	- 2 mL min ⁻¹
Flow rate of other gases used	
Hydrogen	- 30 mL min ⁻¹
Oxygen	- 120 mL min ⁻¹

3.2.4 High Performance Liquid Chromatography (HPLC)

Samples were also analyzed by HPLC (Thermo Separation Product model Spectra System P 2000) equipped with a variable wavelength UV-150 UV VIS detector and a Rheodyne injector (20 μ L loop) and connected to a Datajet reporting integrator. Stationary phase consisted of a Lichrosorb C-18 column (250 mm x 4.6 mm id) and the mobile phase was methanol water (70 + 30, by volume) maintained at a flow rate of 1 mL min⁻¹ with detector wavelength set at 270 nm.

3.3 Spectroscopy

3.3.1 Fourier Transform Infra-red spectroscopy (FT-IR)

Infrared spectra (IR) were recorded on a Nicolet Fourier Transform Infrared spectrophotometer (Model Impact- 700 FT - IR spectrophotometer). The liquid samples were analyzed as thin films and solid samples as KBr disc and a nujol mull.

3.3.2 Nuclear Magnetic Resonance Spectroscopy (^1H -NMR and ^{13}C -NMR)

The proton nuclear magnetic resonance spectra were recorded on a Varian EM 360 L (60 MHz), Bruker 400 AC (400 MHz) and on a Bruker 300 AC (300 MHz) instrument. The solvent used was carbon tetrachloride (CCl_4) and deuterio chloroform (CDCl_3) containing tetramethylsilane (Me_4Si , TMS) as the internal standard. The chemical shifts are expressed in δ values and coupling constant (J) are given in hertz (Hz). The notations used for splitting pattern are s=singlet, d=doublet, t=triplet, q=quartet and m=multiplet.

3.3.3 Gas chromatography - Mass spectroscopy (GC-MS)

Gas chromatography-Mass spectroscopy (GC-MS) was performed on a HRGC-MEGA 2 series gas chromatograph coupled to a FISIONS-TRIO 1000 ion trap mass spectrometer and connected to a Panasonic K x P1150 multimode printer and on a HP 5890 GC coupled with HP 5790 mass selective detector (MSD). The ionization potential was 70 eV. The gas chromatograph was equipped with a OV-101 capillary column (30 m x 0.25 mm i.d., film thickness 0.1-0.15 mm). The column temperature was programmed from 70 to 270⁰C at a

rate of $10^{\circ}\text{C min}^{-1}$. Helium was used as a carrier gas with a flow rate of 2 mL min^{-1} .

3.4 Bioactivity

3.4.1 Fungicidal activity

3.4.1.1 Examination of antagonism of *F. oxysporum*: Dual culture method

A mycelial disc (1.2 cm) obtained from the peripheral region of 5-7 day old cultures of *S. rolfii* Saccardo, *R. bataticola* (Taub.) Butler; *P. aphanidermatum* (Edson) Fitz. *M. phaseolina*, *P. debaryanum* or *R. solani* Nees on PDA, was placed on a fresh PDA plate (3 cm from the centre) and incubated at 28°C for 48 h to initiate growth. Then a 1.2 cm- diam mycelial disc, obtained from the periphery of a 5-7 day old culture of *F.oxysporum* was placed 3 cm away from the inoculum of the pathogen, the plates were incubated at 28°C , the measurements were taken after 7 days. In the control experiment a sterile agar disc (1.2 cm diam) was placed in the dish instead of *F. oxysporum*. At the end of the incubation period, radial growth was measured. Radial growth reduction was calculated in relation to growth of the control as follows

$$\frac{C-T}{C} \times 100 = \% \text{ Inhibition of radial mycelial growth}$$

Where C is radial growth measurement of the pathogen in control and T is the radial growth of the pathogen in the presence of *F. oxysporum*.

3.4.1.2 Fungicidal activity of the extracts

3.4.1.2.1 *A qualitative bioassay using a thin layer chromatography*

Different quantities of crude extracts from *F. oxysporum* liquid culture filtrate and dried mycelial mass were applied to silica gel plates for TLC (60 F254; Merck AG, FRG). The plates were developed in suitable solvent system. For antibiosis bioassay, the pathogenic fungi were grown PDA media. The mycelial mat of pathogenic fungi was transferred to a sterile blender. The sterile V8 broth (200 mL of V8 juice, 3 g of CaCO_3 + 5 g glucose and 800 ml water) was added, providing 100 mL of solution per 15 g of mycelium. To prevent bacterial growth, Streptomycin was added at 500 mg a.i. per liter of medium. The mixture was blended for two minutes at maximum speed and transferred to a sterile spraying bottle. The TLC plates, which were developed in solvent system, were sprayed with the pathogenic fungi suspension and incubated in a plastic box with moist filter paper for 48 h at 20° C. to visualize the zone of growth inhibition; the plates were dipped in an aqueous 4% carbon powder suspension. Carbon adsorbed to the mycelium, whereas the inhibition zones remained clear.

3.4.1.2.2 *Fungicidal activity of the extracts and isolated pure compound*

3.4.1.2.2.1 *A quantitative bioassay by poisoned food technique*

Fungicidal activities of crude extracts obtained by extraction of culture filtrate and mycelia of *F. oxysporum*, and pure compounds/metabolites isolated from crude extracts of culture filtrate and hyphae evaluated by poisoned food technique using potato dextrose agar media (Nene and Thaplyal, 1979) against

six phytopathogenic fungi namely; (i) *S. rolfii* (ii) *M. phaseolina*, (iii) *R. solani* (iv) *R. bataticola*, (v) *P. debaryanum* and (vi) *P. aphanidermatum*

3.4.1.2.2.1 Preparation of PDA media:

The PDA media was prepared as described by Johnston and Booth (1983) using potato (peeled, 200 g), dextrose (anhydrous, 20 g) and Agar (Bacto, 20g). The potatoes (200 g) were peeled, cut into small pieces and boiled with water (600 mL) for 20 min, till the potato pieces became soft. The extract was strained through a muslin cloth and volume was made upto 1000 mL. The weighed quantities of agar-agar (20 g) and dextrose (20 g) were added to the filtrate and the mixture was heated with stirring till uniform media was obtained. The media (65 mL) was transferred to each conical flasks (150 mL). The flask was plugged with non-absorbent surgical cotton. The flasks containing media and Petri plates were sterilized in an autoclave at 15 Psi and 121.5⁰C for half an hour.

3.4.1.2.2.2 Preparation of concentration of metabolites /extracts:

Required quantities (130 mg) for a set of 1000, 500 and 250 ppm concentration(s) of each compound/extract were weighed and dissolved in acetone (2 mL) (for *F. oxy Hyp*, DMSO was used as solvent) and from it 1 mL, 0.5 mL and 0.25 mL was pipetted out in vials and volume was made up to 1 mL by adding acetone to make concentrations of 1000, 500 and 250 ppm, respectively, in 65 mL of media with two replications. Acetone/DMSO (1 mL) was taken in separate vials for using as control. These solutions were added to

the media (65 mL) contained in the conical flasks to obtain the desired concentrations of the chemicals in the media. The media (65 mL) was thoroughly mixed and poured into two Petri dishes (2 replication) under aseptic conditions in a Laminar Flow Chamber. The plates were kept under UV light in the Laminar Flow Chamber for solidification of media. After the solidification of the media, a 5 mm disc of the desired pathogenic fungus containing spores and mycelia was inoculated aseptically to the centre of the Petri dish.

3.4.1.2.2.3 Incubation:

The inoculated Petri dishes were kept in a BOD incubator, maintained at 25⁰C till the fungal growth was almost complete in the control plates. The incubation periods for different pathogenic fungi are given in Table 5.

3.4.1.2.2.4 Recording of observations:

The mycelial growth of fungus (cm) in both treated (T) and control (C) Petri dishes were measured diametrically and the percentage inhibition of growth (I) was calculated by using the following formula -

$$\text{Percentage (\% I) Inhibition} = \frac{C - T}{C} \times 100$$

3.4.1.2.2.5 Calculation of ED₅₀ Values: For calculation of ED₅₀ values (effective dose required for 50 per cent inhibition of growth), the per cent inhibition was converted to corrected per cent inhibition by using the formula –

$$\text{Corrected per cent inhibition} = \frac{\% I - CF}{100 - CF} \times 100$$

Where CF is the correction factor obtained by the equation -

$$\text{Correction factor (CF)} = \frac{9 - C}{C} \times 100$$

Where 9 is the diameter of the Petri dish in cm, C is the diameter of growth of the fungus in control plates. From the concentration (ppm) and corresponding corrected percentage inhibition data of each extract compound, the ED₅₀ (ppm) value was calculated with the help of probit package of MSTATC software using a personal computer (PC).

3.4.2 Nematotoxicity testing

Nematicidal activity of crude extracts obtained by extraction of culture filtrate and mycelia of *F. oxysporum*, and pure compounds/metabolites isolated from these extracts was evaluated against juveniles (J2) of *M. incognita* and *R. reniformis*.

The study was conducted at 29.2°C in B.O.D incubator under laboratory condition. Suspension containing freshly hatched juveniles of *M. incognita* and *R. reniformis* was diluted to give a count of approximately 40-50 juveniles of each per ml. A stock solution (2000 ppm) of the test compounds was prepared by dissolving test compound (50 mg) in appropriate solvent (2 mL) and Tween 80 (2 drops) and the volume was made up to 25 ml with distilled water. Each of the solution was further diluted with water to get effective concentration of 1000, 500, 250 and 125ppm. One ml of nematode suspension was put in a vial over 1 ml solution of double concentration of 1000, 500, 250, 125 and 62.5 ppm. The

drops) and the volume was made up to 25 ml with distilled water. Each of the solution was further diluted with water to get effective concentration of 1000, 500, 250 and 125ppm. One ml of nematode suspension was put in a vial over 1 ml solution of double concentration of 1000, 500, 250, 125 and 62.5 ppm. The loosely capped vials were stored for 24, 48 and 72 h at $30\pm 1^{\circ}\text{C}$. The number of active and inactive nematodes was counted by taking suspension in a counting dish under stereoscopic binocular microscope. After the said period of exposure, the percent mortality of the larvae was worked out on an average of three replicates and corrected for natural mortality according to Abbots' formula (Abbot, 1925).

3.4.3 *Plant growth regulatory activity*

The bioassay was carried out using literature method ⁴ using healthy wheat seeds (Variety – HD 2329) collected from Division of Genetics, IARI, and New Delhi. The 1500, 1000 and 500 ppm concentrations of all the compounds were prepared in acetone. The seeds (25) of equal size were soaked overnight in above solutions of different extracts. These soaked seeds were removed from the aqueous solutions and dried in the air, to give a uniform layer of the extract on the seed coat. These coated seeds (25) were placed in the Petri dishes (9 cm) lined with moist sheet of Whatman No. 1 filter paper and sealed with paraffin. Assays were carried out at 25°C under artificial fluorescent light (8 x 40W) in an incubator during three days, after which germination was scored and the radical length was measured. Seeds were considered germinated if a radical protruded at least 1 mm. The observations were recorded on 3, 5 and 7 day. Each

bioassay was replicated 5 times in a complete randomized design. A control experiment was carried out under the same conditions described using only water. The percent germination, vigor index⁵ and percent phytotoxicity⁶⁻⁷ were calculated as follows.

$$\% \text{ Phytotoxicity} = \frac{\text{Root/ shoot length in control (cm)} - \text{Root/ shoot length in treatment (cm)}}{\text{Root/ shoot length in control (cm)}} \times 100$$

$$\text{Vigor index} = \% \text{ germination} \times \text{Total length of seedling}$$

3.4.4 Herbicidal activity

The *Phalaris minor* seed germination test for culture filtrate/hyphae extracts and some of the isolated pure compounds were carried out using *P. minor* seed. The 100, 250, 500, 1000 and 1500 ppm concentrations of different extracts were prepared in water, minimum solvent and surfactant. The seeds (25) of equal size were soaked overnight in above solutions of different extracts. These soaked seeds were removed from the aqueous solutions and dried in the air, to give a uniform layer of the extract on the seed coat. These coated seeds (25) were placed in the Petri dishes (9 cm dia) containing a moist filter paper at the bottom of the lower plate. Three replications for each treatment were taken. The plates containing seeds were kept in a BOD incubator at $10 \pm 1^{\circ}\text{C}$. The

observations were made at 3, 5 and 7 day. The percent germination, percent phytotoxicity and vigour index were calculated.

3.4.5 Insecticidal activity

The *Spodoptera litura* larvae were collected from the field and were reared in the laboratory on the castor leaves. The culture was maintained at $27\pm 2^{\circ}\text{C}$ and $70\pm 5\%$ RH. The third instars larvae of the insect were then taken for the experiment. The larvae were starved for 6 h and fed on castor leaves (1cm^2) treated with various test materials at different concentrations. Leaves treated only with solvent were used as control. Larvae (10) were released in a jam bottle containing treated leaf. Three replicates were taken for each concentration. Mortality percentage was observed at 24, 48, 72 and 96 h and LD50 was calculated using probit package of MSTATC software.

3.4.6 Synthesis of secondary metabolites

3.4.6.1 Synthesis of fusaric acid

Synthesis of fusaric acid, the major metabolites of the *F. oxysporum* and other related species involves following steps.

3.4.6.1.1 Step 1: Synthesis of Meldrum's acid:

Malonic acid (52g) was taken in a round bottom flask and acetic anhydride (60 ml) was added to it. Concentrated H_2SO_4 (1.5 ml) was added drop wise to it with constant stirring over a magnetic stirrer. The stirring was continued for half an hour and then dry distilled acetone (40 ml) was added with constant shaking at room temperature. The reaction mixture was shaken for one hour and

kept in refrigerator for overnight. The solid obtained was washed with ice-water and dried under vacuum. The white solid thus obtained was recrystallised from acetone-water as shining white crystal (m.p. yield). NMR:

3.4.6.1.2 Step 2: Synthesis of oximinosulfonate of Meldrum's acid:

Meldrum's acid (2.61 g) was taken in a round bottom flask (25 mL), NaNO_2 (1.25g in 10 ml water) and methanol (13ml) was added to it. Mixture was stirred on a magnetic stirrer. Stirring was continued for two hours at room temperature till a deep red solution was obtained. Phosphate buffer (pH 7, 2.5 ml) was added to it and the temperature of the reaction mixture was brought down to 0°C . Tosyl chloride (3.11g) was added to it and stirring was continued for another half an hour at room temperature. The solid thus obtained was filtered and washed repeatedly with cold methanol, dried in desiccator.

3.4.6.1.3 Step 3: Synthesis of a conjugated di-ene:

Potassium tertiary butoxide (1.65 g), piperidine (2.06g) and tetra hydro furan (12 ml) were taken in a round bottom flask and cooled to -65°C . Isoprene (1.50g) was added to it. 1-Bromopropane (2.72 g) was taken in tetra hydro furan (3 ml) and added to the previous mixture. The reaction mixture was stirred for 20 min at -50°C and resulted in a yellow suspension. The content of the flask was heated to 0°C and water (40 ml) was added to it. Then the mixture was extracted

with diethyl ether (3 x 25 ml) and organic layer was collected. The organic layer was washed with 1N HCl (2 x 20 ml) and with a saturated solution of NaCl (30 ml). Again the organic layer was collected and dried over anhydrous sodium sulphate. Solvent was evaporated under reduced pressure on a rotary vacuum evaporator and the residue was purified by chromatographic separation. The column was eluted with pentane and the eluent was collected and concentrated to get the desired product

3.4.6.1.4 Step 4: Synthesis of methyl fusarate: Reaction of oximosulfonate and di-ene system

Oximosulfonate (0.4 g), 2-butyl-1, 3-butadiene (0.13g) and dichloromethane (6 ml) were taken in a round bottom flask and AlCl_3 (0.33g) was added to it. CaCl_2 guard tube was attached with the flask and the content was stirred for 4 hour at -50°C . After the said period, NaOMe (0.19g), N-bromo succinimide (0.17g) and THF:Methanol (1:1) (5 ml) was added and the reaction mixture was stirred for 15 h at room temperature. After the said period, white slurry was obtained.

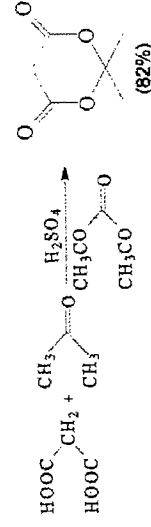
3.4.6.1.5

Step 5: Synthesis of fusaric acid: Hydrolysis of methyl fusarate

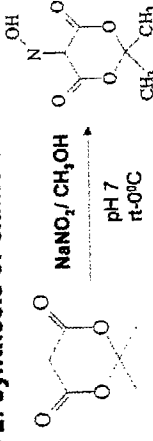
To the white slurry obtained in step 3.4.5.1.4, a methanolic solution of KOH (5N, 15 ml) was added and the content was stirred for one hour at 0°C .

Scheme 2. Synthetic route of Fusaric acid

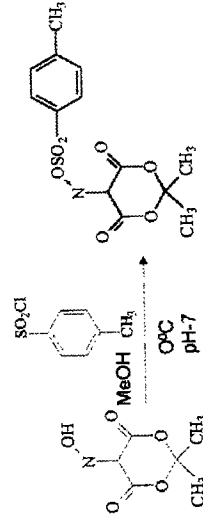
Step 1: synthesis of Meldrum's acid



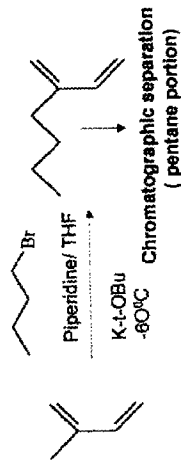
Step 2: synthesis of oxime of Meldrum's acid



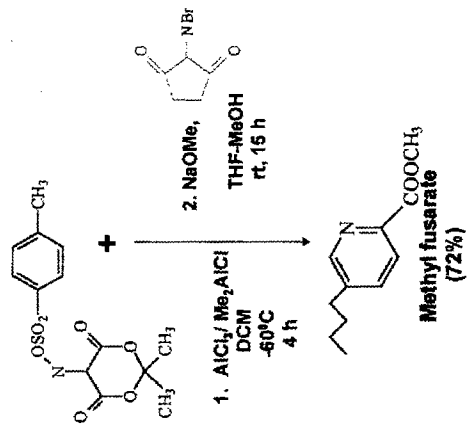
Step 3: Synthesis of oximosulfonate of Meldrum's acid



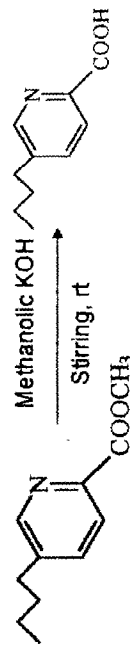
Step 4: Synthesis of a conjugated di-ene system



Step 5: Synthesis of methyl fusarate- cyclo [4+2] pyridine annulation



Step 6: Synthesis of fusaric acid from methyl fusarate



Sublimation of the resultant product gave pure white crystal. The structure of the product obtained was confirmed by IR, NMR and MS spectra.

3.4.6.2 *Synthesis of Fusaric acid analogues*

Substitution at 5 position of picolinic acid and different ester derivatives of fusaric acid were prepared in the same manner as described in 3.2.5.1.1-3.2.5.1.5. for substitution at 5 position, different alkyl bromides were reacted with isoprene and the remaining steps were followed as stated earlier. Different ester analogues of fusaric acid were synthesized by esterification of fusaric acid with suitable alcohol in presence of concentrated H_2SO_4 . Ethyl fusarate was also prepared by using sodium ethoxide instead of sodium methoxide at step 3.2.5.1.4.

3.4.6.3 *Synthesis of palmitic acid derivatives:*

Methanol (20 ml) and concentrated H_2SO_4 (1 ml) was added to palmitic acid (13 g) taken in a round bottom flask and content of the flask was refluxed for 6 h on water bath. The resultant product was distilled under reduced pressure. Similarly, ethyl, isopropyl, propyl, butyl, isobutyl and cyclohexyl esters of palmitic acid were prepared and purified by distillation under reduced pressure..

3.4.7 *Structure activity relationship*

Structure activity relationship (SAR) was studied by using calculated ED_{50} values and chemical structure of 2 and 5-substituted fusaric acid analogues and

T-7448



different alkyl esters of palmitic acids. The effect of different alkyl groups were correlated with the ED₅₀ values against different phytopathogenic fungi.

3.4.8 Preparation of formulation of *F. oxysporum*

3.4.8.1 *Mycelia based dust formulation:*

Dried lyophilized powdered mycelia of *F. oxysporum* (5 g) were mixed with glucose (5g), urea (1g), fly ash (42g), saw dust (38 g) and sucrose (4 g) in a beaker. The content was transferred to a laboratory blender and thoroughly pulverized to get the fine powder.

3.4.8.2 *Fly ash and charcoal based dust formulations.*

Similarly, fly ash and charcoal based mycelia formulation (1%) was prepared by mixing dried mycelia (1 g) and fly ash/charcoal (99 g).

3.4.8.3 *Sawdust based solid formulation*

Dried lyophilized powdered mycelia of *F. oxysporum* (5 g) were mixed with glucose (5g), urea (1g) and remaining sawdust in a beaker. The content was transferred to a laboratory blender and thoroughly pulverized to get the fine powder.

3.4.8.4 *Preparation of mycelia extract based EC formulation:*

Dichloromethane extract of mycelia of *F. oxysporum* (1g) was dissolved in acetone (10 ml) with Tween-80. Volume was made up to 50 ml by addition of distilled water and the content was shaken thoroughly to get the EC formulation.

3.4.8.5 *Preparation of mycelia based pellet formulations*

For pellet formation, 20 g of sodium alginate/potassium alginate was dissolved in 750 ml of distilled water at 40°C, while stirring and then autoclaved

(pH adjusted to 6.6 – 7.0). Concurrently, 50 g of fine powdered wheat bran or kaolin or saw dust (finely powdered) was placed in a blender with 250 ml of distilled water and then autoclaved. The ground filter mat material was then added to the blender container together with alginate solution and then wheat bran / kaolin/ saw-dust (finely powdered) water mixture. The resultant wheat bran/saw dust/ kaolin alginate fungal biomass mixture was comminuted for 30 sec at high speed and then added drop wise from reservoir through tubes, the droplets falling a distance of about 15 cm into 200 ml of a 0.25 M calcium chloride/calcium gluconate (1 to 15% or 2-5%) solution. Gel beads formed immediately upon contact with the gellant solution and were allowed to remain in the gellant solution for 20 min. The gel beads were separated by decanting, spread on a nylon screen, and dried at ambient temperature using a gentle flow of air keeping moisture content 5 to 15%. Drying transformed the gel beads into granules, which were then stored under ambient conditions (30°C).

3.4.8.6 *Preparation of metabolite based pellet formulation*

Major metabolite based pellets formulation was prepared by diluting dichloromethane extract of mycelia 1:3 (v/v) with 1.33% (w/v) of sodium alginate in distilled water to give a total of 4 parts by volume. This mixture had a final sodium alginate concentration of 1.0% and a pH of 6.6 to 7.3. The final pH was approximately 7.0 when kaolin (10%, w/v) was added to the mycelia homogenate-alginate mixture. These mixtures with and without kaolin were palletized by drop wise addition to 0.25 M CaCl_2 in distilled water.

3.4.8.7 Release of metabolites from the alginate matrix:

Metabolites based controlled release formulation of *F. oxysporum* (1g) was taken into a vial and distilled water (5 mL) was added to it. The vials were capped and kept at room temperature for different time period. Blank pellets were used as a control in the similar way. Three replications were maintained for each sample. Samples were drawn periodically; the supernatant liquid was decanted and extracted with chloroform (2 x10ml). The organic layer was collected, dried over anhydrous sodium sulphate and concentrated. The residue thus obtained was dissolved in trimethyl silyl chloride (0.3 mL) and heated for 1 h at 60°C and analyzed by GLC.

3.4.9 Bioefficacy of mycelia based formulation:

3.4.9.1 Pot culture study;

Charcoal and saw dust based mycelia formulation of *F. oxysporum* was tested for its bioefficacy against *P. aphanidermatum* and *P. debaryanum* using tomato as a test plant. Soil (200 g) was sterilized in an autoclave at 120°C at 15psi for 15 minute.

3.4.9.1.1 Inoculum Preparation.

P. aphanidermatum and *P. debaryanum* was used in the experiment. Mycelia mats of *P. aphanidermatum* and *P. debaryanum* were grown in PD broth in 250 ml conical flasks, incubated horizontally at 27°C. After 7 days, the medium was decanted and 200 mL of sterile deionised water was added to each bottle and incubated vertically at 20°C for 3-4 weeks. Chlamydospores were harvested

by rinsing the cultures two or three times with deionised water and homogenizing in a small blender for 1 min. The mycelial suspension then was ground in a glass tissue grinder to further breakup the mycelium. The suspension was filtered through two layers of cheesecloth and sonicated in a water bath for two 30-s periods to disrupt any remaining viable mycelium. Microscopic examination verified that no cytoplasm remained in the mycelial fragments. Inoculum from 10 flasks was thoroughly incorporated into 2.5kg of sterilized by autoclave at 120°C at 15 psi for 15 min, composted soil previously sifted through a 2-mm sieve. The infested soil was incubated in the lab at room temperature (approximately 25°C) for 5-7 days. Sterilized autoclaved, uninfested soil was used as a control in all experiment

3.4.9.1.2 *Treatments*

The experimental treatment for the disease control experiment in the green house was i). uninfested soil, only water added as a check, ii) Infested soil, only water added, iii) the formulated dry mycelia (5 and 10% concentration) was mixed thoroughly in the infested soil.

3.4.9.1.3 *Disease control in green house*

After the 5-7 day incubation period, the infested soil was treated by incorporating 84 mL of 5 and 10% of solid formulation of *F. oxysporum* into 2.5 kg of soil at the rate of 5.0 g of dust in 150cm³ of the soil. The treated soil was placed in double polyethylene bags that were then closed tightly and incubated for 7 days. Metalaxyl was incorporated similarly at the same rates as above. After the incubation period soil from each treatment was placed in twenty 6.6 cm

diameter standard plastic pots, and one 4-6 week old seedling of tomato was transplanted to the soil in each pot. Pots were placed randomly on the green house bench. Disease characteristics (wilting) and mortality was assessed 7, 14, 21, 28 and 35 days after transplanting, and weekly thereafter. The number of symptomless plants was recorded for each treatment at each assay date and expressed in terms of the proportion of symptom less plant. Six trials of the experiment, with all concentrations were repeated.

3.4.9.1.4 *Analysis*

Data from the above experiments in the green house were transformed as the arcsine of the square root of the proportion of the symptom less plant stand. It was analyzed as repeated measure design and analysis of variance determined using MSTATC software (Statistical Package). The significance level was determined before analysis based on the observed variation in plant growth among trials of the experiment due to external green house variable.

3.4.10 *Bioefficacy of metabolite based EC formulation*

3.4.10.1 *Pot culture study*

Metabolites based EC formulations were tested for their herbicidal activity against *P. minor* and *P. hysterothorax*. Seeds of these weeds (10 per pot) were sown and after ten days, 1% and 2.5% formulations were sprayed @ 10 l/ ha. The visual symptom, percent mortality and root and shoot length were recorded.

3.4.11 Bioefficacy of mycelia based dust formulation

3.4.11.1 Field study

Charcoal based mycelia formulation of *F. oxysporum* was tested for its nematicidal activity under the field condition using RBD as an experimental design. The dust formulation was applied @ and mixed thoroughly with soil. Mung bean seeds were sown in field at the spacing of. Germination percentage, nematode count at root zone and yield parameters was recorded.. Control plots and plots treated with commercial formulation of carbofuron @ 2kg ha⁻¹ and 3 kg ha⁻¹ were also maintained along with the treated plots.

3.4.12 Compatibility study of isolated metabolites from *F. oxysporum* with commercial fungicides

Two of the fungicidally most active isolated metabolites were studied for their compatibility with the commercially used organic and inorganic fungicides for control of soil borne phytopathogenic fungi. The fungicides used for the experiment were captan, thiophanate-methyl, mancozeb, carbamorph, borax, sulphur dust and metalaxyl. The fungi used for the study were *S. rolfsii*, *R. bataticola*, *P. aphanidermatum*, *M. phaseolina* and *R. solani*.

3.4.12.1 Biological compatibility study

The fungitoxicity of the commercial fungicides were tested against *F. oxysporum* by poisoned food technique using PDA as the media for study. The growth pattern was recorded and LD₅₀ of the metabolites were calculated using probit package of MSTATC software.

3.4.12.2 *Chemical compatibility study:*

~~—~~ A stock solution (2000ppm) of the two active metabolites of *F. oxysporum* and commercial fungicides listed in section 3.4.11 were prepared in acetone-water. The resultant solution of mixture of two metabolites (1000 ppm) was prepared by mixing at 1:1 ratio. To this solution each and every fungicides were mixed at 1:1 ratio to get a resultant solution containing 500 ppm of both isolated compounds from fungi and 1000 ppm of commercial fungicide. Control solution of each fungicide (1000 ppm) and mixture of two active compounds (1000 ppm) were also kept. The samples were taken in a capped vial and stored at room temperature. Samples were drawn periodically and analysed by GC and HPLC. For each day, control and three replications containing mixture of fungicide and isolated compounds were maintained. The chemical compatibility was studied as a function of stability of isolated compounds and fungicides in mixture as compared to control with progress of time.

3.4.13 *Preparation of reagents required for derivatization/ intermediates etc*

Reagents such as nitrosomethyl urea, diazomethane, toluene-p-sulphonyl chloride, N-bromosuccinimide were prepared according to literature methods (Vogel).

Table 5. Incubation periods for pathogenic fungi used

Fungus	Incubation period
<i>Sclerotium rolfsii</i>	4
<i>Sclerotium sclerotiorum</i>	5-6
<i>Rhizoctonia solani</i>	5-6
<i>Rhizoctonia bataticola</i>	4-5
<i>Pythium aphanidermatum</i>	6-7
<i>Alternaria alternata</i>	7-8

4. Results and Discussions

4. *Fusarium oxysporum*

4.1 *Antagonism of F. oxysporum*

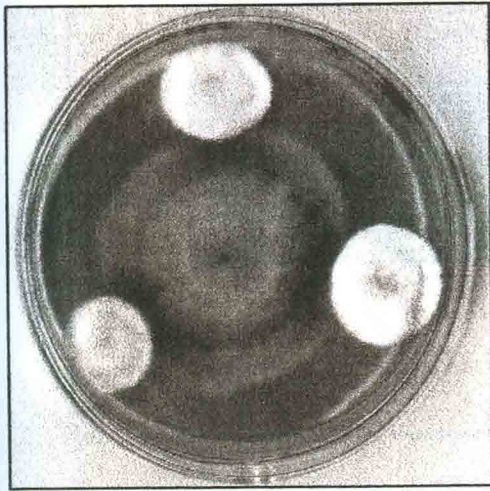
Fungicidal activity of *F.oxysporum* was evaluated by dual culture method against pathogenic fungi such as *Sclerotium rolfsii* Saccardo, *Rhizoctonia bataticola* (Taub.) Butler, *Pythium aphanidermatum* (Edson) Fitz. *Macrophomina phaseolina*, *Pythium debaryanum* and *Rhizoctonia solani* Nees. It increased the zone of inhibition, decreased sclerotia production and germination of the pathogen compared with the control. It was capable of lysing the mycelium of *Sclerotium rolfsii*, and *Rhizoctonia solani*. It also suppressed the growth of the pathogens and their capacity to colonize the area where the pathogen had grown. Moreover, all of the sclerotia developed were infected with the antagonist and did not germinate when seeded in PDA. The hyaline mycelium of *F. oxysporum* grew on the contact zone of pathogens. Gradually, this zone turned light pink and progressively antagonist proceeded forward till the colony of pathogen was completely overgrown. The fluffy growth of the antagonist intermixed with the mycelium of the pathogens. The intermingling pathogen in mycelium remained visible upto 10 days of incubation. This type of inhibition was observed with *P. debaryanum*, *R. bataticola* or *M. phaseolina* , but not on *Sclerotium rolfsii*. High degrees of antibiosis were observed against *S.*

sclerotiorum, which was marked as the formation of wide halozones. Antibiosis was also observed against *M. phaseolina*, *R. bataticola*, and *P. debaryanun* (Figure1). This confirms that non-pathogenic *F. oxysprum* is able to contro growth of a number of pathogenic fungi.

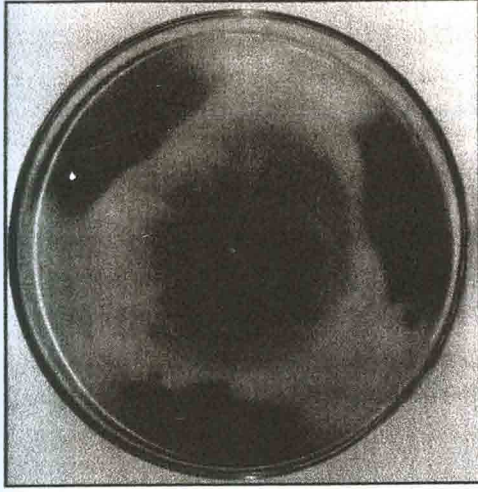
4.2 Selection of Culture media for Mass Culturing

4.2.1 Growth of *F. oxysporum* in different Culture media

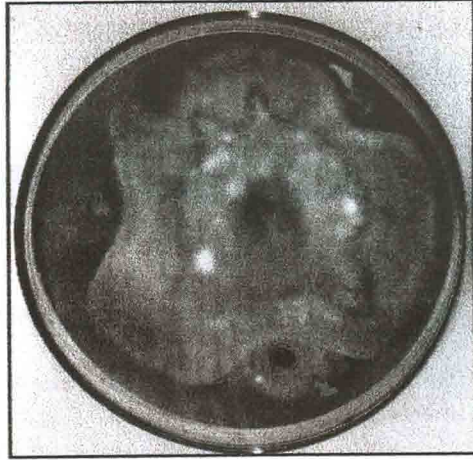
Synthetic media such as Ala-2, Potato Dextrose agar, Nutrient Agar, Yems media, Luria Agar, Malt Extract, BGA, Czepek dox and Rose Bengal have been tried for mass culturing of *F. oxysporum*. These media were inoculated with a mycelial disc of *F. oxysporum* and incubated in a BOD at 27⁰C for seven days. After seven days, radial growth of mycelial was measured in each media (Table 6). The maximum radial growth was observed in ALA II (8.9 cm) followed by SYP (8.7cm,), PDA (8.5 cm) and Luria broth (8.1cm) culture media. Whereas only 7.7 cm radial growth was observed in Nutrient agar, 5.1 cm in Czepek dox and 4.2 cm in malt extract media (Figure 2). Very less radial growth was observed in media such as Richard's media, rose Bengal and BGA II culture media. The difference in radial growth along with the intensity of the mat thickness and color proved that the choice of culture media has a prominent role in the growth behavior of *F. oxysporum*. (Figure 3)



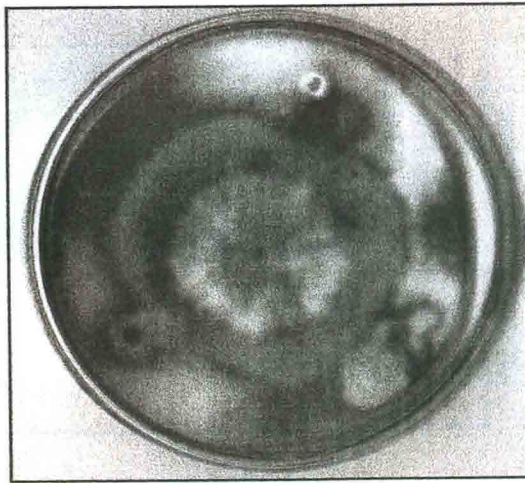
Sclerotium rolfsii



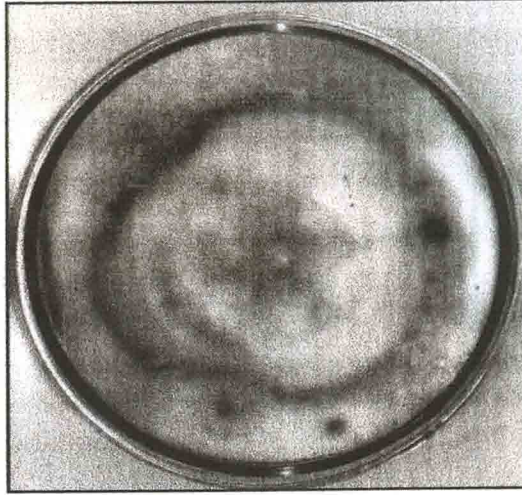
Macrophomina phaseolina



Pythium debaryanum



Rhizoctonia bataticola



Sclerotium sclerotiorum

Fig 1. Antagonistic activity of *Fusarium oxysporum* against r phytopathogenic fungi- dual culture study.

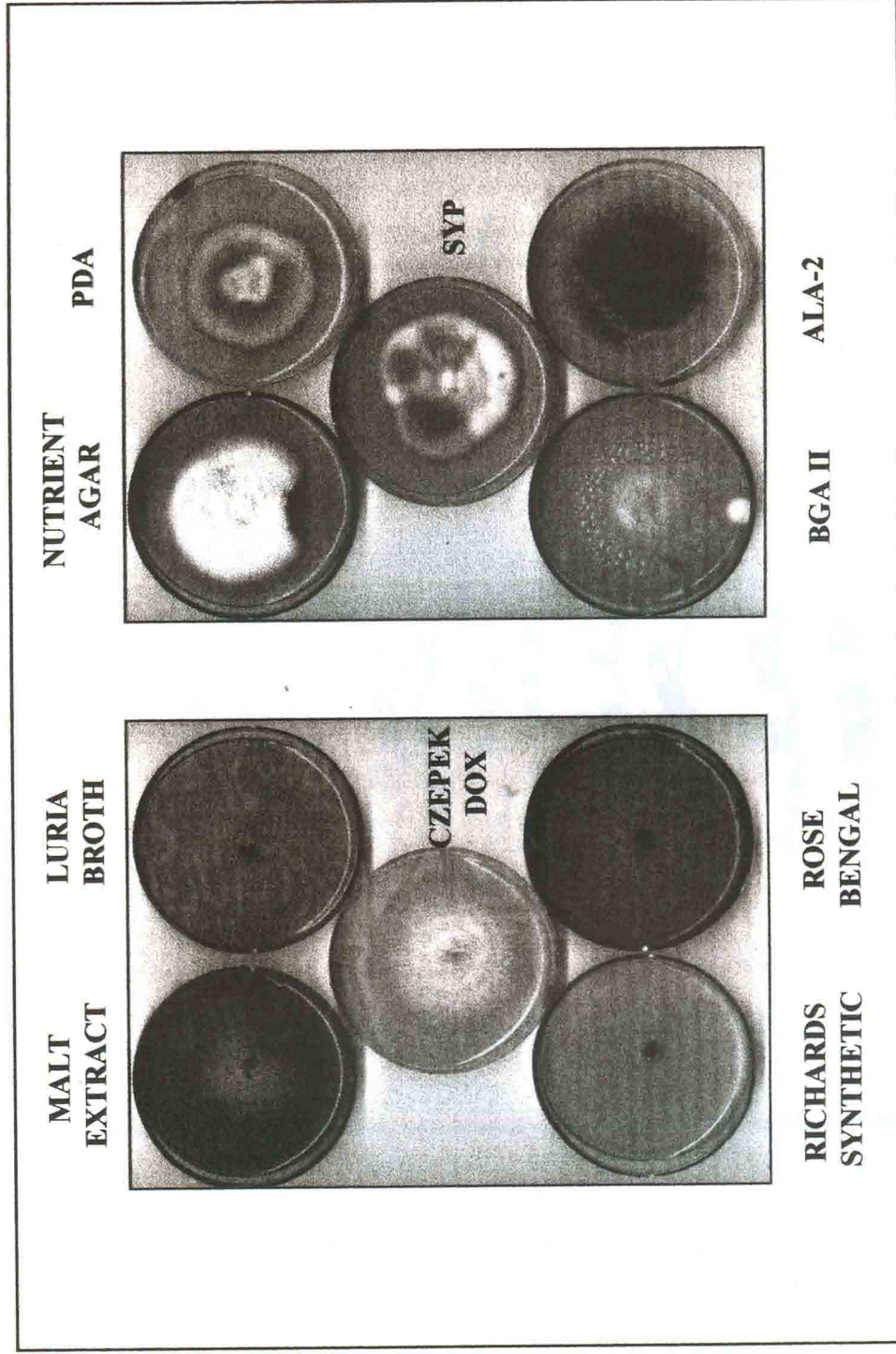


Fig 2. Effect of different media on growth of *Fusarium oxysporum*

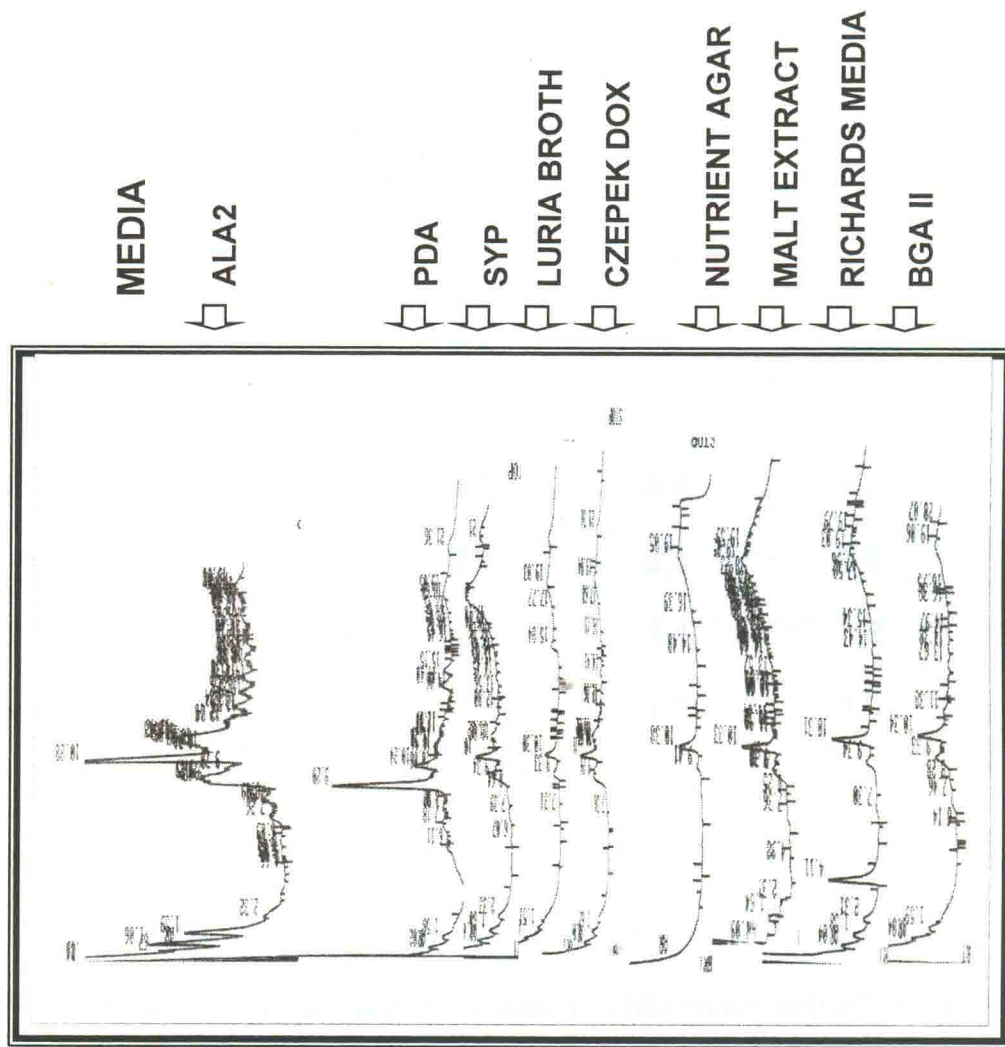


Fig 3: Chromatogram showing production of toxins by *F. oxysporum* in different media

Table 6. Radial Growth of *Fusarium oxysporum* in different culture media

Culture media	Radial growth of <i>F. oxysporum</i> (cm)			
	R1	R2	R3	Average
Ala-2	9.0	8.9	9.0	8.9
SYP	8.6	8.7	8.8	8.7
PDA	8.6	8.4	8.5	8.5
Luria Broth	8.1	8.2	8.1	8.1
Nutrient Agar	8.0	7.7	7.5	7.7
Vems media	6.0	6.5	6.3	6.2
Czepek dox	5.2	5.0	5.0	5.1
Malt Extract	4.0	4.5	4.3	4.2
Richard's media	2.1	2.3	2.0	2.1
BGAII	1.3	1.4	1.2	1.2
Rose Bengal	0.9	1.0	1.1	1.0

4.2.2 Effect of culture media on production of different toxins

The fungal mycelium was gently scrapped from a 7d old culture of *F. oxysporum* and extracted with chloroform. Chloroform extract after drying over sodium sulphate was concentrated and dissolved in 1ml of ethyl acetate and analyzed by GC-FID (Section 3.2.3). GLC chromatogram (Figure 3) showed the formation of different amount and different toxins in different media by *F.*

oxysporum. Maximum number and concentration of toxins/ secondary metabolites were observed in ALA II media followed by PDA and SYP media (Figure 2) when inoculated with *F. oxysporum* after seven days of incubation. Total of ten different toxins were observed from *F. oxysporum* (Figure 4). Out of these, nine different toxins were formed in ALA II media and 8 in PDA and Richard's media. Whereas minimum concentration of these toxins were observed in Luria Agar, Rose Bengal, Nutrient Agar, yem, Richard's and Malt Extract media. We can conclude that growth of a biocontrol agent, production and amount of secondary metabolites is dependent upon the culture media. Therefore selection of a media is very important.

4.2.3 Fungicidal activity of *F. oxysporum* cultured in different media

Residues obtained after extraction of fungal mat (scrapped from each media) with chloroform, were tested for fungicidal activity against *P. aphanidermatum* *in vitro* by poisoned food technique at different concentrations. The average percent inhibition of three replicates was calculated for each concentration and was corrected by Abbott's formula (Abbott; 1925). The ED₅₀ values for each media extract were calculated statistically by probit analysis (Finney; 1971). Maximum inhibition of *P. aphanidermatum* was shown by fungal mat extract of *F.oxysporum* when grown in ALA-II (ED₅₀ = 35ppm) (Figure 5) followed by PDA (ED₅₀ =40 ppm) and SYP (ED₅₀ =45 ppm) media. In the case of Malt Extract, Luria, Nutrient Agar and Richard media the LD₅₀ ranged between 80- 100 ppm (Figure 5). Minimum inhibition was observed when *F. oxysporum* was cultured in BGA II and Rose Bengal media (ED₅₀ = 140-150 ppm) (Figure5).

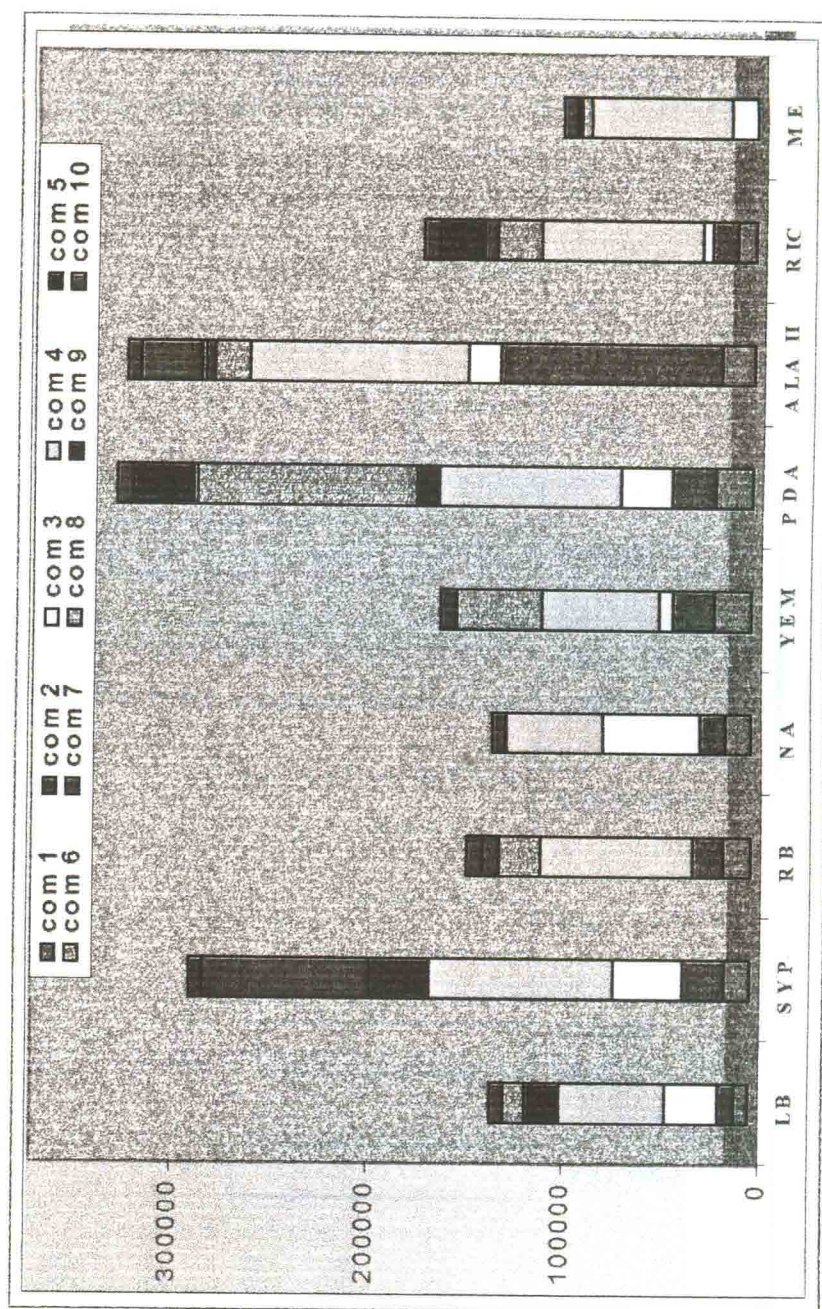


Fig 4. Effect of media on production of secondary metabolites

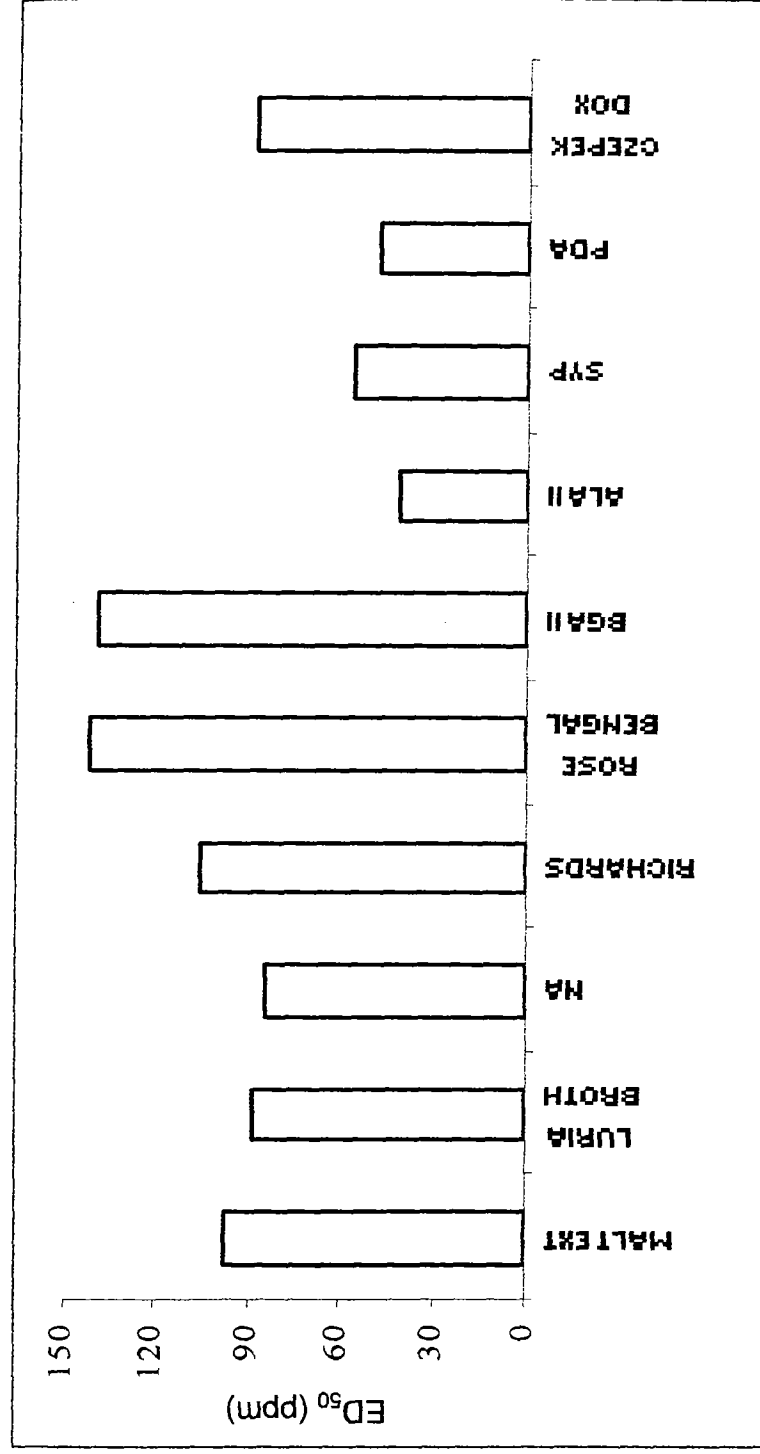


Fig 5. Correlation of antifungal activity of extracts obtained from different media inoculated with *Fusarium oxysporum* against *Pythium aphanidermatum*

We can conclude based on this study that the best media for radial growth of non-pathogenic *F. oxysporum*, toxin production and inhibition against pathogenic fungi can be ALA-II, PDA and SYP. In case if we consider the economics of production at a large scale, the best and cheap media will be PDA, which is at par with ALA-II and SYP media. Therefore further studies were carried out using PDA media.

4.2.4 Effect of days of incubation in PDA media on release of toxins

Metabolite production and fungicidal activity by *F. oxysporum* was measured by growing *F. oxysporum* in shake flasks (1000ml) containing PD broth (250 ml). The batches of *F. oxysporum* were harvested at different time interval of 5, 9, 15 and 21 days. The culture filtrate was separated from mycelial mass by filtering through muslin cloth and extracted with dichloromethane, whereas mycelia was dried and extracted with methanol. Dichloromethane and methanol were dried by distilled under reduced pressure and analyzed by GLC and HPLC, respectively.

Methanol extract of mycelia when analyzed by HPLC (6a) showed an increase in the production of toxins/metabolites from 5 days sample up to 15 days. The 5d sample showed production of one major metabolite at Rt 2.36 min, where as 9th day sample showed two metabolites with Rt 1.42 and 2.36 min with trace amount of toxins at Rt 3.78 and 6.16 min. The 15th day sample showed decrease in the concentration of metabolites, which appeared, at Rt 1.42 and at Rt 2.35 min, with increase in concentration of a metabolite at Rt 6.22 min. After

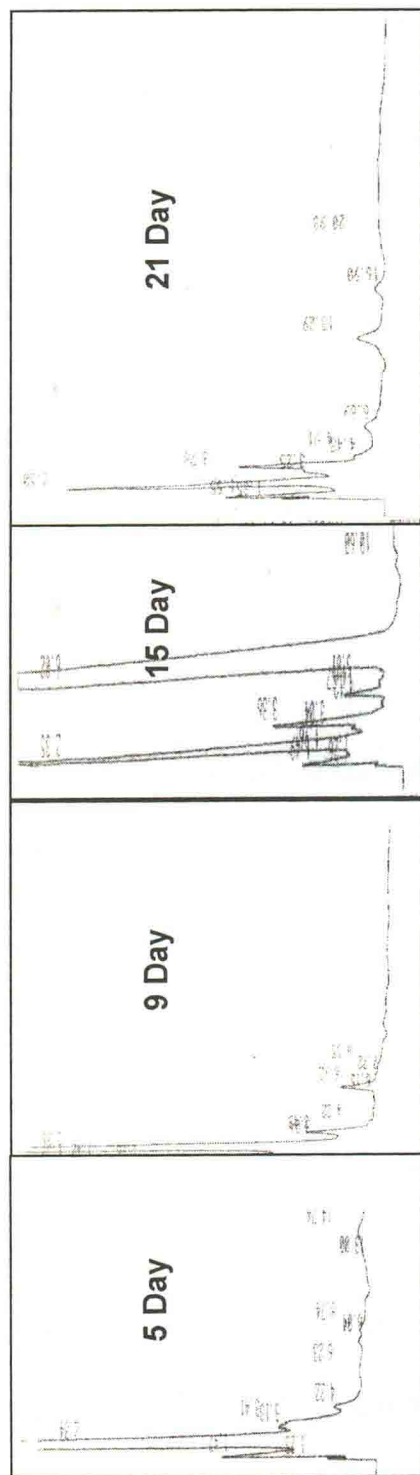


Fig 6a. Days of incubation vs formation of secondary metabolites in PD broth (Methanol extract, By HPLC)

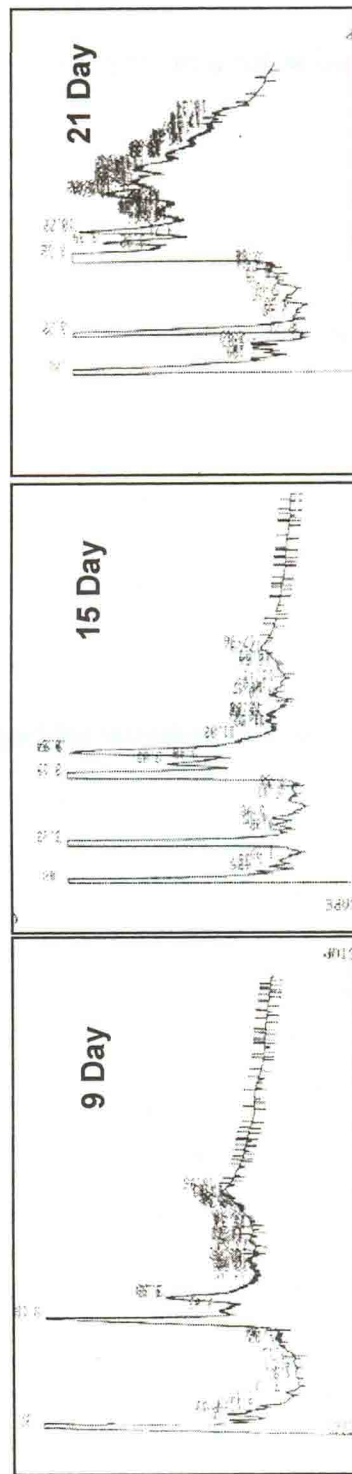


Fig 6b. Days of incubation vs formation of secondary metabolites in PD broth (Dichloromethane extract, By GLC)

and mycellia suspension. The flasks were incubated at $28 \pm 1^{\circ}\text{C}$ for 15 days. At the end of the incubation period the culture was harvested by filtering through two layers of muslin cloth. The culture filtrate was stored at 0°C until use. The mycelia mass was dried either by lyophilization or by drying in a fume cupboard.

4.4 Extraction

4.4.1 Culture filtrate

The liquid culture filtrate was then extracted by liquid partition with an equal volume of hexane (0.06 g L^{-1}), dichloromethane (0.17 g L^{-1}) and ethyl acetate (0.24 g L^{-1}) (Scheme 1). The combined organic layer was dried over anhydrous sodium sulphate and then evaporated on a rotary evaporator at $40\text{--}50^{\circ}\text{C}$ under reduced pressure. A portion of the concentrated residue was retained for biological studies and remaining used for isolation and purification of metabolites. (Scheme 1)

4.4.2 Mycelial mass

The hyphae separated by filtering the culture filtrate of 15 d old *F. oxysporum* culture broth were dried under shade for 7 days. The dried hyphae/biomass were powdered and extracted by soxhlet using solvents such as dichloromethane and methanol. The solvents were combined and evaporated separately under reduced pressure at $40\text{--}50^{\circ}\text{C}$. A portion of the concentrated residue was retained for biological activity and remaining was used for isolation of metabolites. (Scheme 1)

Table 7 a. Percent growth inhibition of various fungi caused by Dichloromethane culture filtrate extract of *Fusarium oxysporum* and its ED₅₀ value

Fungi	Dose (ppm)	Average % Growth Inhibition ± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Rhizoctonia bataticola</i>	500	71.0 ± 0.10	$Y = 3.04 + 0.91X$	0.56	2.14	141.1
	250	56.3 ± 0.36				
	125	47.4 ± 0.36				
	62.5	38.7 ± 0.16				
<i>Macrophomina phaseolina</i>	500	67.3 ± 0.40	$Y = 3.21 + 0.83X$	0.13	2.15	142.8
	250	57.4 ± 0.31				
	125	49.6 ± 0.55				
	62.5	37.5 ± 0.21				
<i>Scelerotium rolfsii</i>	500	69.7 ± 0.44	$Y = 3.35 + 0.78X$	0.49	2.09	124.8
	250	56.5 ± 0.50				
	125	51.0 ± 0.02				
	62.5	41.0 ± 0.20				
<i>Rhizoctonia solani</i>	500	67.4 ± 0.26	$Y = 3.10 + 0.88X$	0.46	2.16	145.8
	250	60.3 ± 0.67				
	125	45.3 ± 0.58				
	62.5	38.2 ± 0.49				
<i>Pythium aphanidermatum</i>	500	80.3 ± 0.67	$Y = 4.03 + 0.73X$	0.84	1.32	21.1
	250	71.9 ± 0.68				
	125	63.7 ± 0.58				
	62.5	56.3 ± 0.30				
<i>Pythium debaryanum</i>	31.25	53.0 ± 0.21	$Y = 4.04 + 0.65X$	0.78	1.47	29.9
	500	86.4 ± 0.17				
	250	76.1 ± 0.46				
	125	69.7 ± 0.36				
	62.5	63.9 ± 0.61				
	31.25	56.0 ± 0.55				

Table 7 b. Percent growth inhibition of various fungi caused by Ethyl acetate culture filtrate extract of *Fusarium oxysporum* and its ED₅₀ value

Fungi	Dose (ppm)	Average % Growth Inhibition ± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Rhizoctonia bataticola</i>	500	78.3 ± 0.46	$Y = 2.04 + 1.39X$	0.62	2.11	130.55
	250	65.2 ± 0.23				
	125	52.2 ± 0.20				
	62.5	30.7 ± 0.66				
<i>Macrophomina phaseolina</i>	500	90.1 ± 0.66	$Y = 0.80 + 1.95X$	5.70	2.14	138.9
	250	66.4 ± 0.32				
	125	38.8 ± 0.25				
	62.5	30.5 ± 0.89				
<i>Sclerotium rolfsii</i>	500	36.6 ± 0.40	$Y = 2.44 + 0.84X$	0.90	3.03	1083
	250	32.0 ± 0.15				
	125	23.1 ± 0.21				
	62.5	13.1 ± 0.21				
<i>Rhizoctonia solani</i>	500	67.4 ± 0.36	$Y = 0.90 + 1.65X$	2.53	2.47	300.3
	250	38.4 ± 0.40				
	125	29.5 ± 0.50				
	500	80.7 ± 0.67				
<i>Pythium aphanidermatum</i>	250	72.7 ± 0.67	$Y = 4.17 + 0.60X$	0.425	1.36	22.28
	125	65.1 ± 0.32				
	62.5	61.0 ± 0.10				
	31.25	54.1 ± 0.26				
<i>Pythium debaryanum</i>	500	78.1 ± 0.65	$Y = 4.21 + 0.57X$	0.44	1.38	24.43
	250	72.1 ± 0.10				
	125	63.3 ± 0.46				
	62.5	58.3 ± 0.42				
	31.25	54.1 ± 0.26				

4.5 Bioactivity of solvent extracts

4.5.1 Fungicidal activity

4.5.1.1 Culture filtrate extracts

The residues obtained after extraction of culture filtrate with dichloromethane and ethyl acetate were tested for fungicidal activity *in vitro* by means of poisoned food technique using potato-dextrose-agar-media (PDA) against *R. bataticola*, *M. phaseolina*, *S. rolfsii* (Saccardo,) Butler, *R. solani*, *P. aphanidermatum* (Edson) Fitz., and *P. debaryanum* at 500, 250, 125, 62.5 and 31.25 ppm concentrations. Fungitoxicity was evaluated in terms of corrected percent inhibition (CPI) and ED₅₀ value (Table 7). The dichloromethane extract was found to inhibit 38.7, 37.5, 41.0, 38.2, 56.3 and 63.9 per cent growth of *R. bataticola*, *M. phaseolina*, *S. rolfsii*, *P. aphanidermatum* and *P. debaryanum*, respectively at 62.5 ppm with ED₅₀s at 141.1, 142.0, 124.8, 145.8, 21.1 and 29.9 ppm, respectively (Table 7). Dichloromethane extract was found very active against *P. aphanidermatum* (21.1 ppm) and *P. debaryanum* (29.9 ppm) (Table 7). Ethyl acetate extract was found to inhibit 65.2, 66.4, 32.0, 38.4, 72.7 and 72.1 per cent growth of *R. bataticola*, *M. phaseolina*, *S. rolfsii*, *P. aphanidermatum* and *P. debaryanum*, respectively at 250 ppm with calculated ED₅₀s of 130.55, 138.9, 108.3, 300.3, 22.28 and 24.13 ppm, respectively (Table 7). Both culture filtrate extracts (dichloromethane and ethyl acetate) were found most effective against *P. aphanidermatum* and *P. debaryanum* (Figure 8a)

4.5.1.2 Mycelia extract

Hexane, dichloromethane and methanol extracts of mycelial mass were tested for antifungal activity. Hexane extract was found to inhibit 38.5, 26.7, 54.6, and 52.7 per cent growth of *R. bataticola*, *M. phaseolina*, *P. aphanidermatum* and *P. debaryanum*, respectively at 31.25 ppm with calculated ED₅₀s of 82.32, 271.8, 23.9 and 28.1 ppm, respectively. Average percent growth, regression equation, and ED₅₀ are reported in Table 8. The dichloromethane extract of mycelia was found most active against *R. bataticola* (ED₅₀ 24.5 ppm), *P. aphanidermatum* (ED₅₀ 28.40 ppm) and *P. debaryanum* (ED₅₀ 27.7 ppm) as compared to *M. phaseolina* (ED₅₀ 75.8 ppm), *S. rolfsii* (ED₅₀ 182.6ppm), and *R. solani* (ED₅₀ 104 ppm)(Table). The methanol extract was found to inhibit 60.00, 42.22, 40.00, 53.33, 57.78 and 60.00 percent growth of *R. bataticola*, *M. phaseolina*, *S. rolfsii*, *R. solani*, *P. aphanidermatum* and *P. debaryanum* at 125 ppm, respectively (Table 8, Figure 8b). It was found less effective than hexane and dichloromethane extract of mycelial mass of *F. oxysporum*. It shows that secondary metabolites/ toxins responsible for fungicidal activity are non polar compounds in nature.

4.5.2 Insecticidal activity

4.5.2.1 Culture filtrate and mycelia mass extracts

Insecticidal activity of dichloromethane and ethyl acetate extracts of culture filtrate and hexane and dichloromethane extracts of mycelia were tested

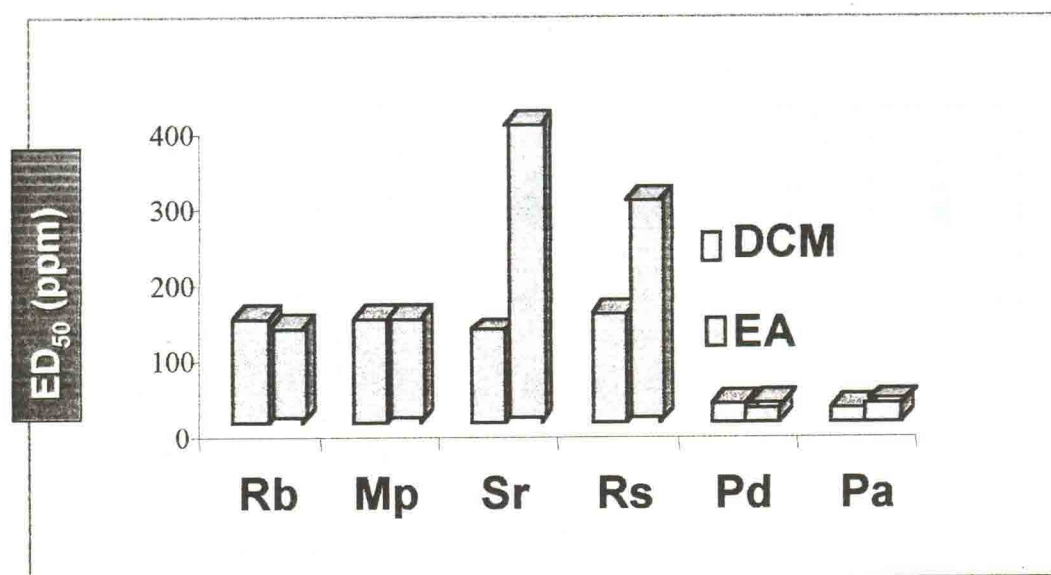


Fig 8a. Fungicidal activity of various solvent extracts of Culture filtrate of *Fusarium oxysporum*

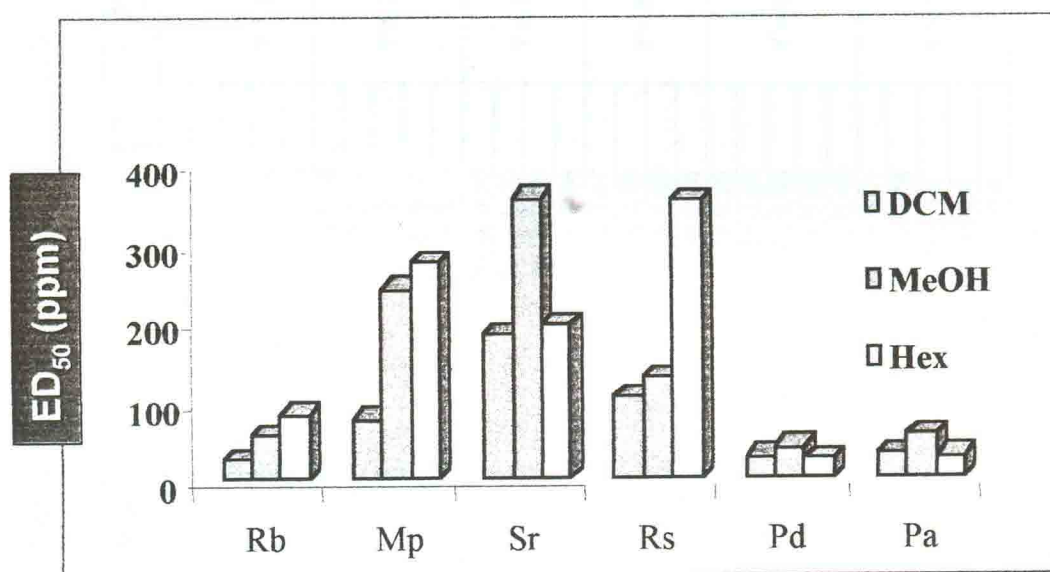


Fig 8b. Fungicidal activity of various solvent extracts of mycelial mass of *Fusarium oxysporum*

Table 8 a. Percent growth inhibition of various fungi caused by Hexane hyphal extract of *Fusarium oxysporum* and its ED₅₀ value

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Rhizoctonia bataticola</i>	500	67.7± 0.2	$Y = 3.78 + 0.637X$	0.25	1.91	82.32
	250	63.7± 0.81				
	125	54.7± 0.58				
	62.5	47.6± 0.61				
	31.25	38.5± 0.69				
<i>Macrophomina phaseolina</i>	500	57.2± 0.46	$Y = 3.34 + 0.680X$	0.063	2.43	271.8
	250	48.2± 0.75				
	125	41.0± 0.31				
	62.5	32.2± 0.35				
	31.25	26.7± 0.15				
<i>Sclerotium rolfsii</i>	500	60.4± 0.55	$Y = 3.55 + 0.632X$	0.036	2.28	194.5
	250	52.2± 0.30				
	125	45.8± 0.15				
	62.5	37.5± 0.66				
	500	45.6± 0.61				
<i>Rhizoctonia solani</i>	250	39.7± 0.58	$Y = 4.18 + 0.237X$	2.18	3.14	2598.3
	125	32.2± 0.60				
	62.5	38.9± 0.25				
	500	86.7± 0.25				
	250	74.6± 0.59				
<i>Pythium aphanidermatum</i>	125	70.6± 0.60	$Y = 3.95 + 0.759X$	1.21	1.38	23.9
	62.5	62.2± 0.60				
	31.25	54.6± 0.49				
	500	85.1± 0.81				
	250	74.5± 0.65				
<i>Pythium debelianum</i>	125	67.5± 0.4	$Y = 3.89 + 0.762X$	0.716	1.44	28.1
	62.5	60.4± 0.4				
	31.25	52.7± 0.4				
	500	85.1± 0.81				
	250	74.5± 0.65				

Table 8 b. Percent growth inhibition of various fungi caused by Dichloromethane hyphal extract of *Fusarium oxysporum* and its ED₅₀ value

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500	82.22 ± 1.21	$Y = 4.01 + 0.68X$	1.79	1.45	28.40
	250	74.44 ± 1.03				
	125	62.22 ± 0.09				
	62.5	57.78 ± 0.72				
	31.25	54.44 ± 0.63				
<i>Pythium deborianum</i>	500	81.11 ± 0.57	$Y = 4.04 + 0.66X$	1.04	1.44	27.70
	250	74.44 ± 0.62				
	125	64.44 ± 0.67				
	62.5	56.67 ± 1.02				
	31.25	54.44 ± 0.04				
<i>Rhizoctonia bataticola</i>	500	74.44 ± 0.26	$Y = 4.30 + 0.50X$	0.03	1.38	24.50
	250	68.89 ± 0.01				
	125	64.44 ± 1.03				
	62.5	57.78 ± 0.97				
	31.25					
<i>Macrophomina phaseoli</i>	500	70.00 ± 1.20	$Y = 3.68 + 0.69X$	0.98	1.88	75.80
	250	64.44 ± 0.33				
	125	60.00 ± 0.17				
	62.5	45.56 ± 0.91				
	31.25	38.89 ± 0.72				
<i>Sclerotium rolfsii</i>	500	58.89 ± 0.13	$Y = 3.88 + .49X$	0.01	2.26	182.6
	250	52.22 ± 0.12				
	125	46.67 ± 0.21				
	62.5	41.11 ± 0.76				
	500	74.44 ± 0.82				
<i>Rhizoctonia solani</i>	250	67.78 ± 0.01	$Y = 2.77 + 1.10X$	3.25	2.01	104
	125	60.00 ± 0.19				
	62.5	35.56 ± 0.93				
	31.25					

Table 8 c. Percent growth inhibition of various fungi caused by Methanol hyphal extract of *Fusarium oxysporum* and its ED₅₀ value

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500	78.89±0.62	$Y = 3.68 + 0.76X$	0.97	1.72	52.88
	250	70.00±0.63				
	125	57.78±0.92				
	62.5	51.11±1.13				
	31.25	45.56±1.05				
<i>Pythium debelianum</i>	500	78.89±0.57	$Y = 3.88 + 0.69X$	1.18	1.60	39.96
	250	72.22±0.62				
	125	60.00±0.93				
	62.5	53.33±0.02				
	31.25	50.00±0.11				
<i>Rhizoctonia bataticola</i>	500	72.22±1.02	$Y = 3.23 + 0.89X$	0.75	1.98	96.50
	250	67.78±1.03				
	125	60.00±0.29				
	62.5	52.22±0.27				
	31.25	43.33±0.52				
<i>Macrophomina phaseoli</i>	500	57.78±0.63	$Y = 3.60 + 0.58X$	1.49	2.38	239.60
	250	47.78±1.02				
	125	42.22±0.91				
	62.5	34.44±0.22				
	31.25					
<i>Sclerotium rolfsii</i>	500	54.44±0.33	$Y = 3.43 + 0.62X$	0.07	2.54	344.00
	250	45.56±0.11				
	125	40.00±0.27				
	62.5	32.22±0.29				
	500	72.22±0.36				
<i>Rhizoctonia solani</i>	250	63.33±0.44	$Y = 2.74 + 1.07X$	0.99	2.10	126.7
	125	53.33±0.47				
	62.5	34.44±0.59				
	31.25					

against 2nd instar larvae of *Spodoptera litura*. Culture extracts were found more effective than hyphae extracts (Table 9, Figure 9).

4.5.3 Nematicidal activity

4.5.3.1 Culture filtrate and mycelia extracts

Nematicidal activity of culture filtrate (dichloromethane, ethyl acetate extracts) and mycelial extracts (dichloromethane, methanol and hexane extracts) was evaluated against J2 of *R. reniformis* and *M. incognita* after 24, 48 and 72 hours of incubation period. Hexane extract was found effective among the different hyphal extracts tested after 24 h of incubation, (ED₅₀ 188 and 218 ppm, against *R. reniformis* and *M. incognita*, respectively) Among the different culture filtrate extracts, ethyl acetate extract showed better nematicidal activity than dichloromethane extract. (ED₅₀ 249 and 240 ppm) against both *R. reniformis* and *M. incognita*, respectively. Average mortality, regression equation and LD₅₀ are reported in Table 10. Hexane extract of hyphae showed 97.7 percent mortality at 500 ppm as compared 48.1 and 25.6 percent mortality with methanol and dichloromethane extracts, respectively at 500 ppm against *R. reniformis*. Both dichloromethane and ethyl acetate extracts of culture filtrate showed 79.9 and 73.9 percent mortality at 500 ppm respectively after 24 hours (Table 10).

Hexane extract of mycelia showed 99.8 percent mortality even after 48 and 72 hours of incubation against both the nematodes (Table 10) (ED₅₀ 132 – 188). Thus it was observed that non-polar fraction contains toxins, which are responsible for nematicidal activity.

Table 9. Biological activity of various hyphal and culture filtrate extracts of *Fusarium oxysporum* and its LD₅₀ value against 2nd instar larvae of *Spodoptera litura*

EXTRACT	Concentration (ppm)	Avg. mortality (%) $\pm$ S.D.	Regression equation	χ^2	Log LD ₅₀	LD ₅₀ (ppm)
Dichloromethane (hypha)	2000	58.2 $\pm$ 0.26	Y = 3.26 + 0.57X	0.84	3.05	1113.6
	1000	45.7 $\pm$ 0.26				
	500	41.2 $\pm$ 0.40				
	250	37.4 $\pm$ 0.42				
	125	29.3 $\pm$ 0.20				
Dichloromethane (culture filtrate)	2000	91.5 $\pm$ 0.15	Y = 2.07 + 1.33X	1.29	2.19	154.60
	1000	87.4 $\pm$ 0.50				
	500	78.1 $\pm$ 0.79				
	250	58.4 $\pm$ 0.23				
	125	55.1 $\pm$ 0.40				
Ethyl acetate (Culture filtrate)	2000	95.5 $\pm$ 0.61	Y = 2.27 + 1.40X	4.79	1.93	86.78
	1000	95.8 $\pm$ 0.15				
	500	83.3 $\pm$ 0.58				
	250	79.5 $\pm$ 0.40				
	125	55.1 $\pm$ 0.40				
Hexane (hyphae)	2000	37.4 $\pm$ 0.50	Y = 1.58 + 0.91X	1.57	3.74	5505
	1000	20.7 $\pm$ 0.50				
	500	29.3 $\pm$ 0.20				
	250	12.4 $\pm$ 0.11				

*Avg. Mortality is the average of three replicates.

Table 10 a. Nematotoxic effect of *Fusarium oxysporum* extracts against J2 of *Rotylenchulus reniformis* at 24 hours

Extract	Dose(ppm)	Average mortality(%) $\pm$ S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
Dichloromethane (Culture filtrate)	1000	78.0 $\pm$ 0.10	Y=0.21+1.95X	11.3	2.46	290.13
	500	79.9 $\pm$ 0.15				
	250	44.3 $\pm$ 0.58				
	125	24.1 $\pm$ 0.31				
	62.5	9.3 $\pm$ 0.27				
Dichloromethane (Hyphae)	1000	49.0 $\pm$ 0.50	Y=-0.80+1.92X	1.52	3.01	1036.9
	500	25.6 $\pm$ 0.79				
	250	14.1 $\pm$ 0.42				
	125	2.30 $\pm$ 0.45				
	62.5	1.25 $\pm$ 0.33				
Ethyl acetate (Culture filtrate)	1000	99.1 $\pm$ 0.90	Y=-1.42+2.82X	7.22	2.39	248.89
	500	3.9 $\pm$ 0.38				
	250	47.8 $\pm$ 0.29				
	125	49.8 $\pm$ 0.27				
	62.5	8.1 $\pm$ 0.32				
Methanol (Hyphae)	1000	58.3 $\pm$ 0.47	Y=0.73+1.52X	1.57	2.80	638.6
	500	48.1 $\pm$ 0.42				
	250	27.1 $\pm$ 0.11				
	125	14.4 $\pm$ 0.02				
	62.5	5.10 $\pm$ 0.49				
Hexane (Hyphae)	1000	99.9 $\pm$ 0.15	Y=-2.92+3.48X	15.4	2.27	188.20
	500	97.7 $\pm$ 0.21				
	250	59.9 $\pm$ 0.15				
	125	19.5 $\pm$ 0.10				
	62.5	10.4 $\pm$ 0.50				

Table 10 b. Nematotoxic effect of Fusarium oxysporum extracts against J2 of *Meloidogyne incognita* at 24 hours

Extract	Dose(ppm)	Average mortality(%) $\pm$ S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
Dichloromethane (Culture filtrate)	1000	79.3 $\pm$ 0.35	Y=0.26+1.91X	3.85	2.48	304.0
	500	73.1 $\pm$ 0.98				
	250	43.5 $\pm$ 1.22				
	125	22.0 $\pm$ 0.62				
	62.5	8.70 $\pm$ 0.20				
Dichloromethane (Hyphae)	1000	56.3 $\pm$ 0.49	Y=-0.85+1.96X	4.62	2.97	942.5
	500	22.0 $\pm$ 0.15				
	250	14.4 $\pm$ 0.40				
	125	6.02 $\pm$ 0.12				
	62.5	0.48 $\pm$ 0.45				
Ethyl acetate (Culture filtrate)	1000	99.8 $\pm$ 0.26	Y=-1.66+2.79X	10.26	2.38	240.5
	500	80.3 $\pm$ 1.05				
	250	41.9 $\pm$ 0.06				
	125	22.2 $\pm$ 0.27				
	62.5	8.49 $\pm$ 0.38				
Methanol (Hyphae)	1000	67.3 $\pm$ 0.20	Y=-1.34+2.53X	26.1	2.51	324.1
	500	54.1 $\pm$ 0.21				
	250	32.0 $\pm$ 0.10				
	125	21.0 $\pm$ 0.10				
	62.5	4.70 $\pm$ 0.10				
Hexane (Hyphae)	1000	99.9 $\pm$ 0.11	Y=-2.89+3.37X	22.7	2.33	217.8
	500	95.2 $\pm$ 0.15				
	250	45.3 $\pm$ 0.61				
	125	16.3 $\pm$ 0.66				
	62.5	9.03 $\pm$ 0.11				

Table 10 c. Nematotoxic effect of Fusarium oxysporum extracts against J2 of *Rotylenchulus reniformis* at 48 hours

Extract	Dose(ppm)	Average mortality(%)±S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
Dichloromethane (Culture filtrate)	1000	96.2±0.20	Y=1.16+2.45X	7.02	2.51	326.36
	500	62.0±0.10				
	250	28.3±0.10				
	125	16.1±0.26				
	62.5	8.23±0.25				
Dichloromethane (Hyphae)	1000	85.2±0.31	Y=-0.11+2.07X	1.87	2.47	397.3
	500	72.2±0.44				
	250	38.2±0.31				
	125	23.0±0.36				
	62.5	8.29±0.29				
Ethyl acetate (Culture filtrate)	1000	90.6±0.75	Y=-0.52+2.29X	0.88	2.41	257.58
	500	74.1±0.31				
	250	49.6±0.57				
	125	26.1±0.15				
	62.5	6.13±0.70				
Methanol (Hyphae)	1000	99.4±0.95	Y=-2.91+3.42X	8.02	2.31	205.6
	500	94.7±0.15				
	250	55.9±0.15				
	125	19.3±0.23				
	62.5	7.40±0.61				
Hexane (Hyphae)	1000	99.8±0.21	Y=-2.76+3.41X	22.39	2.27	188.7
	500	99.8±0.10				
	250	50.6±0.15				
	125	27.1±0.20				
	62.5	9.27±0.15				

Table 10 d. Nematotoxic effect of Fusarium oxysporum extracts against J2 of *Meloidogyne incognita* at 24 hours

Extract	Dose(ppm)	Average mortality(%±S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
Dichloromethane (Culture filtrate)	1000	95.9±0.65	Y=-0.63+2.32X	6.89	2.42	264.7
	500	70.6±0.71				
	250	40.3±0.58				
	125	23.9±0.67				
	62.5	9.97±0.21				
Dichloromethane (Hyphae)	1000	86.1±0.58	Y=0.37+1.97X	8.45	2.35	223.0
	500	82.7±0.55				
	250	49.3±0.36				
	125	37.1±0.15				
	62.5	10.1±0.20				
Ethyl acetate (Culture filtrate)	1000	94.0±0.90	Y=-1.25+2.58X	7.34	2.42	264.2
	500	70.0±0.85				
	250	57.7±0.76				
	125	16.1±0.31				
	62.5	5.20±0.20				
Methanol (Hyphae)	1000	99.6±0.55	Y=-2.56+3.25X	14.24	2.32	211.8
	500	94.2±0.55				
	250	47.1±0.23				
	125	21.2±1.0				
	62.5	8.40±0.15				
Hexane (Hyphae)	1000	99.9±0.05	Y=-2.81+3.43X	21.62	2.27	188.1
	500	99.8±0.20				
	250	52.4±0.55				
	125	25.1±0.15				
	62.5	9.80±0.15				

Table 10 e. Nematotoxic effect of Fusarium oxysporum extracts against J2 of *Rotylenchulus reniformis* at 72 hours

Extract	Dose(ppm)	Average mortality(%±S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
Dichloromethane (Culture filtrate)	1000	89.5±0.35	$Y = -0.879 + 1.85X$	19.3	2.20	166.7
	500	78.7±1.02				
	250	68.4±0.45				
	125	54.1±0.32				
	62.5	10.1±0.32				
Dichloromethane (Hyphae)	1000	94.1±0.26	$Y = 1.05 + 2.59X$	6.21	2.34	217.1
	500	88.3±0.57				
	250	54.3±0.21				
	125	20.9±0.96				
	62.5	11.2±0.33				
Ethyl acetate (Culture filtrate)	1000	99.6±0.55	$Y = -0.71 + 2.43X$	10.5	2.35	225.2
	500	73.9±0.66				
	250	47.8±0.29				
	125	31.0±0.20				
	62.5	10.1±0.55				
Methanol (Hyphae)	1000	99.9±0.23	$Y = -2.74 + 3.08X$	25.5	2.51	327.5
	500	68.1±0.50				
	250	23.2±0.68				
	125	11.2±0.66				
	62.5	5.10±0.20				
Hexane (Hyphae)	1000	99.9±0.05	$Y = -2.15 + 3.37X$	6.30	2.12	132.8
	500	99.7±0.20				
	250	80.7±0.98				
	125	39.3±0.31				
	62.5	18.2±0.10				

Table 10 f. Nematotoxic effect of Fusarium oxysporum extracts against J2 of *Meloidogyne incognita* at 24 hours

Extract	Dose(ppm)	Average mortality(%) $\pm$ S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
Dichloromethane (Culture filtrate)	1000	90.2 $\pm$ 0.78	Y=0.68+1.97X	10.97	2.18	152.7
	500	86.4 $\pm$ 0.46				
	250	71.6 $\pm$ 0.55				
	125	50.2 $\pm$ 0.62				
	62.5	14.4 $\pm$ 0.45				
Dichloromethane (Hyphae)	1000	94.6 $\pm$ 0.15	Y=1.05+2.61X	8.26	2.31	207.8
	500	91.5 $\pm$ 0.32				
	250	52.5 $\pm$ 0.15				
	125	24.6 $\pm$ 0.42				
	62.5	11.7 $\pm$ 0.20				
Ethyl acetate (Culture filtrate)	1000	99.6 $\pm$ 2.55	Y=-0.79+2.37X	23.3	2.44	276.6
	500	66.9 $\pm$ 0.87				
	250	32.1 $\pm$ 0.93				
	125	23.1 $\pm$ 0.36				
	62.5	11.2 $\pm$ 0.31				
Methanol (Hyphae)	1000	99.6 $\pm$ 0.36	Y=-1.77+2.71X	18.9	2.50	316.6
	500	61.0 $\pm$ 0.20				
	250	31.0 $\pm$ 0.12				
	125	17.1 $\pm$ 0.20				
	62.5	5.23 $\pm$ 0.31				
Hexane (Hyphae)	1000	99.9 $\pm$ 0.10	Y=-2.15+3.37X	6.41	2.12	132.6
	500	99.8 $\pm$ 0.16				
	250	80.6 $\pm$ 0.64				
	125	39.4 $\pm$ 0.32				
	62.5	18.2 $\pm$ 0.15				

4.5.4 Plant growth regulatory activity

4.5.4.1 Culture filtrate

Culture filtrate extracts were tested for their effects on wheat seed germination. Dichloromethane and ethyl acetate extracts showed 90 and 95 percent germination respectively at 100-ppm concentration as compared to control (88 %). At 1500-ppm concentration, the germination percent was found to be 58 as compared to 88 in the control. It was observed that at low concentration the germination percent was more than control, but as the concentration increased from 250 to 1500 ppm, there was reduction in germination percent. (Figure 10 a) Similar observations were recorded in the case of shoot length and root length. Shoot and root length with dichloromethane extract at 100 ppm was found to be 13.1 and 4.1 cm as compared to control 13.1 and 4.6 cm respectively. Whereas at higher concentrations (250 and above) there was reduction in root and shoot length. (Table 11). Vigour index at 100 ppm was found to be 1591 as compared to control 1557. Whereas at higher concentrations vigour index varied from 1334 to 644. Dichloromethane extract showed phytotoxicity at higher concentrations (Table 11, Figure 10 b)

Ethyl acetate extract was found more effective up to 500 ppm concentration (95-93 percent germination). Increase in root and shoot length also was observed as compared to control. It did not show phytotoxicity and vigour index ranged from 2109 to 2204 (Table 11, Figure 10 c)

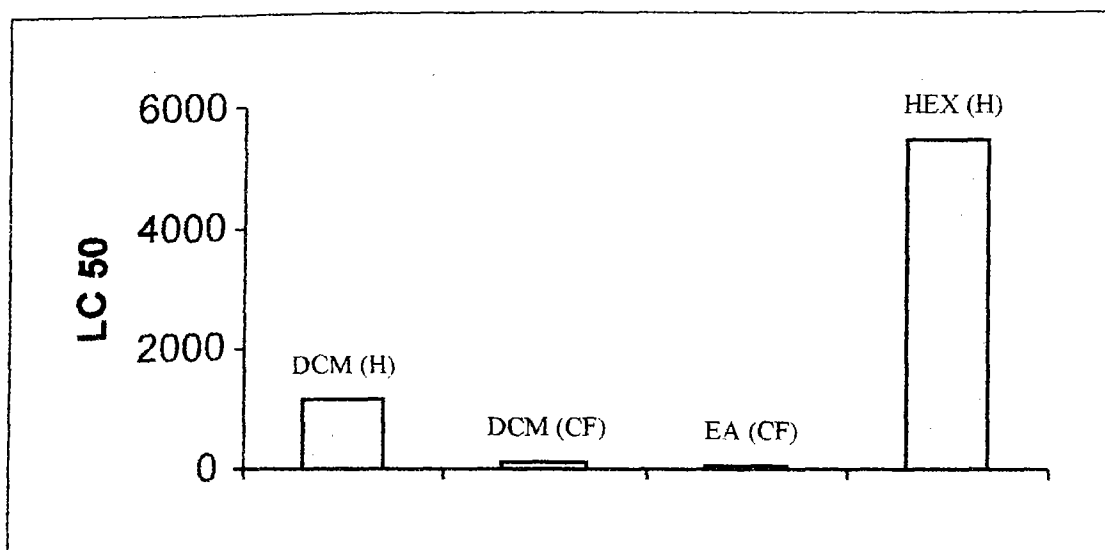


Fig 9. Insecticidal activity of various solvent extracts of Culture filtrate of *Fusarium oxysporum*

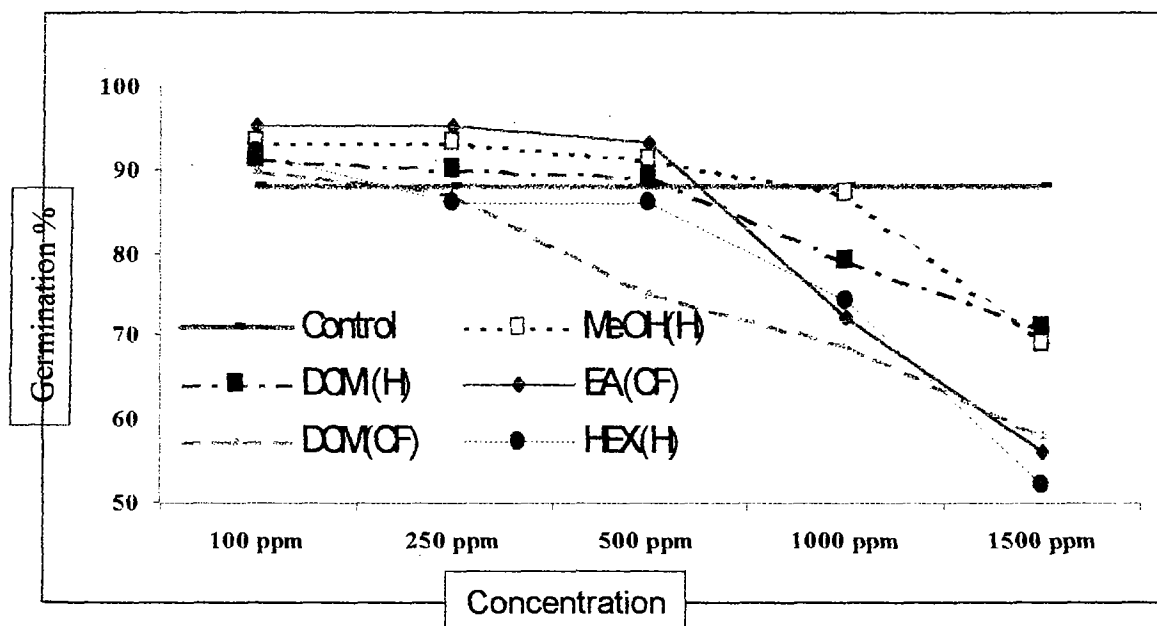


Fig 10 a. Effect of various solvent extracts of Culture filtrate of *Fusarium oxysporum* on germination of wheat

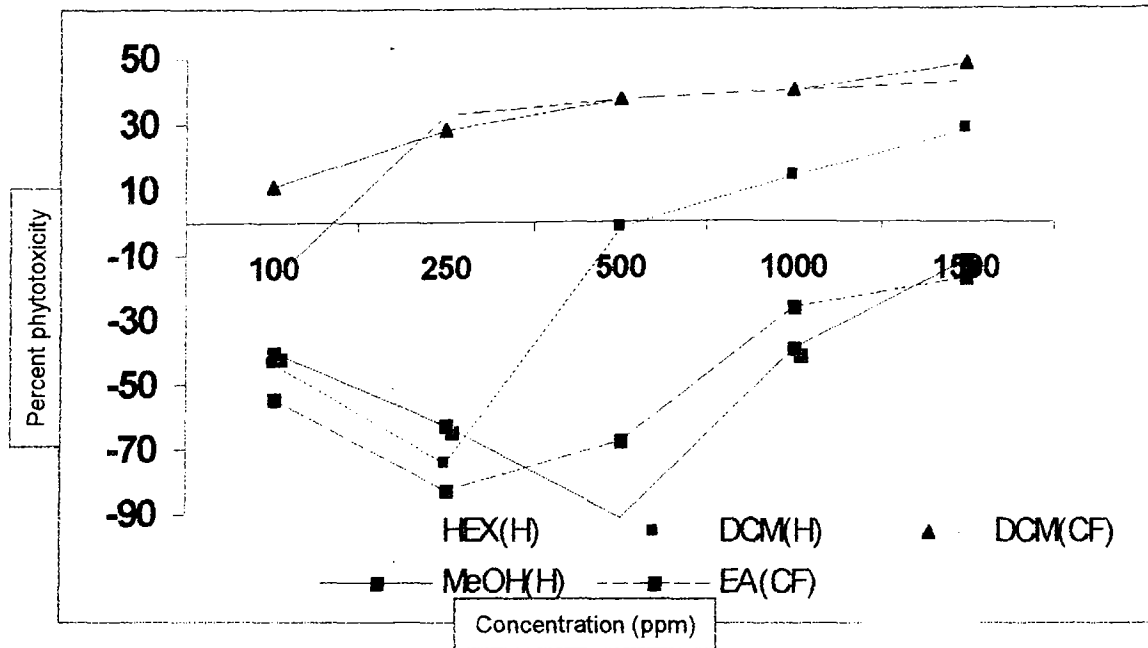


Fig 10 b. Effect of various solvent extracts of *Fusarium oxysporum* on % phytotoxicity of wheat

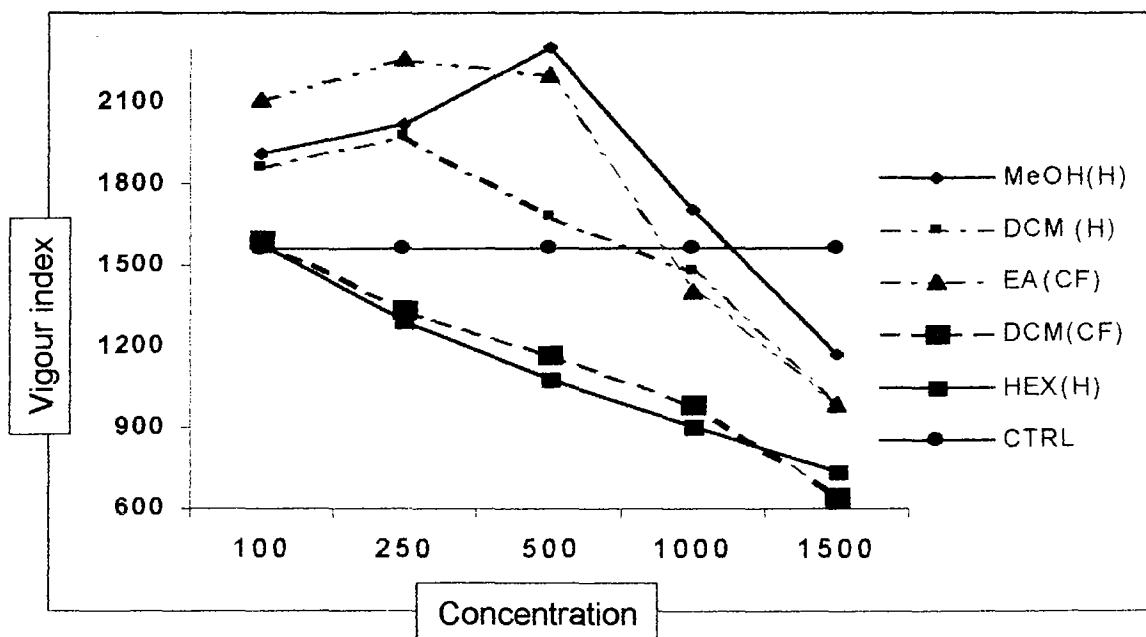


Fig 10 c. Effect of various solvent extracts of *Fusarium oxysporum* on vigour index of wheat

Table 11. Plant growth regulatory activity of different culture filtrate extracts of *Fusarium oxysporum* on wheat seeds

Extract	Concentration (ppm)	Germination % (Mean $\pm$ S.D.)	Shoot length (cm) (Mean $\pm$ S.D.)	Root length (cm) (Mean $\pm$ S.D.)	Phytotoxicity (%)	Vigour index
Dichloromethane	Control	88 $\pm$ 1.20	13.1 $\pm$ 0.28	4.6 $\pm$ 0.21	00.00	1557.6
	100	90 $\pm$ 0.96	13.1 $\pm$ 0.89	4.1 $\pm$ 0.33	10.86	1591.6
	250	87 $\pm$ 1.12	12.1 $\pm$ 0.61	3.3 $\pm$ 0.48	28.26	1333.0
	500	75 $\pm$ 0.83	10.7 $\pm$ 0.61	2.9 $\pm$ 0.13	36.95	1169.6
	1000	69 $\pm$ 0.72	10.5 $\pm$ 0.24	2.7 $\pm$ 0.15	39.85	984.20
	1500	58 $\pm$ 0.21	10.0 $\pm$ 1.10	2.3 $\pm$ 0.06	48.55	644.80
Ethyl acetate	Control	88 $\pm$ 0.29	13.1 $\pm$ 0.72	4.6 $\pm$ 0.12	00.00	1557.6
	100	95 $\pm$ 0.71	15.1 $\pm$ 0.85	7.1 $\pm$ 0.31	-54.34	2109.0
	250	95 $\pm$ 1.21	15.4 $\pm$ 0.84	8.4 $\pm$ 0.02	-82.60	2261.0
	500	93 $\pm$ 1.18	15.9 $\pm$ 1.20	7.7 $\pm$ 0.09	-67.39	2204.1
	1000	72 $\pm$ 0.03	13.7 $\pm$ 0.35	5.8 $\pm$ 0.01	-26.08	1404.0
	1500	56 $\pm$ 0.23	12.1 $\pm$ 0.47	5.4 $\pm$ 0.07	-17.39	985.60

*Germination %, Shoot length and Root length are the average of three replicates.

4.5.4.2 *Mycelia extract*

Methanol, dichloromethane and hexane extracts of mycelia were tested for plant growth activity at 1500, 100, 500, 250 and 100ppm concentrations. The percent germination at 500 – 100 ppm concentration was found to be 93-87 percent in the case of methanol, dichloromethane and hexane extracts as compared to control (88%). A slight increase in shoot length and root length was also observed as compared to control (Table 12). No phytotoxicity was recorded with methanol and dichloromethane extracts (Table 12). Whereas a decrease in root and shoot length was recorded in the case of hexane extract at all the tested concentrations. Phytotoxicity was observed at higher concentrations with hexane extract. A higher value of vigour index was observed with all extracts as compared to control (Table 12).

4.5.5 *Herbicidal activity against Parthenium hysterophorous*

4.5.5.1 *Culture filtrate and mycelia extracts*

Different culture filtrate and mycelia mass extracts were tested against *P. hysterophorous* at different concentrations. All the extracts showed a significant inhibition of seed germination of *P. hysterophorous* as compared to control at all test concentrations. Methanol extract showed the maximum germination inhibition followed by ethyl acetate culture filtrate and dichloromethane culture filtrate extracts (IC_{50} 180-600 ppm). Whereas hexane and dichloromethane extracts of mycelia showed germination inhibition at higher concentrations. (Figure 11, Table 13)

Table 12. Plant growth regulatory activity of different mycellial extracts of *Fusarium oxysporum* on wheat seeds

Extract	Concentration (ppm)	Germination % (Mean $\pm$ S.D.)	Shoot length (cm) (Mean $\pm$ S.D.)	Root length (cm) (Mean $\pm$ S.D.)	Phytotoxicity (%)	Vigour index
Methanol	Control	88 $\pm$ 1.30	13.1 $\pm$ 0.29	4.6 $\pm$ 0.43	00.00	1557.6
	100	93 $\pm$ 0.29	14.1 $\pm$ 0.31	6.4 $\pm$ 0.61	-39.85	1906.5
	250	93 $\pm$ 0.74	14.1 $\pm$ 1.10	7.5 $\pm$ 0.21	-63.04	2018.1
	500	91 $\pm$ 0.89	15.5 $\pm$ 0.96	8.7 $\pm$ 1.40	-90.57	2304.0
	1000	87 $\pm$ 1.13	13.0 $\pm$ 0.78	6.4 $\pm$ 0.02	-39.13	1696.5
	1500	69 $\pm$ 1.20	11.7 $\pm$ 0.22	5.1 $\pm$ 0.09	-11.59	1166.1
Dichloromethane	Control	88 $\pm$ 0.96	13.1 $\pm$ 0.24	4.6 $\pm$ 0.13	00.00	1557.6
	100	91 $\pm$ 1.12	13.7 $\pm$ 0.78	6.6 $\pm$ 0.41	-43.47	1856.4
	250	90 $\pm$ 0.65	13.8 $\pm$ 0.56	8.0 $\pm$ 0.36	-74.63	1971.0
	500	89 $\pm$ 0.06	14.1 $\pm$ 0.38	4.7 $\pm$ 0.17	-2.173	1673.2
	1000	79 $\pm$ 0.56	14.7 $\pm$ 1.04	3.9 $\pm$ 0.19	13.76	1477.3
	1500	71 $\pm$ 0.98	10.6 $\pm$ 0.93	3.3 $\pm$ 0.72	28.26	986.90
Hexane	Control	88 $\pm$ 1.15	13.1 $\pm$ 0.42	4.6 $\pm$ 0.84	00.00	1557.6
	100	92 $\pm$ 0.12	12.1 $\pm$ 0.36	5.4 $\pm$ 1.21	-17.39	1856.4
	250	86 $\pm$ 0.58	11.7 $\pm$ 0.11	3.1 $\pm$ 0.17	32.60	1971.0
	500	86 $\pm$ 0.22	11.4 $\pm$ 0.17	2.9 $\pm$ 0.19	36.95	1673.2
	1000	74 $\pm$ 0.19	10.3 $\pm$ 1.20	2.7 $\pm$ 0.01	39.85	1477.3
	1500	52 $\pm$ 1.03	10.0 $\pm$ 1.19	2.6 $\pm$ 0.02	42.75	986.90

*Germination %, Shoot length and Root length are the average of three replicates.

Table 13. Herbicidal activity of various extracts of *Fusarium oxysporum* against *Phalaris minor*

Extract	Dose(ppm)	Average %Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
Hexane(H)	1500	42.02±0.98	Y=0.55+1.28X	4.15	3.45	2865.76
	1000	20.68±0.54				
	500	18.4±1.10				
	250	10.3±0.73				
	100	2.29±0.24				
DCM(H)	1500	39.10±1.03	Y=0.81+1.20X	1.95	3.454	2845.9
	1000	25.3±0.73				
	500	20.7±0.12				
	250	8.00±0.09				
	100	4.59±1.17				
DCM(CF)	1500	55.10±0.015	Y=1.35+1.17X	3.79	3.09	1242.46
	1000	41.40±0.05				
	500	32.2±1.24				
	250	26.4±0.36				
	100	6.89±0.73				
Ethyl acetate(CF)	1500	63.2±0.86	Y=2.38+0.929X	2.23	2.80	644.52
	1000	52.9±1.51				
	500	51.7±0.41				
	250	35.60±0.54				
	100	20.68±0.62				
Methanol(CF)	1500	75.9±0.23	Y=2.51+0.93X	6.11	2.65	456.06
	1000	58.6±0.81				
	500	43.6±0.76				
	250	41.4±0.44				
	100	29.8±1.09				

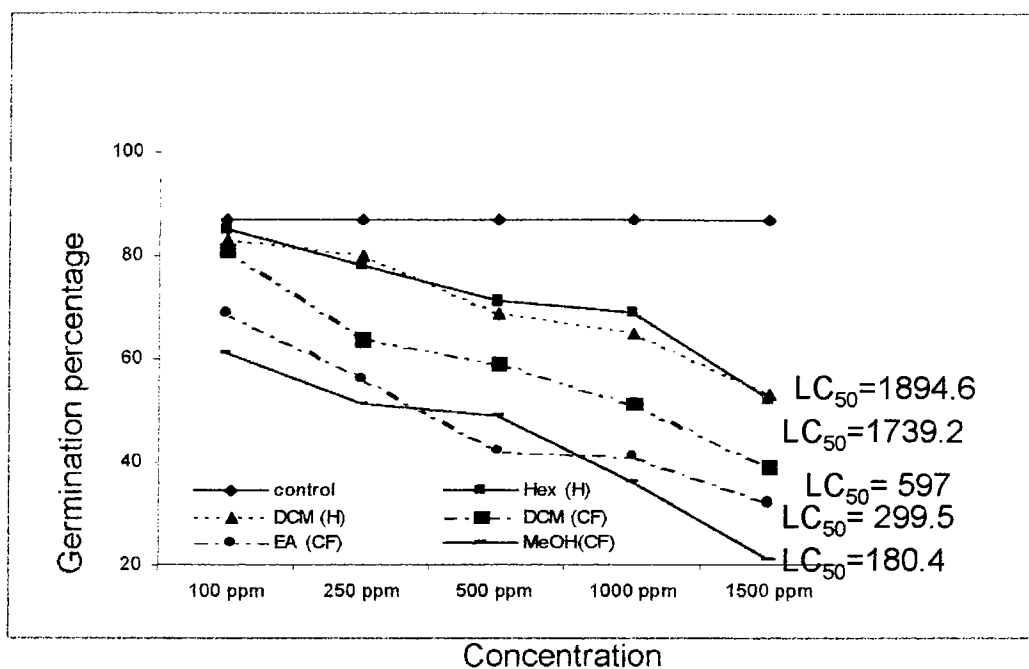


Fig 11. Effect of various solvent extracts of *Fusarium oxysporum* on Germination inhibition of *Parthenium hysterophorus*

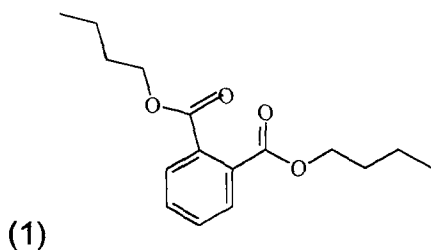
4.6 Isolation of secondary metabolites

4.6.1 Culture filtrate extract

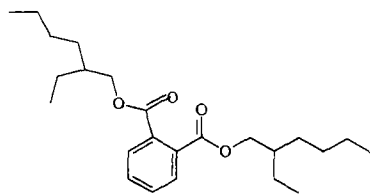
4.6.1.1 Dichloromethane extract

The dichloromethane extract of culture filtrate of *F. oxysporum*, showed the presence of a number of metabolites, when analyzed by GLC (using flame ionization detector, and operating conditions as: column temperature 70⁰C to 270⁰C @ 5⁰C min⁻¹, with detector and injector port temperatures 300⁰C) and TLC. These metabolites were separated by column chromatography over silica gel, eluting successively with hexane, hexane: dichloromethane, dichloromethane and dichloromethane: ethyl acetate in different ratios and identified by FT-IR, NMR and mass spectroscopy.

Residue (200g) was subjected to column chromatography.. Elution of column with hexane and dichloromethane (75:25 v/v) gave viscous oil, which was further purified by preparative thin layer chromatography (5mg). FT-IR of the metabolites showed a band at 1750cm⁻¹ due to C=O stretching. The mass spectrum showed molecular ion peak at m/z 278 with fragment ion peaks at m/z 167 and a base peak at m/z 149. (Figure 12 a). On the basis of mass spectroscopy it was tentatively identified as dibutyl phthalate (1)



A pale yellow viscous oil was obtained by elution of column with hexane and dichloromethane (50:50, v/v) and purified by preparative TLC (8 mg). FT-IR of the compound showed a band at 1750 cm^{-1} due to C=O stretching. Its mass spectrum showed a molecular ion peak at m/z 390 (Figure 12 b) with fragment ion peaks at m/z 279, m/z 167 and base peak at m/z 149. On the basis of mass spectroscopy it was tentatively identified as bis (2-ethylhexyl) phthalate (2).



(2)

First five fractions of column when eluted with dichloromethane and ethyl acetate (9:1, v/v) gave a yellow liquid, which was purified by preparative TLC (R_t 0.21, hexane: ethyl acetate 9: 1, v/v) (14 mg). FT-IR spectrum showed a broad band at 3422 cm^{-1} due to secondary OH group. The ^1H and ^{13}C - NMR values are given in Table 22. ^1H -NMR spectrum showed the presence of two singlet at δ 1.10 and δ 1.15 for 3 proton each indicating the presence of two methyl groups (2 CH_3 groups) Besides it showed two singlets corresponding to two proton each indicating presence of CH_2 (sporol). It was identified sporol by co comparison of ^1H - and ^{13}C -NMR with literature reported (Corley *et. al.*, 1986).

Next ten fractions of column when eluted with dichloromethane and ethyl acetate (9:1, v/v) gave a yellow liquid, which was purified by preparative TLC (R_t 0.15, hexane: ethyl acetate 9: 1, v/v) (20 mg). FT-IR spectrum showed a broad

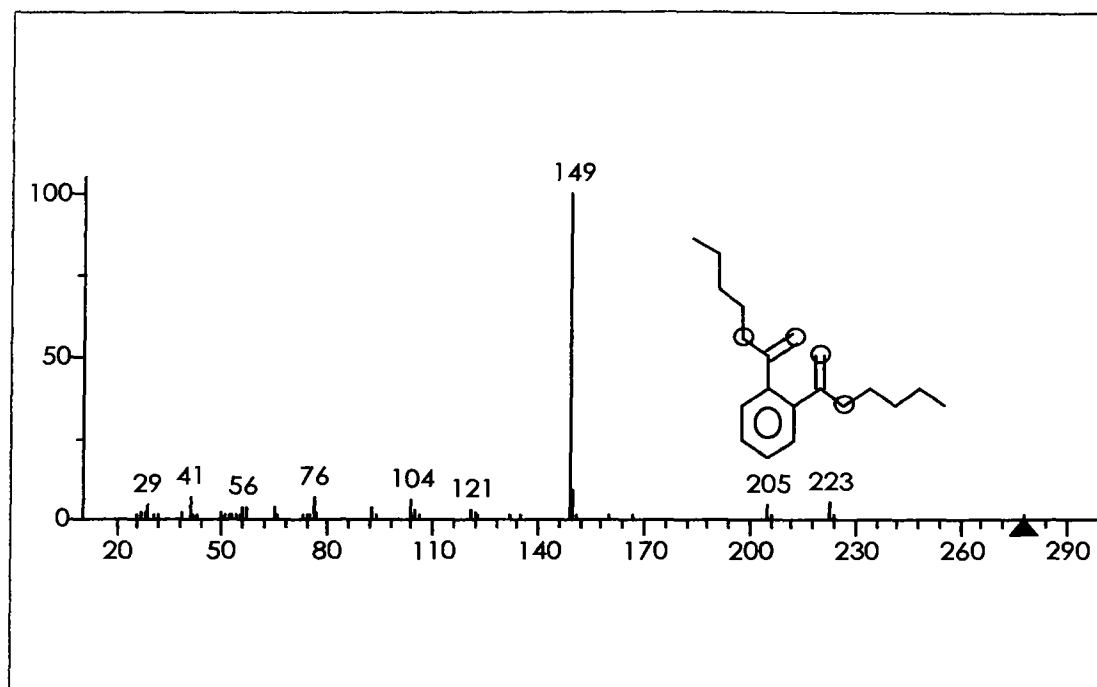


Fig 12a. Mass spectrum of Dibutyl phthalate

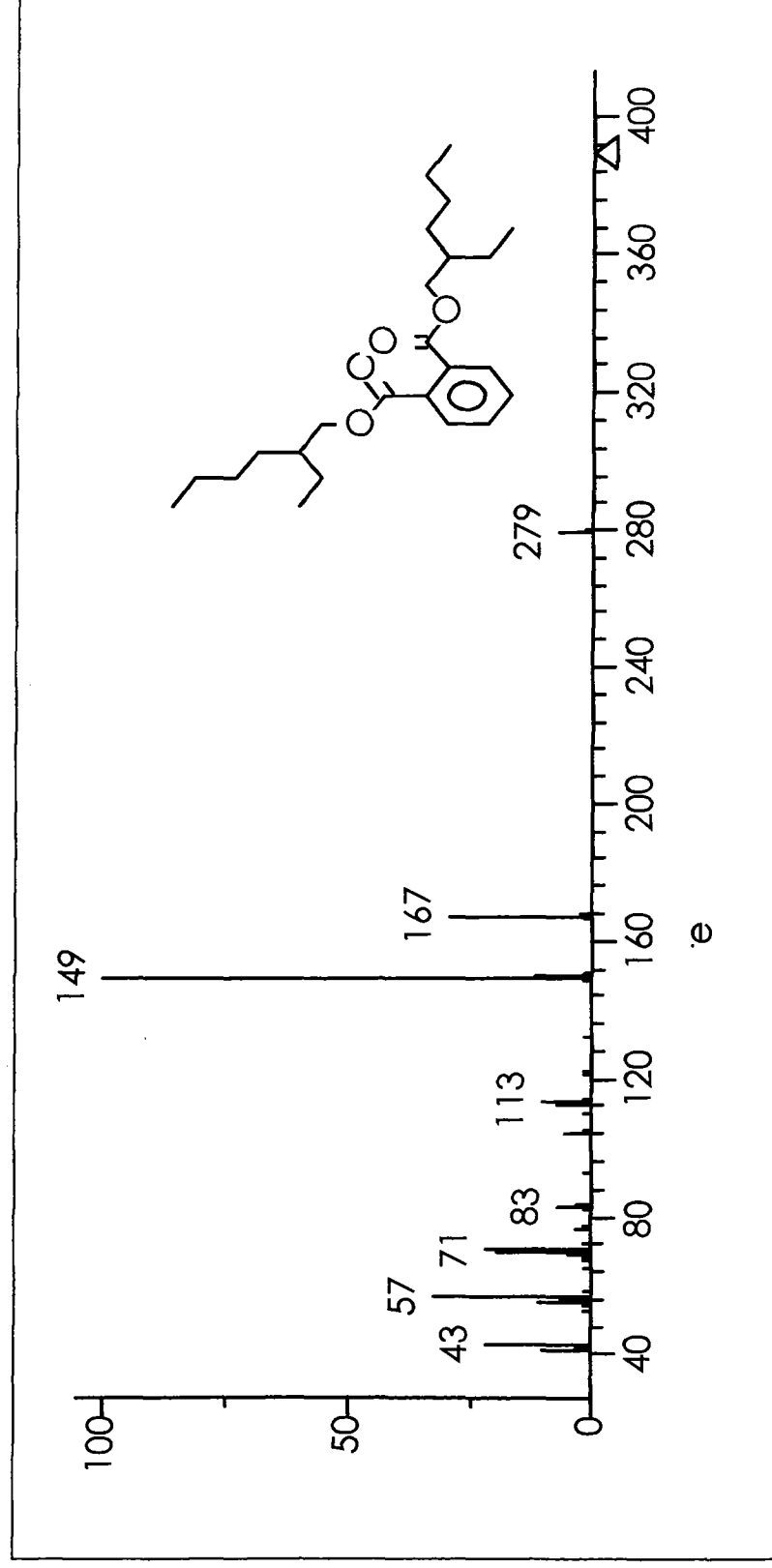
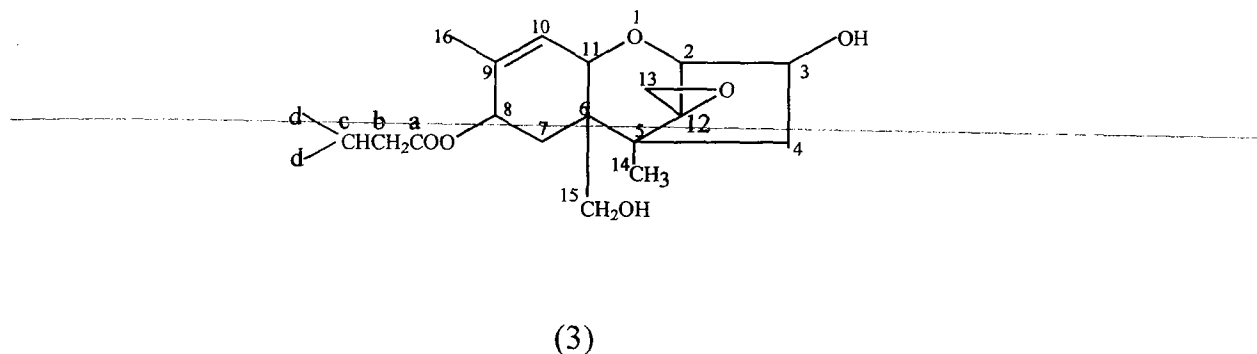


Fig 12b . Mass spectrum of Bis-(2-ethyl hexyl phthalate)

band at 3422 cm^{-1} due to OH group and a band at 1726 due to $\text{C}=\text{O}$ stretching. The ^1H and ^{13}C - NMR values are given in Table 14, Figure 13. On the basis of ^1H and ^{13}C - NMR and co- comparison with the literature reported, it was identified as sporotrichiol (3).



Further elution of column with dichloromethane and ethyl acetate (8;2 v/v) gave a colorless solid, which was further purified by preparative TLC using MeOH- H_2O (7:3, v/v) (2mg). The ^1H - and ^{13}C - NMR data is reported in Table 15, Figure 14. FT-IR showed the presence of a ketonic group at 1730 cm^{-1} and a broad band at 3370 cm^{-1} due to hydroxyl. Presence of ketonic group was further confirmed by presence of singlet at δ 216 in ^{13}C -NMR spectrum. ^{13}C -NMR showed the presence signals at δ 135.2 and 122 indicating unsaturation in the ring. On the basis of ^1H and ^{13}C -NMR and co comparison with literature reported, it was identified as sambucoid (4) (Corley et.al, 1986).

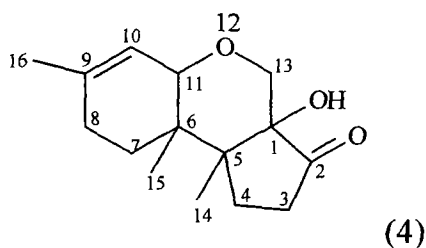
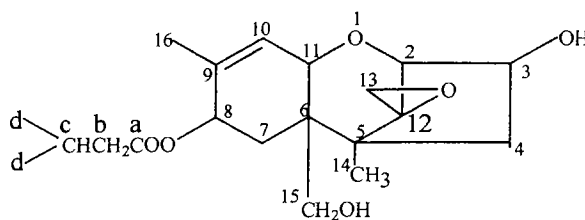


Table 14. Spectral details of Sporotrichol



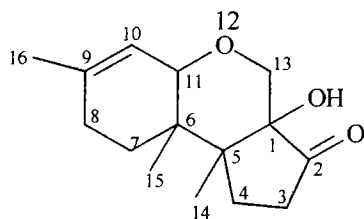
Yellow liquid

IR $V_{\max}$ (KBr) (cm^{-1}) = 3422(-OH), 1726 (-COO)

NMR data:

C	^1H	^{13}C
1		
2	3.5, d	79.6, d
3	4.5, m	69.1, d
4	2.1, m	42.1, d
5		45.8, s
6		43.3, s
7	1.96, d	26.8, t
8	5.5, d	68.4, d
9		135.7, s
10	5.8, d	125.4, d
11	4.2, d	68.1, d
12		65.1, s
13	2.57	48.5
14	0.87, s	12.4
15	3.5	63.1, t
16	1.75, s	20.4,

Table 15. Spectral details of Sambucoin



white crystals

NMR data:

C	¹ H
1	
2	
3	2.26, 1H, J= 7.9
4	1.74, 1H, J= 13.8
5	
6	
7	1.43, 2H, J= 13.4 Hz
8	1.8, 2H, m,
9	
10	5.18, 1H
11	3.94, 1H
12	
13	3.42, 2H, J= 11.2
14	1.13, 3H
15	0.63, 3h
16	1.64, 3H

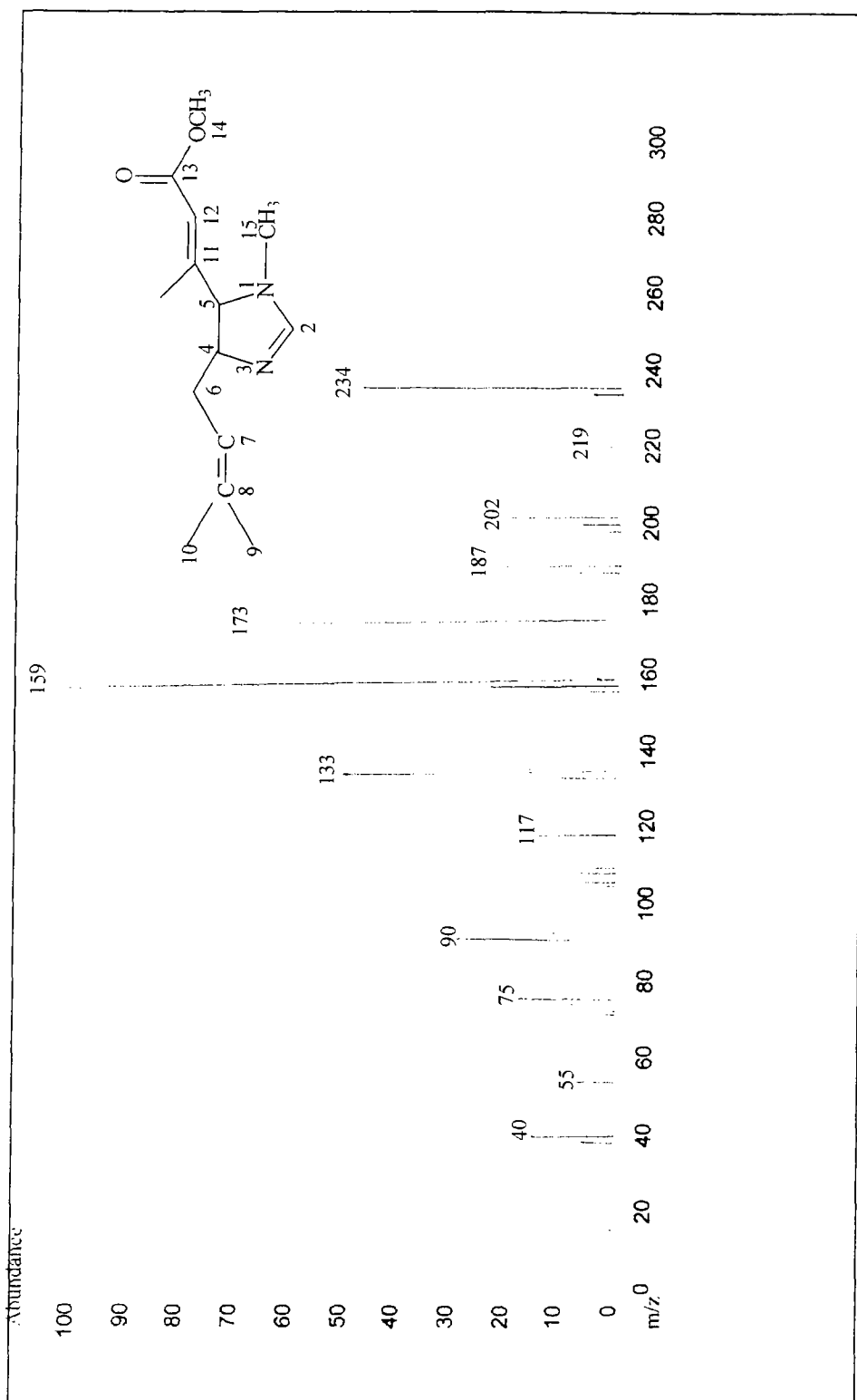


Fig 13. Mass spectrum of Sporotrichiol

OP 21 in DMSO

Current Data Parameters
NAME Apr14-2004
EXPNO 1010
PROCNO 1

F2 - Acquisition Parameters
Date_ 20040415
Time 9.11
INSTRUM av500
PROBHD 5 mm BBO BB-1H
PULPROG zgpg
TD 65536
SOLVENT DMSO
NS 16
DS 0
SWH 8992.806 Hz
FIDRES 0.137219 Hz
AQ 3.6439073 sec
RG 128
DW 55.600 usec
DE 6.00 usec
TE 292.9 K
D1 1.00000000 sec
d12 0.00002000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec

===== CHANNEL f1 =====
NUC1 1H
P1 9.00 usec
PL1 -2.00 dB
PL9 59.00 dB
SFO1 500.1317212 MHz

F2 - Processing parameters
SI 32768
SF 500.1300007 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

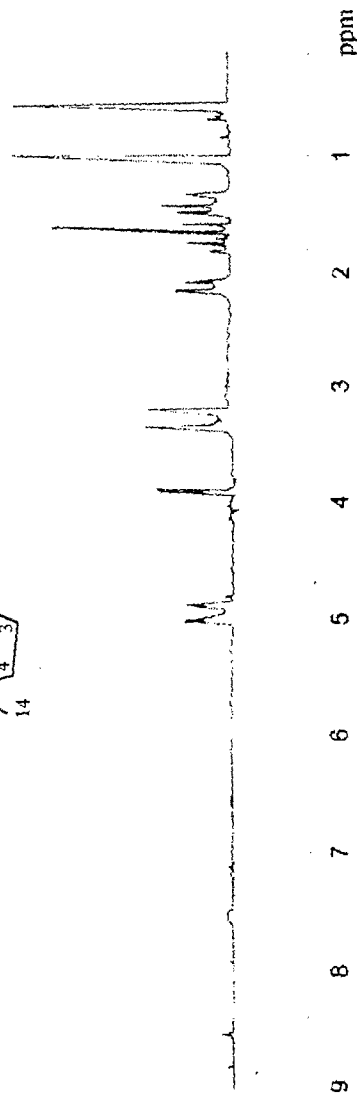
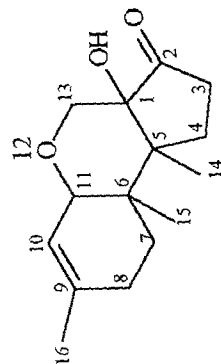


Fig 14a: ¹H-NMR spectrum of Sambucoside

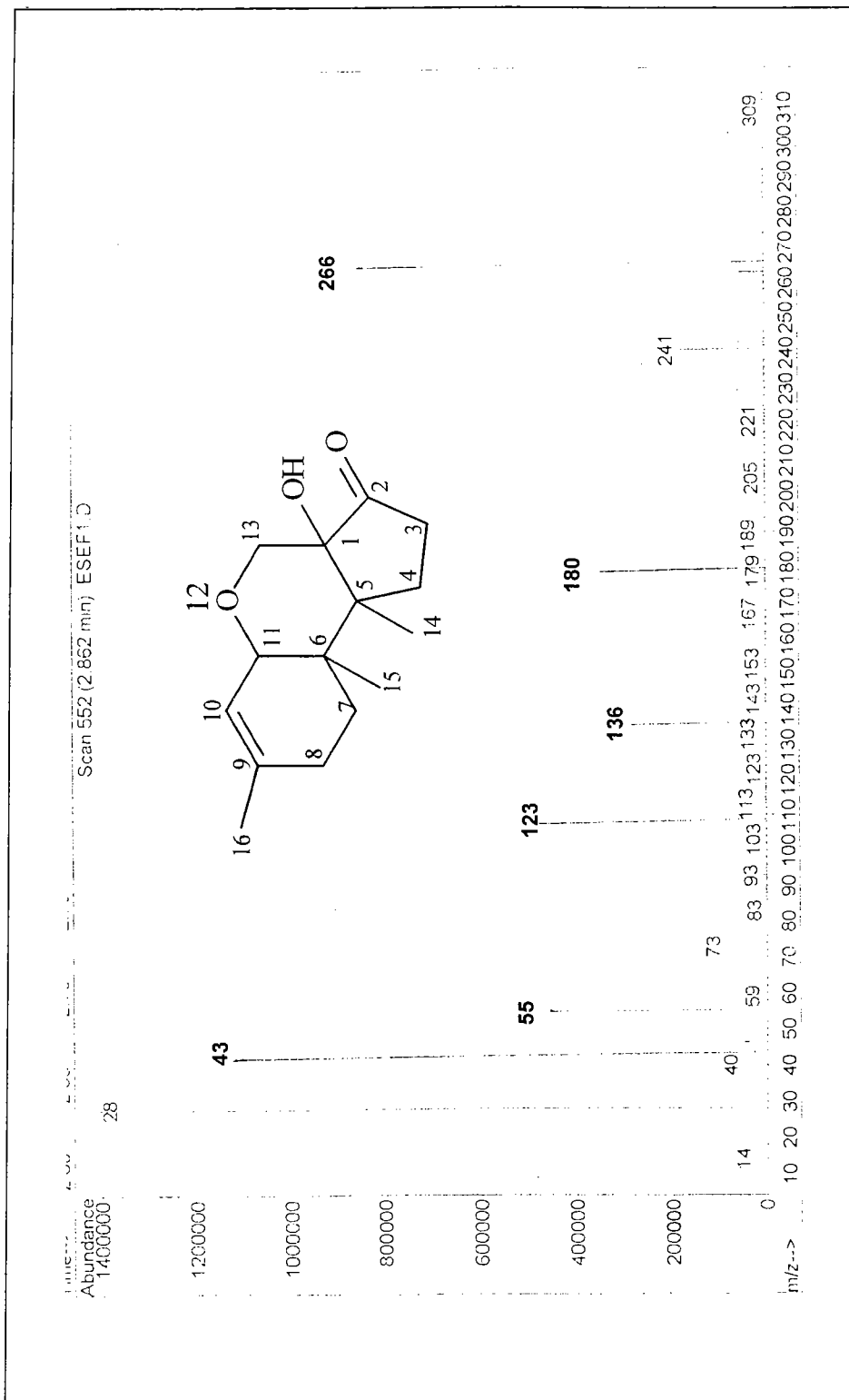
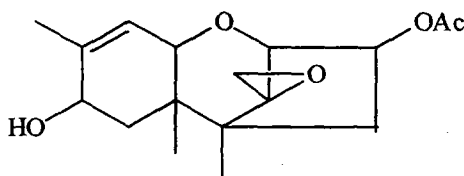


Fig 14b. Mass spectrum of sambucoin

4.6.1.2 Ethyl acetate extract

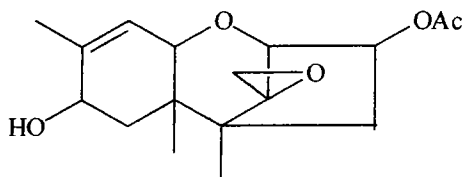
The ethyl acetate extract of culture filtrate showed the presence of a number of metabolites, when analyzed by GLC (using flame ionization detector, and operating conditions as: column temperature 70°C to 270°C @ 5°C min⁻¹, with detector and injector port temperatures 300°C) and TLC. These metabolites were separated by column chromatography over silica gel, eluting successively with hexane, hexane: dichloromethane, dichloromethane and dichloromethane: ethyl acetate in different ratios and identified by FT-IR, NMR and mass spectroscopy.

Residue (5.00g) was subjected to column chromatography. Elution of column with hexane and dichloromethane (75:25 v/v) gave viscous oil, which was further purified by preparative thin layer chromatography (5mg). Mass spectrum showed a molecular ion peak at m/z 266 (M^+ , 1%) with fragment ion peaks at m/z 207 (6%), m/z 182 (28%), m/z 161 (56%), 123 (89%) and with base peak at m/z 109 (100%). The data for ^1H and ^{13}C NMR is given in Table 16, Figure 15. By co-comparison of its spectral data with that reported in the literature (McCormick and Hohn, 1997), it was identified as 8-hydroxyisotrichodermol (5).



(5)

Table 16. Spectral details of 8 – hydroxyisotrichodermol



Prominent mass fragments:

$m/z = 266$ (M^+), 207, 182(3), 161(6), 217(17), 123(89), 109(100), 95(42)

NMR data:

C	^1H (δ)	^{13}C
1		
2	3.48, 1H, d, $J = 4.6$	79.8
3	4.47, 1H, m	71.5
4a,b	2.2, 2H, m	41
5		46
6		39.6
7a	2.2, 1H, dd	33.6
7b	1.7, 1H, d, $J = 14.3$	33.6
8	4.12, 1H, d, $J = 5.5$	68.1
9		138.8
10	5.62, 1H, d, $J = 5.8$	121.9
11	4.19, 1H, d, $J = 5.8$	69.4
12		65.7
13a	3.06, 1H, d, $J = 4$	48.7
13b	2.87, 1H, d, $J = 4$	48.7
14	0.76, 3H, s	11.2
15	0.98, 3H, s	19.1
16	1.87, 3H, s	20.6

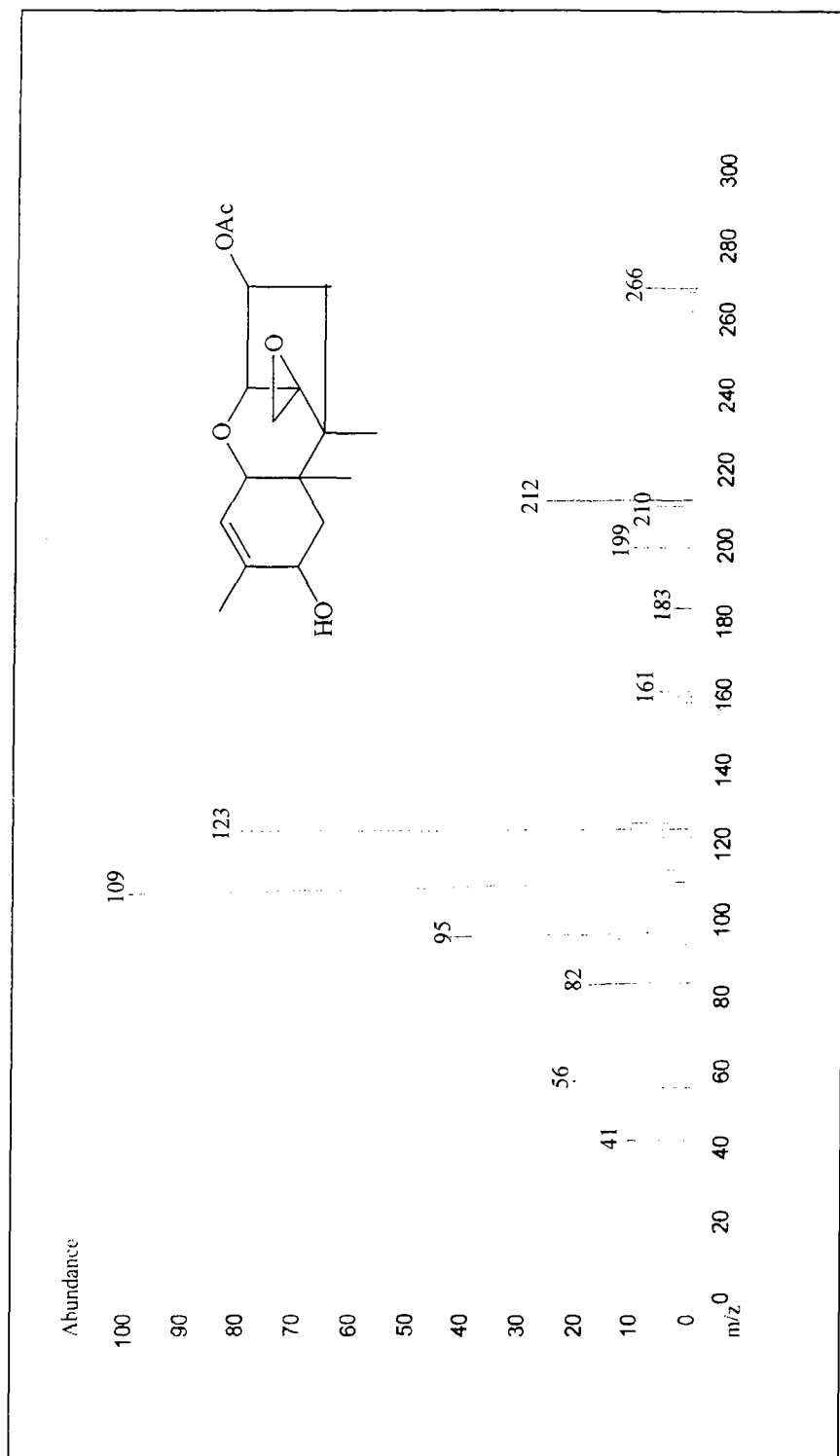
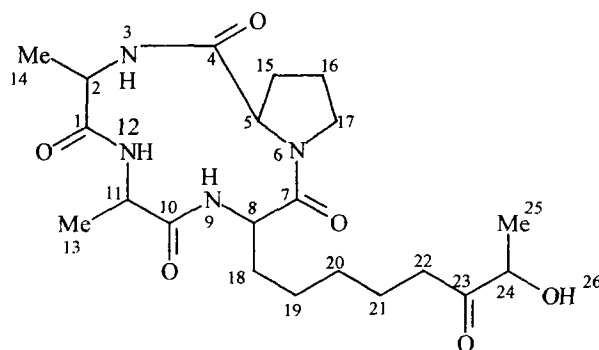


Fig 15 . Mass spectrum of 8-hydroxy isotrichodermol

Elution of column with dichloromethane and ethyl acetate (95:5, v/v) gave a colourless solid, which was further purified by preparative TLC (2 mg, mp. 112-116 Lit. 114-116°C). Its mass spectrum showed m/z (rel. int.): 438 $[M]^+$ (6), 395 (4), 393 (4), 366 (7), 326 (12), 181 (14), 172 (74), 131 (16), 70 (100). Compound (6) was assigned the molecular formula $C_{21}H_{34}N_4O_6$, with seven double bond equivalents, on the basis of its mass and NMR spectra. Interpretation of the NMR data suggested a structure derived from discrete amino acid units. Three amide carbonyl resonances (r, s and t) were identified in the ^{13}C NMR spectrum (170–174), Table 17, Figure 16. However, the intensity of the most downfield signal (carbon t at C 173.68) was approximately double the other two, suggesting the presence four peptidic carbonyl groups in total. Consistent with the carbonyl analysis, four amino acid α -methine groups—m, n, o and p—were identified (respectively at C 47.36, 48.03, 51.86, and 57.82). Two of these three were shown to be alanyl units by connection of their α -methines (m and n) to methyl groups (respectively α and β), which manifested doublet resonances in the ^1H NMR spectrum. The third amino acid possessed an α -methine (p) linked to a contiguous series of three methylenes (f, g and i) and was, therefore, shown to be a prolyl unit. Inspection of the remaining NMR data disclosed the presence of 5 methylene units (e, h, i, j and k), a strongly deshielded carbonyl centre (C 212.36), a methyl group (d, doublet resonance at δH 1.36), and a carbinol-like methine centre (q at C 72.59). It was identified as JM-47 (6), (cyclo{ l-alanyl- d-alanyl-[(2S,9S)-2-amino-9-hydroxy-8-oxodecanoyl]- d-prolyl}), by co-comparison of its spectral that that reported in the literature (Jiang et al. 2002).

Table 17. Spectral details of JM-47



Prominent mass fragments

m/z = 639(45, M^+), 596(22), 556 (22), 496 (12), 456 (8), 409 (21), 296(42), 196 (64), 169 (100)

NMR data:

C	^1H
1	
2	4.45, dq, $J = 10.3$, $J = 6.8$
3	7.40, s
4	
5	4.68, dd, $J = 7.9$, $J = 2.2$
6	
7	
8	4.76, m
9	7.35, s
10	
11	4.55, dq, $J = 9.6$, $J = 7$
12	7.20, s
13	1.26, d, $J = 6.9$
14	1.30, d, $J = 6.9$
15	1.84, m
16	1.92, m
17	3.5-3.9 m
18	1.5, m
19	1.30, m
20	1.30, m
21	1.61, m
22	2.47, m
23	
24	4.21, dq, $J = 4.5$, $J = 7.1$
25	1.36, d, $J = 7.1$
26	7.35, s

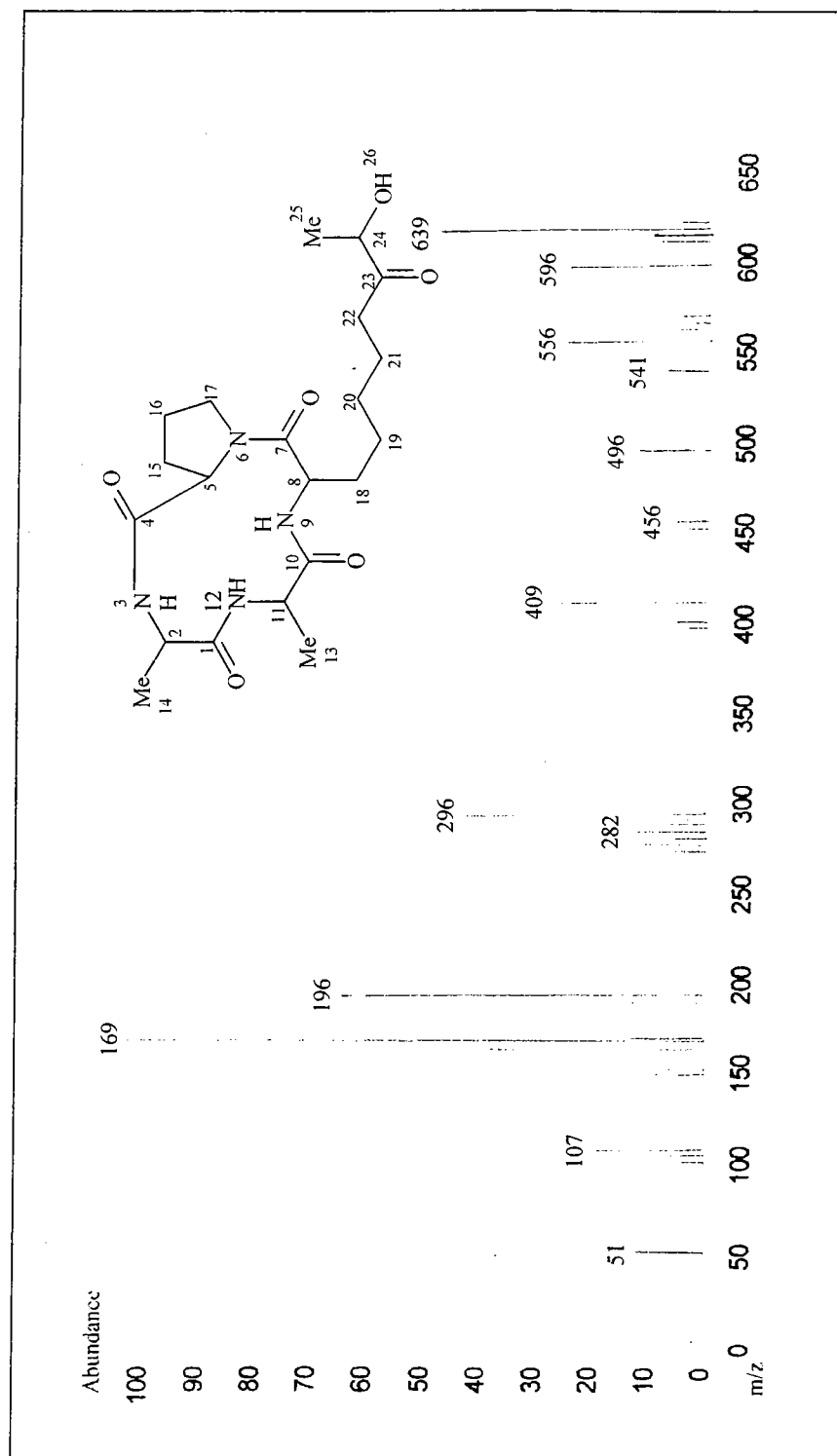
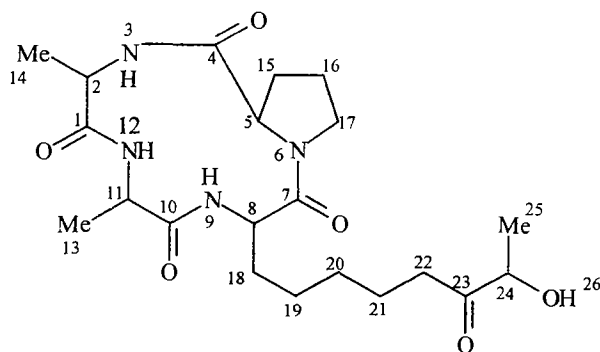
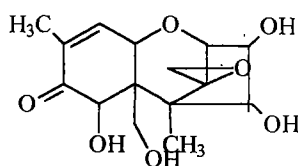


Fig 16 . Mass spectrum of JM-47



(6)

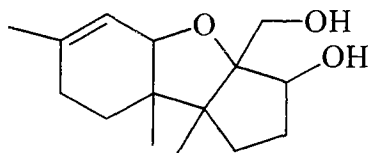
Elution of column with dichloromethane ethyl acetate (9:1) gave a colourless solid, which was purified by crystallization from methanol (mp. 220-224°C, yield 8mg) UV max (methanol) 218, slight soluble in water, but completely soluble in polar organic solvents. Mass spectrum showed a molecular ion peak at m/z 312 (M^+). With fragment ion peaks at m/z 247 (40%), and m/z 189 (30%). By co-comparison of its physiochemical properties and spectral data it was identified as nivalenol (3", 4B, 7")-12, 13-epoxy-3, 4, 7, 15-tetrahydroxytrichothec-9-en-8-one (7).



(7)

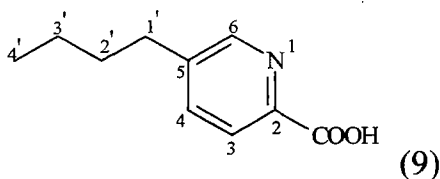
Elution of column with dichloromethane and ethyl acetate (6:4, v/v) gave a colourless solid, which was further purified by preparative TLC. It was identified a trichothecene with molecular weight 354 ($C_{17}H_{22}O_8$). Its mass spectrum showed a

molecular ion peak at m/z 354 (M^+). Based on spectral analysis and comparison with reported trichothecenes, it was identified as fusarenon X (trichothec-9-en-8-one, 4-(acetyloxy)-12,13-epoxy-3, 7,15-trihydroxy) (8)



(8)

Elution of column with dichloromethane and ethyl acetate (5:5, v/v) gave a colourless solid, further purified by crystallization (mp. 95-98°C, 50 mg). The mass spectrum showed molecular ion peak at m/z 179 (M^+) with fragment ion peak at m/z 152(80%). The spectral data is given in Table 18, Figure 17. On the basis of $^1\text{H-NMR}$ it was identified as 5-butylpicolinic acid (9) (Yabuta et.al., 1934).



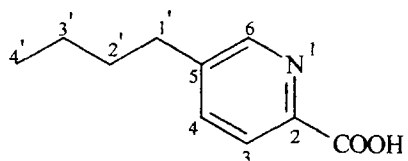
(9)

4.6.2 Mycelia extract

4.6.2.1 Hexane extract

The hexane extract of dried mycelia of *F. oxysporum*, showed the presence of a number of metabolites, when analyzed by GLC (using flame ionization detector, and operating conditions as: column temperature 70°C to 270°C @ 5°C min⁻¹, with detector and injector port temperatures 300°C).

Table 18. Spectral details of Fusaric acid



m.p. 99⁰C - 100⁰C

IR $V_{\max}$ (KBr) (cm⁻¹) = 2960, 2930, 1765, 1410

NMR data:

C	¹ H	¹³ C
1		
2		143
3	8.14, 1H, d, J= 8	122
4	7.75, 1H, dd, J= 8 Hz, J= 2.1 Hz	138
5		141
6	8.44, 1H, d, J= 1.5 Hz	145
1'	2.7, 2H, t, J= 7.6 Hz	14
2'	1.60-1.7, 2H, m	23.2
3'	1.35-1.4, 2H, J =7.3 Hz	31.2
4'	0.95, t, 3h, J= 7.3 Hz	39.0
COOH-H	11.8 (broad)	169

01/01/97 10:00:00
 01/01/97 10:00:00
 01/01/97 10:00:00
 01/01/97 10:00:00

F2 - Acquisition Parameters
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 Time 18.06
 INSTRUM spect
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 PULPROG zgpg30
 TD 32768
 SOLVENT DMSO
 NS 78
 DS 8
 SWH 36735.633 Hz
 FIDRES 0.876942 Hz
 AQ 0.5702132 s
 RG 32768
 DW 17.400 ns
 DE 7.11 ns
 TE 300.0 K
 D1 3.00000000 s
 d11 0.03000000 s
 ===== CHANNEL F1 =====
 NUC1 13C
 P1 6.40 ns
 PL1 0.00 dB
 SFO1 100.6227903 MHz
 ===== CHANNEL F2 =====
 CPDPRG2 db
 NUC2 1H
 PL2 120.00 dB
 PL12 27.70 dB
 SFO2 400.1317580 MHz
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 SF 100.6123143 MHz
 WDW EM
 SSB 0
 LB 3.00 Hz

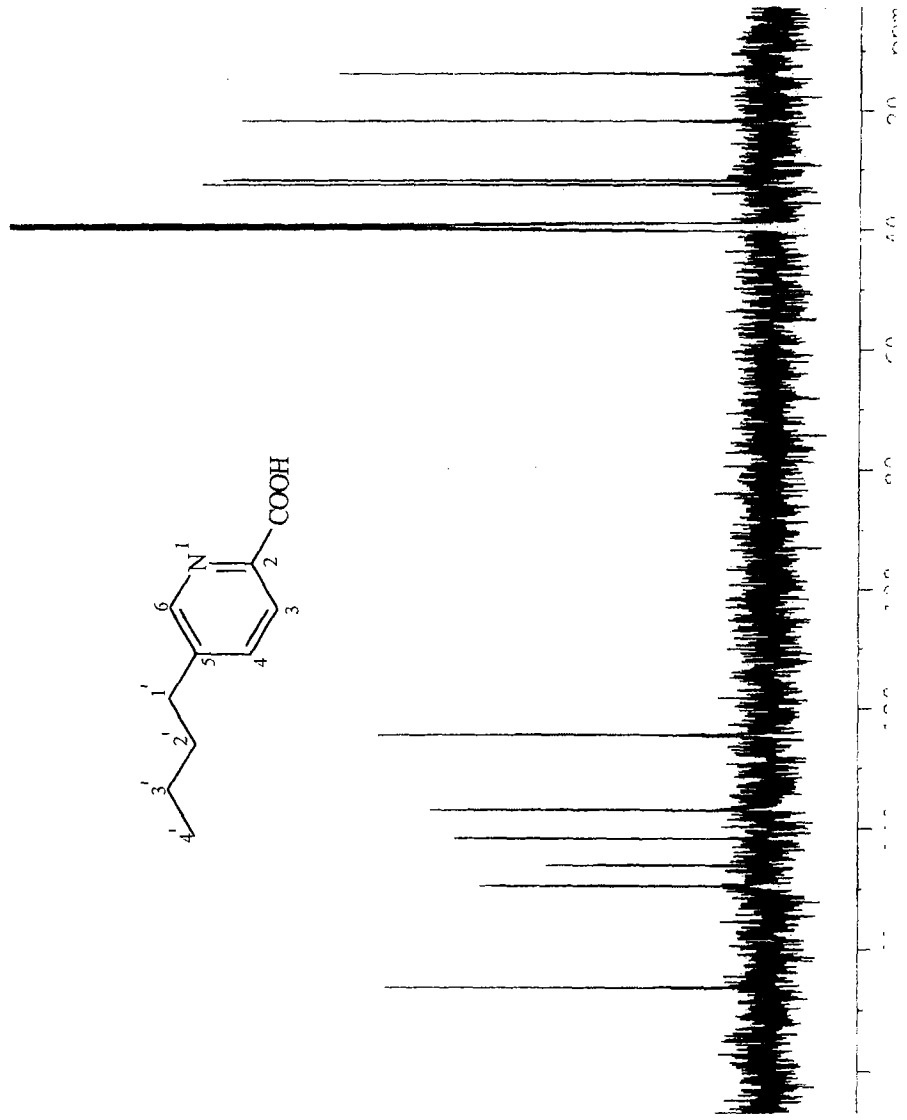
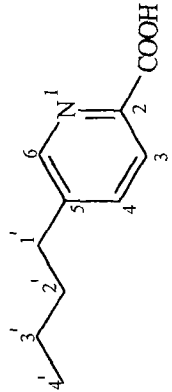


Fig 17b . ¹³C-NMR spectrum of Fusaric acid

These compounds were not separated but identified on the basis of GC-MS as nonadecane (M^+263), 1-tetradecanol (M^+ , 196), methyl hexadecanoate (M^+ ,270), butyl hexadecanoate (M^+ ,312), 2- methyl pentadecane (M^+ 228) and methyl octadecanoate (M^+298), 4- methyl-4-hydroxy pentanone (M^+ 101) by cocomparison of their mass spectrum with literature reported. Besides hydrocarbon such as decane, didecane, tetradecane and nonadecane and fatty acids like palmitic acid and higher aliphatic acids were identified by GC-MS and co-chromatography with standards by GLC. (Table 19, Figure 18-20)

4.6.2.2 Dichloromethane extract

The dichloromethane extract of dried mycelia mass of *F. oxysporum*, showed the presence of a number of metabolites, when analyzed by GLC (using flame ionization detector, and operating conditions as: column temperature 70⁰C to 270⁰C @ 5⁰C min⁻¹, with detector and injector port temperatures 300⁰C) and TLC. These metabolites were separated by column chromatography over silica gel, eluting successively with hexane, hexane: dichloromethane, dichloromethane and dichloromethane: ethyl acetate in different ratios and identified by FT-IR, NMR and mass spectroscopy.

Elution of column with hexane gave a non polar compounds (4 mg each). This was identified as cyclobutane di carbonitrile by mass spectral analysis(M^+ 56) (Figure 21)

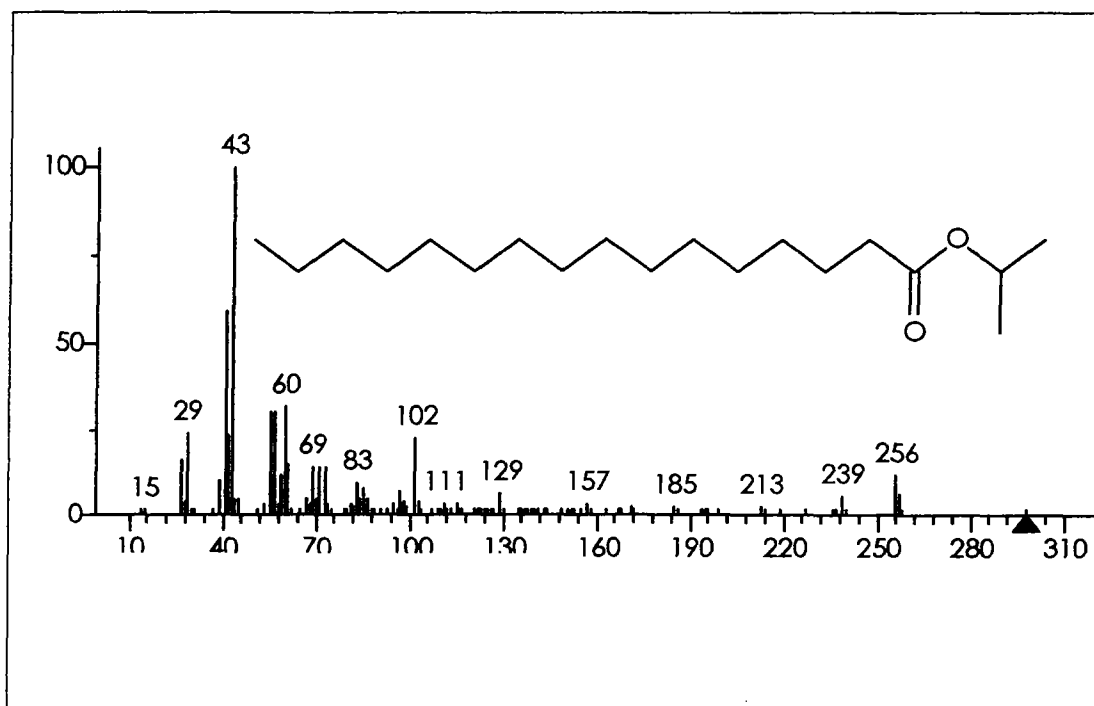


Fig 18d . Mass spectrum of Isopropyl palmitate

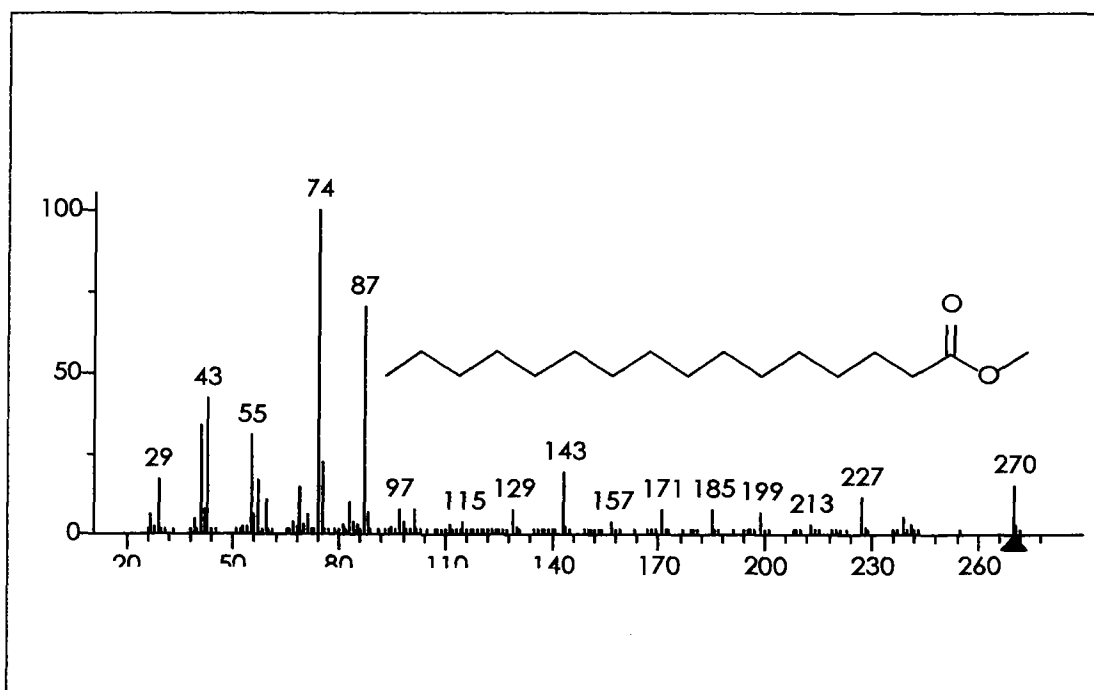


Fig 18a. Mass spectrum of Methyl hexadecanoate

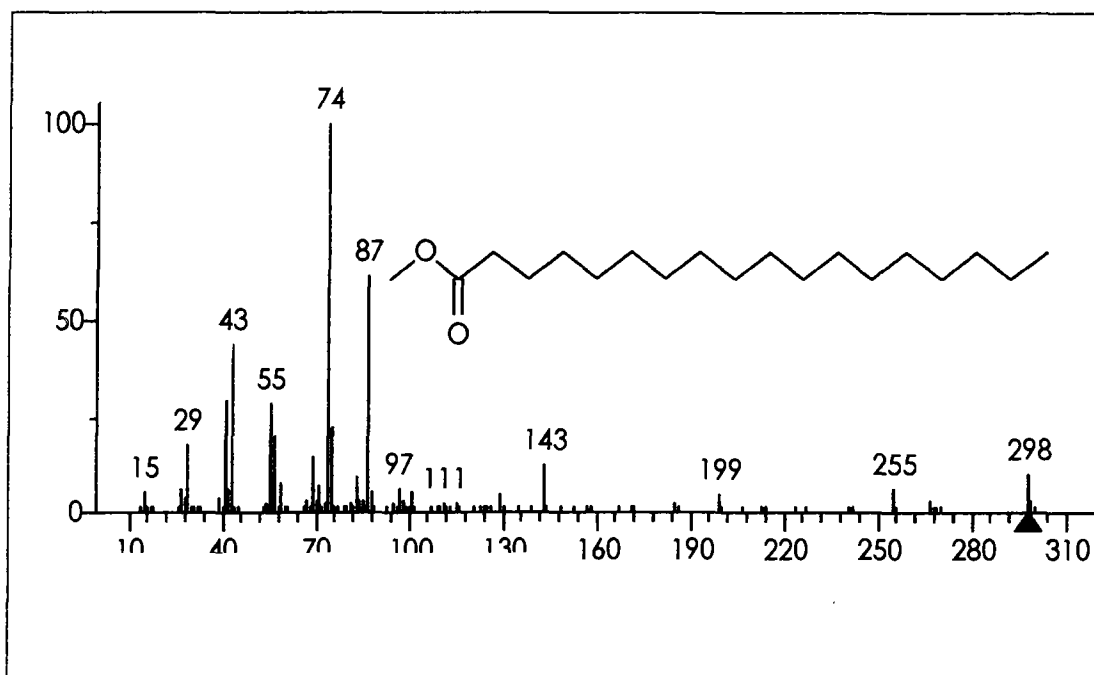


Fig 18b. Mass spectrum of Methyl octadecanoate

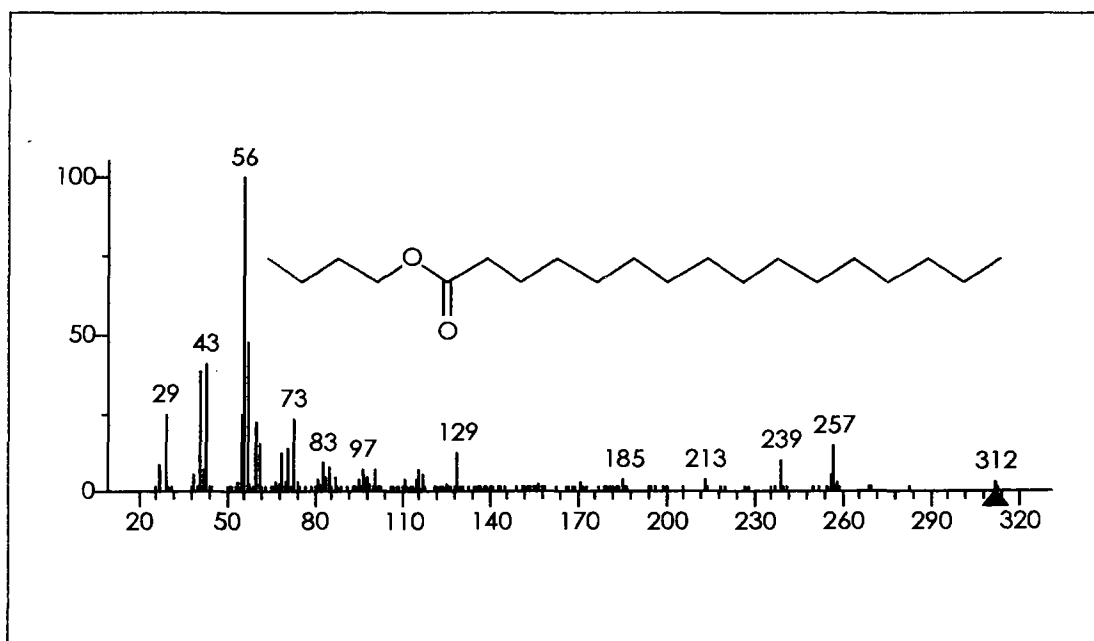


Fig 18c. Mass spectrum of butyl hexadecanoate

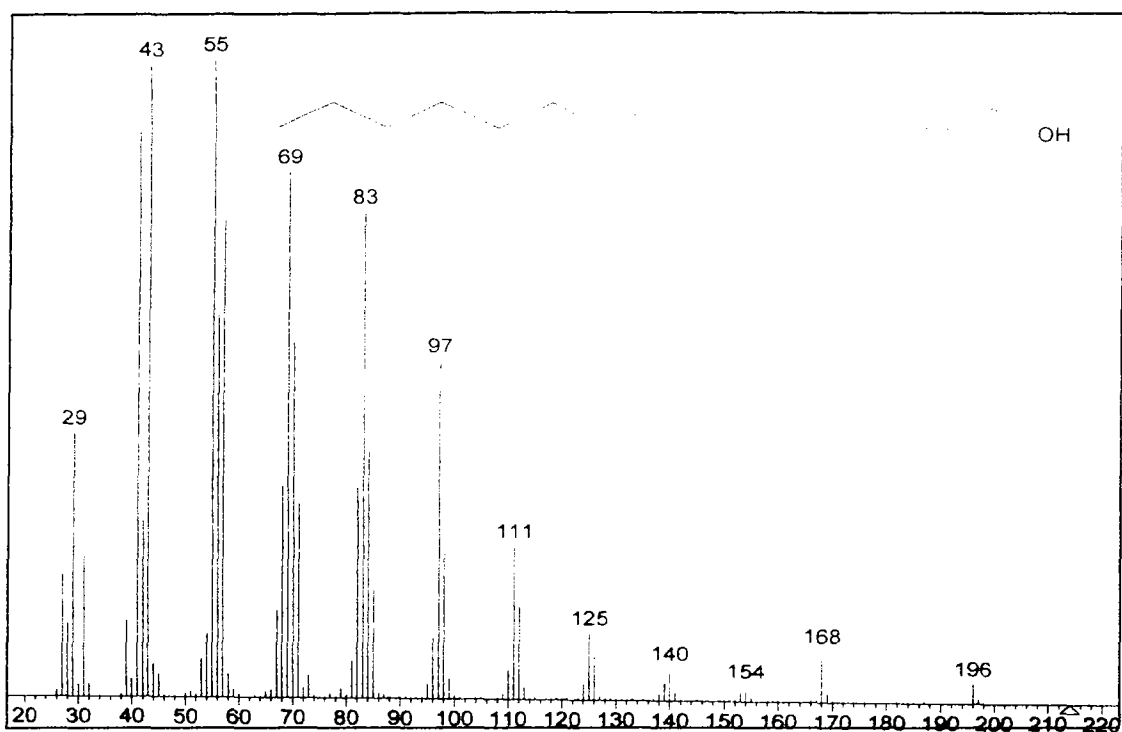


Fig 19a . Mass spectrum of 1-Tetradecanol

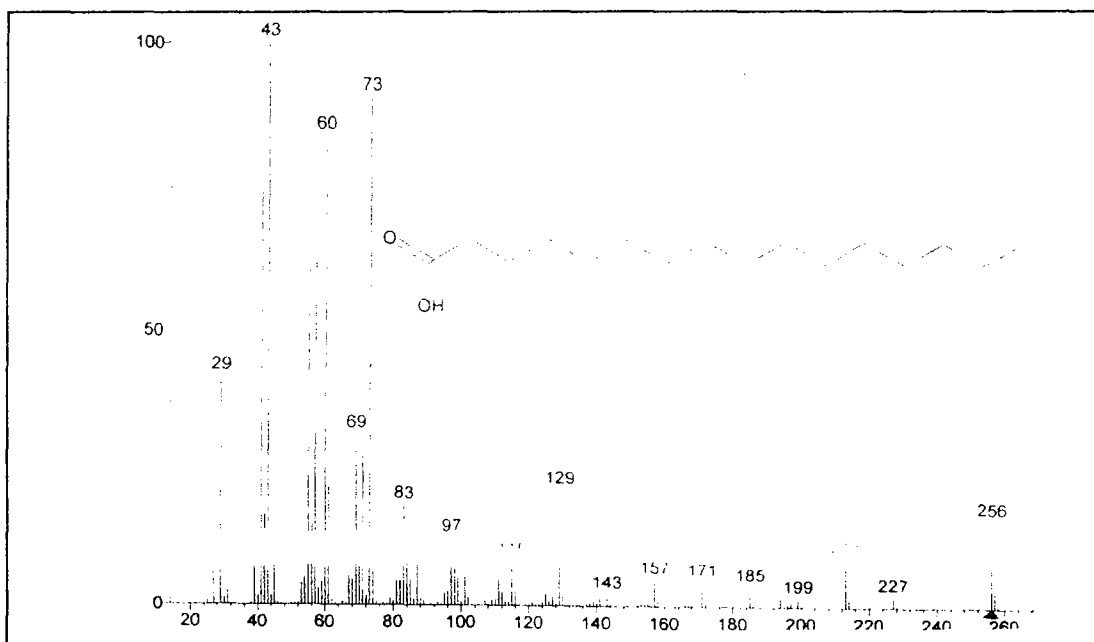


Fig 19b. Mass spectrum of hexadecanoic acid

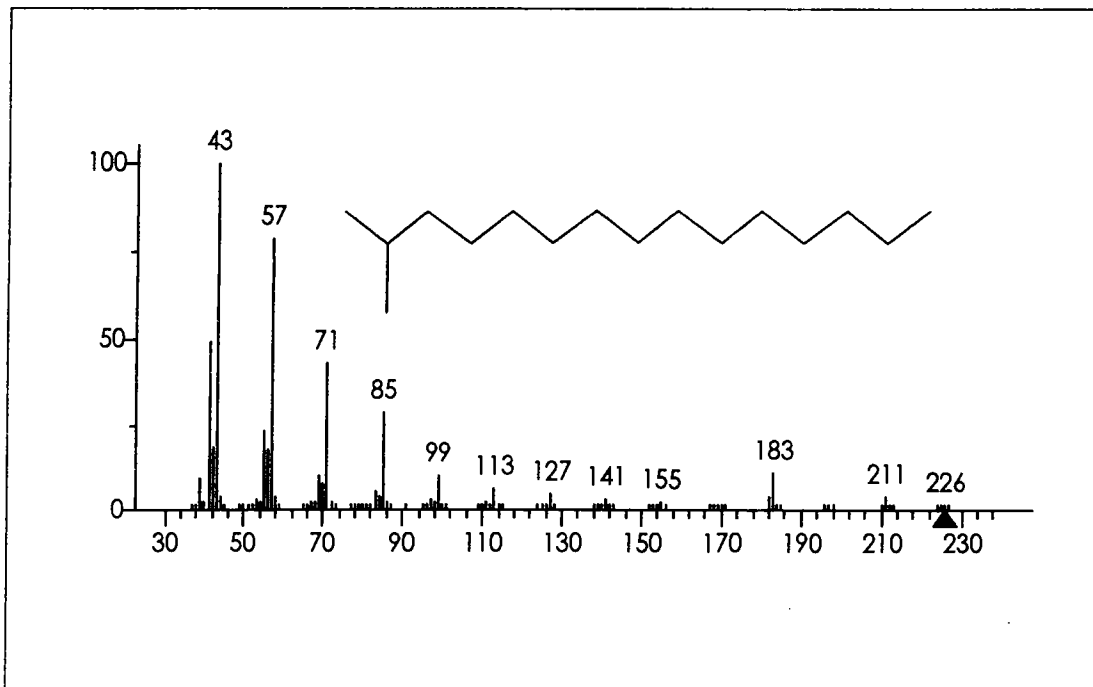


Fig 19c. GCMS spectra of 2- Methyl pentadecane

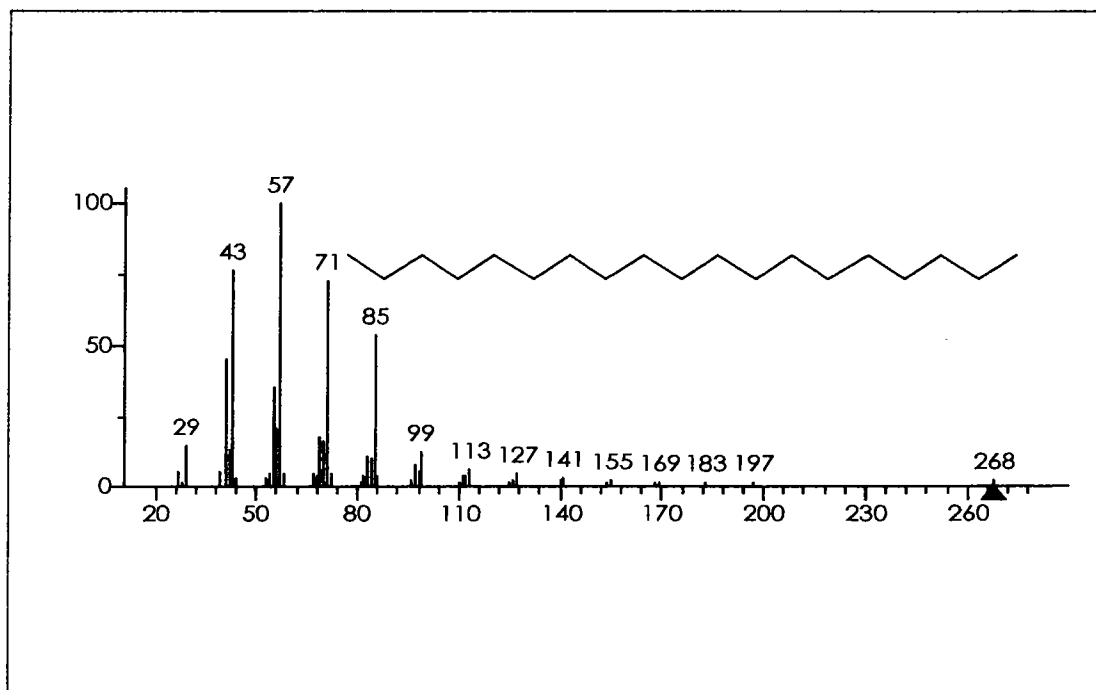


Fig 19d. GCMS spectra of nonadecane

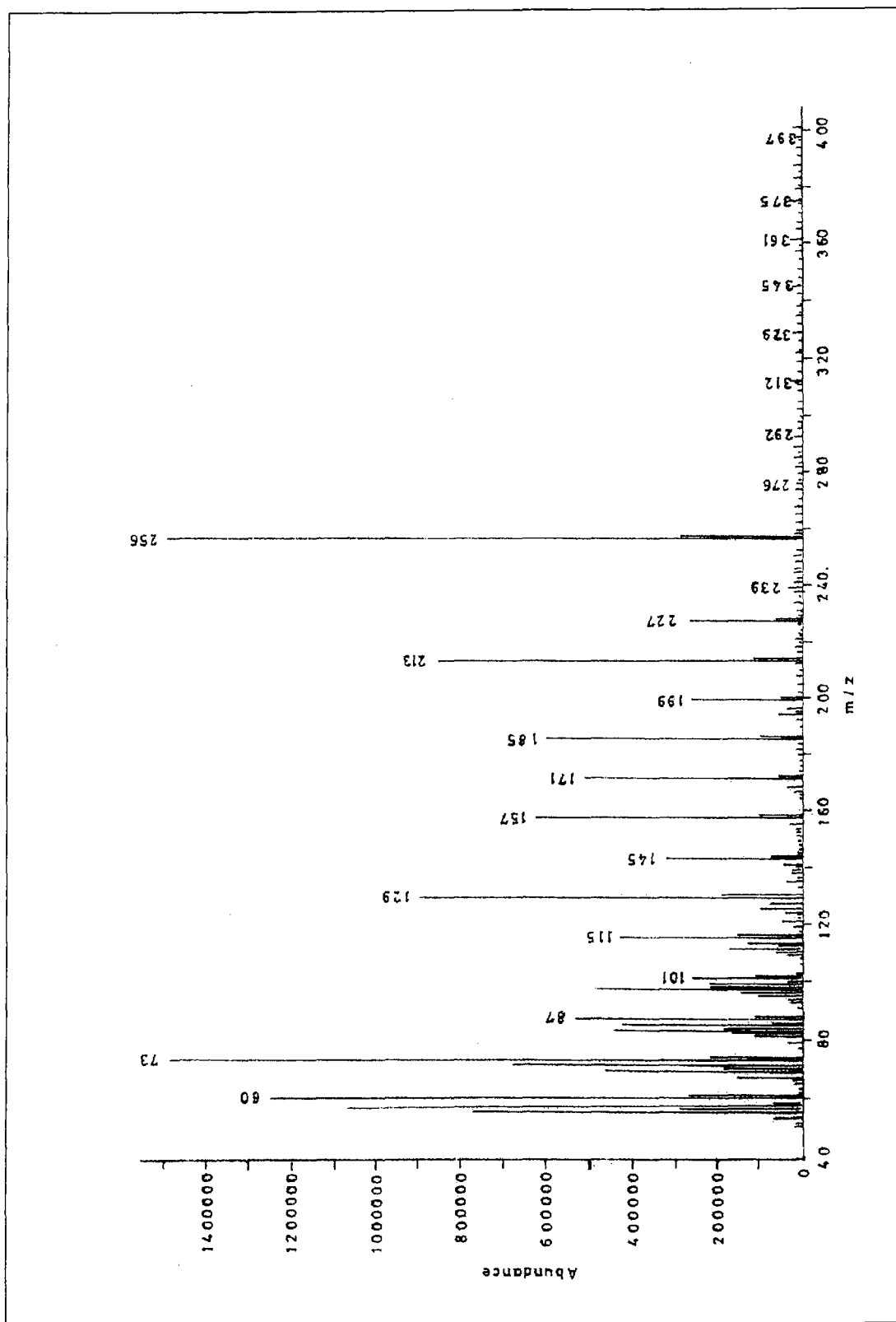


Fig 20 a. Mass spectrum of Palmitic acid

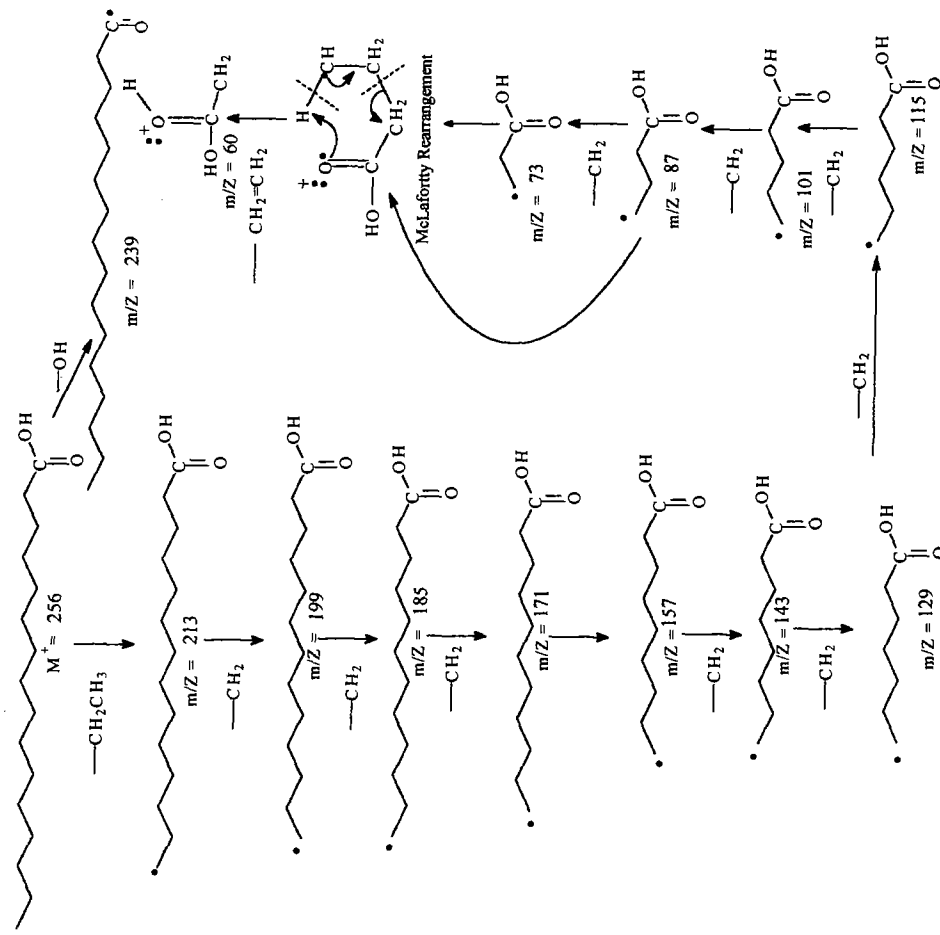
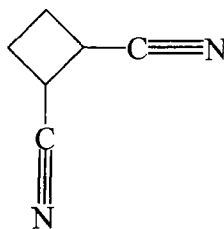
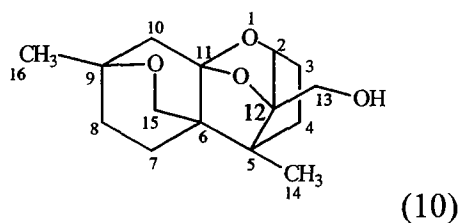


Figure 20b. Mass fragmentation pattern of Palmitic acid



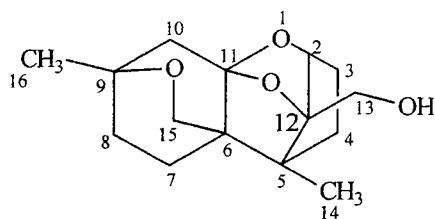
cyclobutane di carbonitrile

First five fractions of column when eluted with dichloromethane and ethyl acetate (9:1, v/v) gave a yellow liquid, which was purified by preparative TLC (Rt 0.21, hexane: ethyl acetate 9: 1, v/v) (14 mg). FT-IR spectrum showed a broad band at 3422 cm^{-1} due to secondary OH group. It was identified sporol (10) by co comparison of ^1H - and ^{13}C -NMR with metabolite isolated from culture filtrate extract. (Table 20, Figure 22)



Next five fractions of column when eluted with dichloromethane and ethyl acetate (9:1, v/v) gave a pale yellow liquid, which was purified by preparative TLC (Rt 0.34, hexane: ethyl acetate 8: 2, v/v) (14 mg). The FT-IR spectrum showed absorptions at $1740\text{--}1760\text{ cm}^{-1}$ ($\text{C}=\text{O}$) and $1630\text{--}1640$ and $1550\text{--}1560\text{ cm}^{-1}$ ($\text{C}=\text{C}$). ^1H -NMR spectrum showed spin-spin coupling of the ring protons giving rise to a doublet of doublets at δ 6.30 and 7.23 ppm. It also indicated a

Table 20. Spectral details of Sporol



Yellowish liquid

IR $V_{\max}$ (KBr) (cm^{-1}) = 3422(OH)

NMR data:

C	^1H	^{13}C
1		
2	4.5, d	76.3, d
3	1.47, m	41.2, d
4	1.23, m	32.0, d
5		46.6, s
6		47.3, s
7	1.75, m	25.0, t
8	1.6, m	41.2, t
9		70.3, s
10	1.83,	44.5, t
11		106.5, s
12		91.5, s
13	3.8	64.4, t
14	1.1	15.4, q
15	3.8	68.4, t
16	1.15	25.7, q

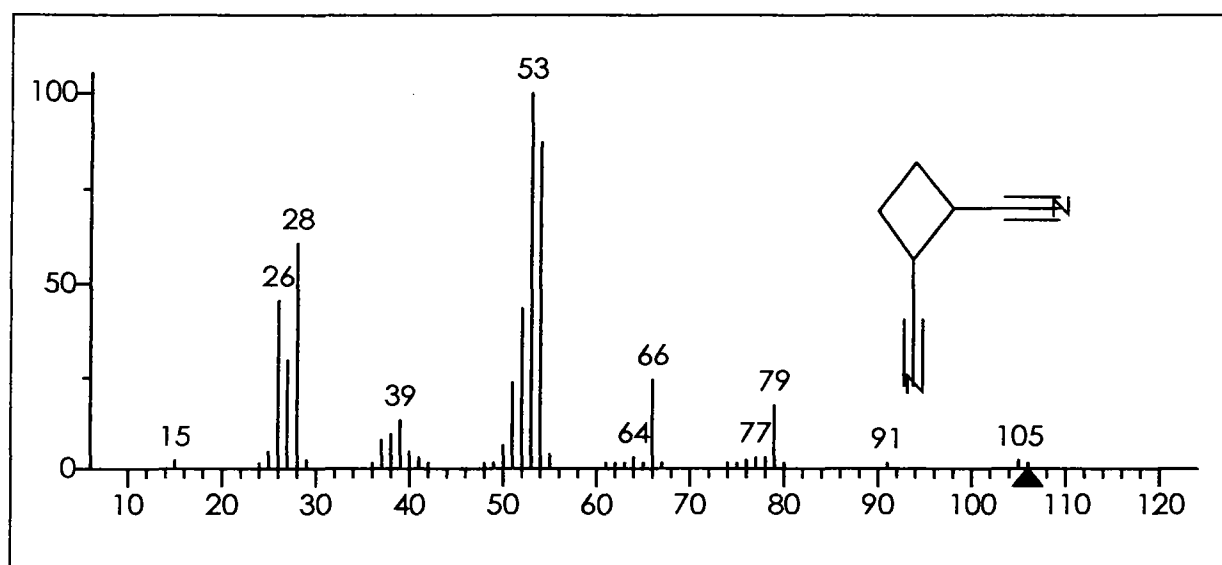


Fig 21a. Mass spectrum of. 1,2-Cyclobutanedicarbonitrile

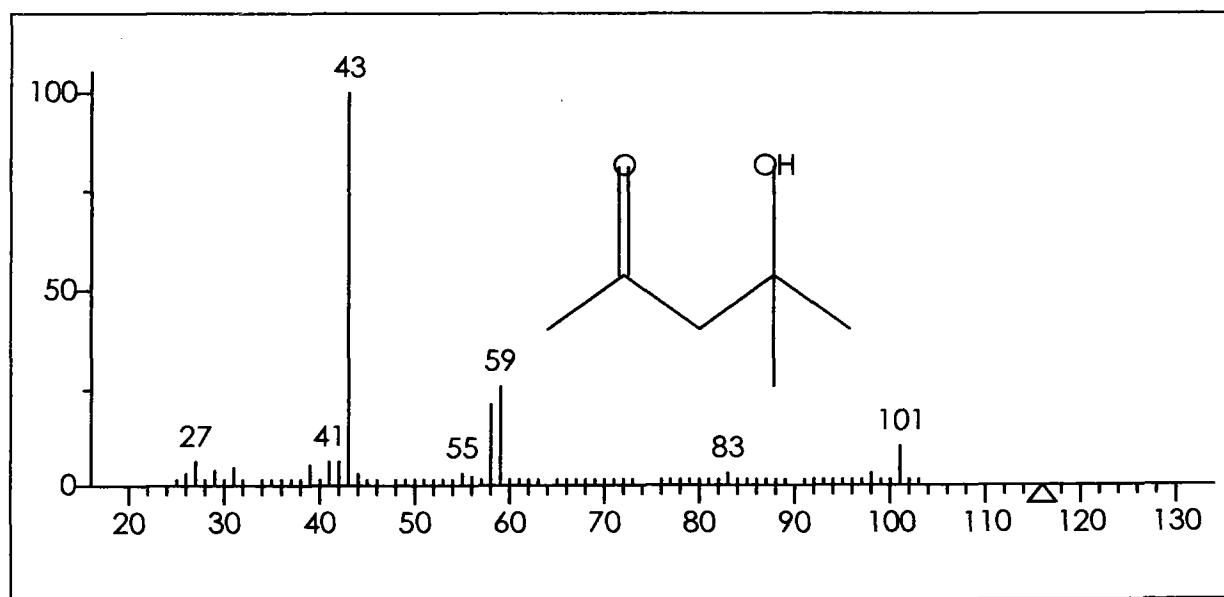


Fig 21b. Mass spectrum of. 4-hydroxy-4-methyl pentanone

SR-6

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PROCNO 1

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Time 5.51

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SWH 10330.578
FIDRES 0.157632
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RG 362
DW 48.400
DE 6.00
TE 292.0
D1 2.00000000
MORFST 0.00000000
MORWK 0.01500000

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P1 9.00
PL1 -2.00
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PC 1.00

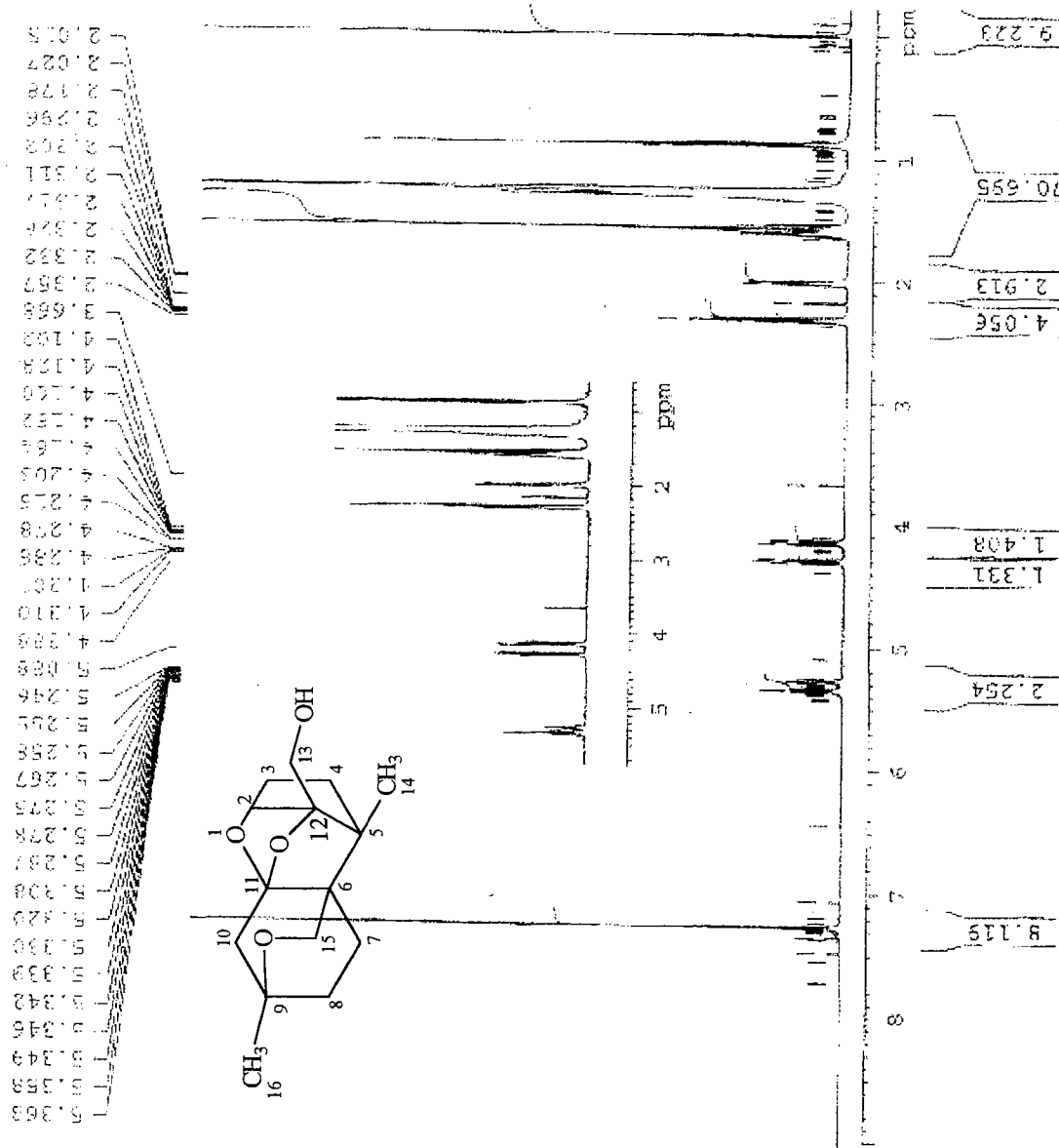
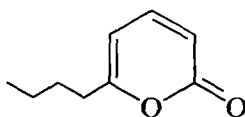


Fig 22 . ¹H-NMR spectrum of Sporol

normal aliphatic chain and three vinyl protons typical of an ABX spin system (Figure 23). The mass spectrum showed most abundant ion arising from cleavage adjacent to the ring oxygen m/z 95 (α -cleavage of the side chain and m/z 110 (β -cleavage with hydrogen transfer). Besides, fragment ions were observed at m/z 152, 138, 122, 110, 107, 95, 82, 81 and 69. The mass spectra and fragment pattern is shown in Figure 23. It was identified as 6-butyl- α -pyrone (11)



(11)

Further elution of column with dichloromethane and ethyl acetate (8:2 v/v) gave a colorless solid, which was further purified by preparative TLC using MeOH-H₂O (7:3, v/v) (2mg). Based on its ¹H- and ¹³C- NMR it was identified as sambucoin (4) which has also been reported from dichloromethane extract of culture filtrate.

Elution of column with dichloromethane and ethyl acetate (5:5, v/v) gave a colourless solid, which was further purified by crystallization from methanol (95%) (5mg). Its FT-IR showed a band at 1868 due to C=O, 1704 and 1630 cm⁻¹. UV max was recorded as 300 nm (methanol). The ¹H and ¹³C- NMR is reported in Table 21. ¹³C-NMR showed the presence of unsaturated carbons (138.5, 135.13, and 134.14) and a carbonyl carbon (168.49). On the basis of spectral

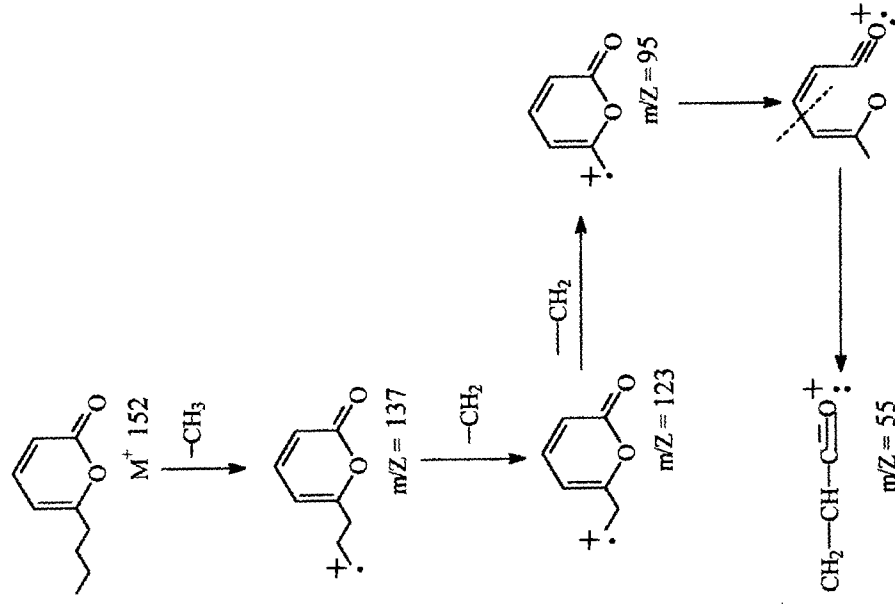
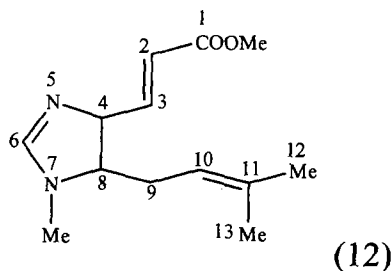
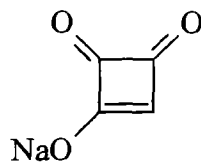


Figure 23. Mass fragmentation pattern of 6-Butyl- α -pyrone

analysis and co comparison with literature reported, it was identified as fungerin (12)



Elution of column with dichloromethane and ethyl acetate (4:6) gave a polar colorless solid, which was purified by crystallization from methanol (90%), mp 150-153°C, UV max 229 and 260 nm in methanol. Its mass spectrum showed a molecular ion peak at m/z 96 (M^+). It was identified as moniliformin (1-hydroxycyclobut-1-ene-3, 4-dione) (Figure 24) (13).



4.6.2.3 Methanol extract

The methanol extract of dried mycelia mass of *F. oxysporum*, showed the presence of a number of polar metabolites, when analyzed by GLC (using flame ionization detector, and operating conditions as: column temperature 70°C to 270°C @ 5°C min⁻¹, with detector and injector port temperatures 300°C) and TLC. These metabolites were separated by column chromatography over silica gel, eluting successively with hexane, hexane: dichloromethane,

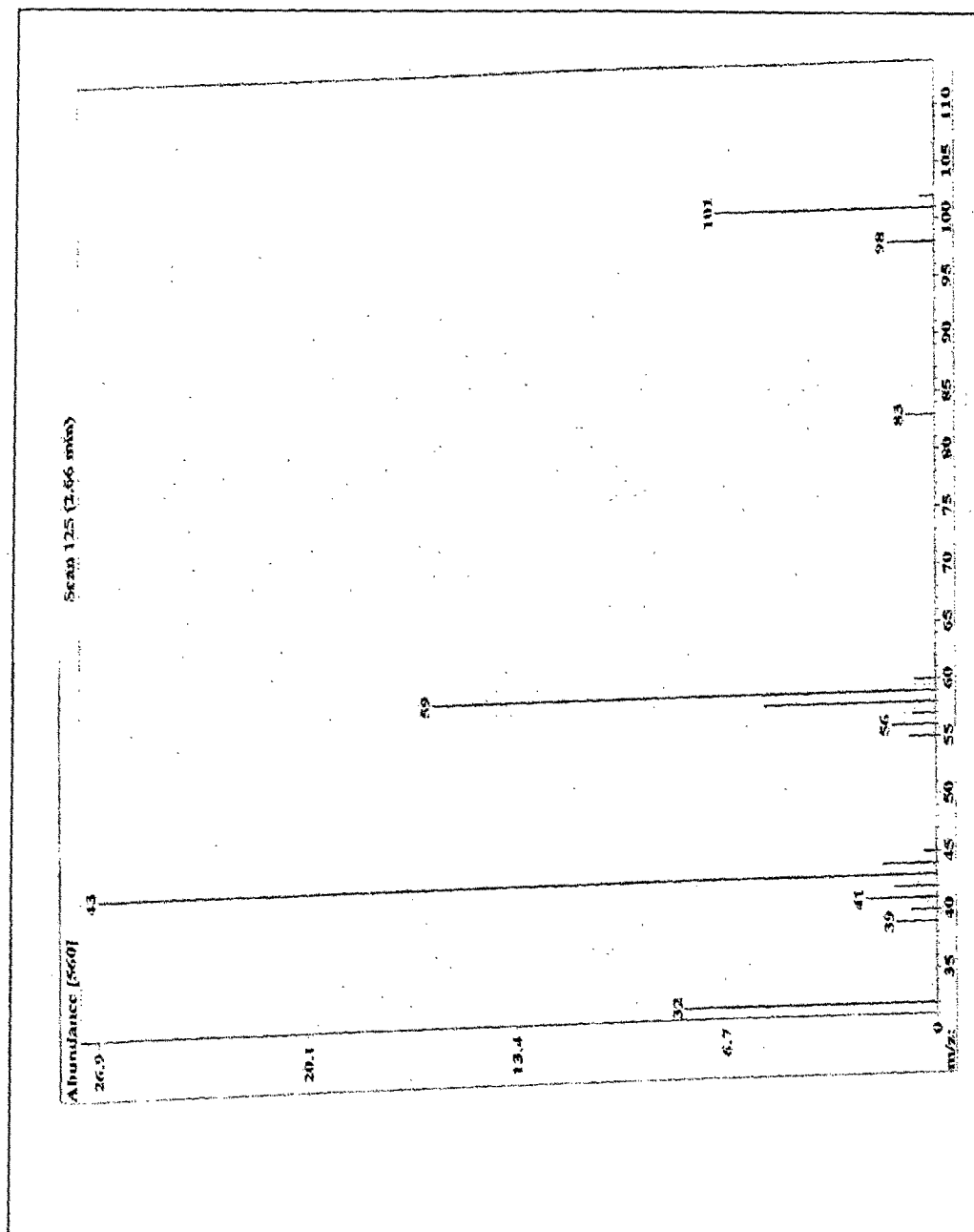


Fig 24. Mass spectrum of Moniliformin

NIVALENOL

Prominent mass fragments:

471(100), 459 (40) , 279 (36), 307(38), 635(20)

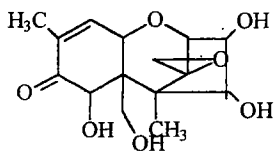
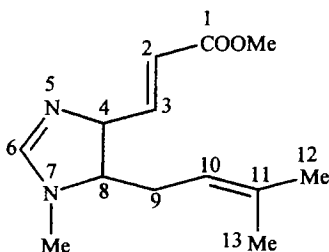


Table 21. Spectral details of Fungerin



IR $V_{\max}$ (KBr) (cm^{-1}) = 2775, 1704, 1630

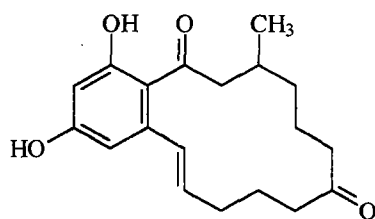
UV $\lambda_{\max}$ (Methanol)= 300 nm

NMR data:

C	^1H	^{13}C
1		168.49
2	6.5, d, J = 15 Hz	114.04
3	7.6, d, J = 15 Hz	134.3
4		134.14
5		
6	7.4 , s	138.5
7		
8		134.2
9	3.4, d, J= 7 Hz	22.3
10	5.0, m	119.31
11		1334.30
12	1.76	25.57
13	1.72	17.92
OMe	3.7, s	52
NMe	3.5, s	31.64

dichloromethane and dichloromethane: ethyl acetate in different ratios and identified by FT-IR, NMR and mass spectroscopy.

Elution of column with dichloromethane and acetone (7:3, v/v) gave a colorless crystalline compound, further purified by crystallization from methanol. UV maxima 236 ($\epsilon=29,7000$, 274 $\epsilon=13,909$) and 316 ($\epsilon=6,020$) in methanol. FT-IR showed bands at 1780 and 1750 cm^{-1} due to C=O stretching and 3340 cm^{-1} for hydroxyl group. (Figure 25) ^1H and ^{13}C NMR showed the presence of an aromatic ring. Based on co-comparison of NMR spectra with reported structure, it was identified zearalenone (14) a β -resorcylic acid lactone (6-(10-hydroxy-6-oxo-trans-1-undecenyl)- β -resorcylic lactone) (Baldwin et. al 1962).



(14)

Elution of column with dichloromethane and acetone (5:5, v/v) gave colourless solid, which was further purified by crystallization from hexane (25mg) mp. 148-152 lit. 152-152 $^{\circ}\text{C}$. Mass spectrum showed a molecular ion peak at m/z 466 (M^+). Based on its spectral data it was identified as T-2 toxin (3'', 4b,8')-12,13-epoxytrichothec-9-ene-3,4,8,15-tetrol-4,15diacetate-8-(3-methylbutanoate) (15).

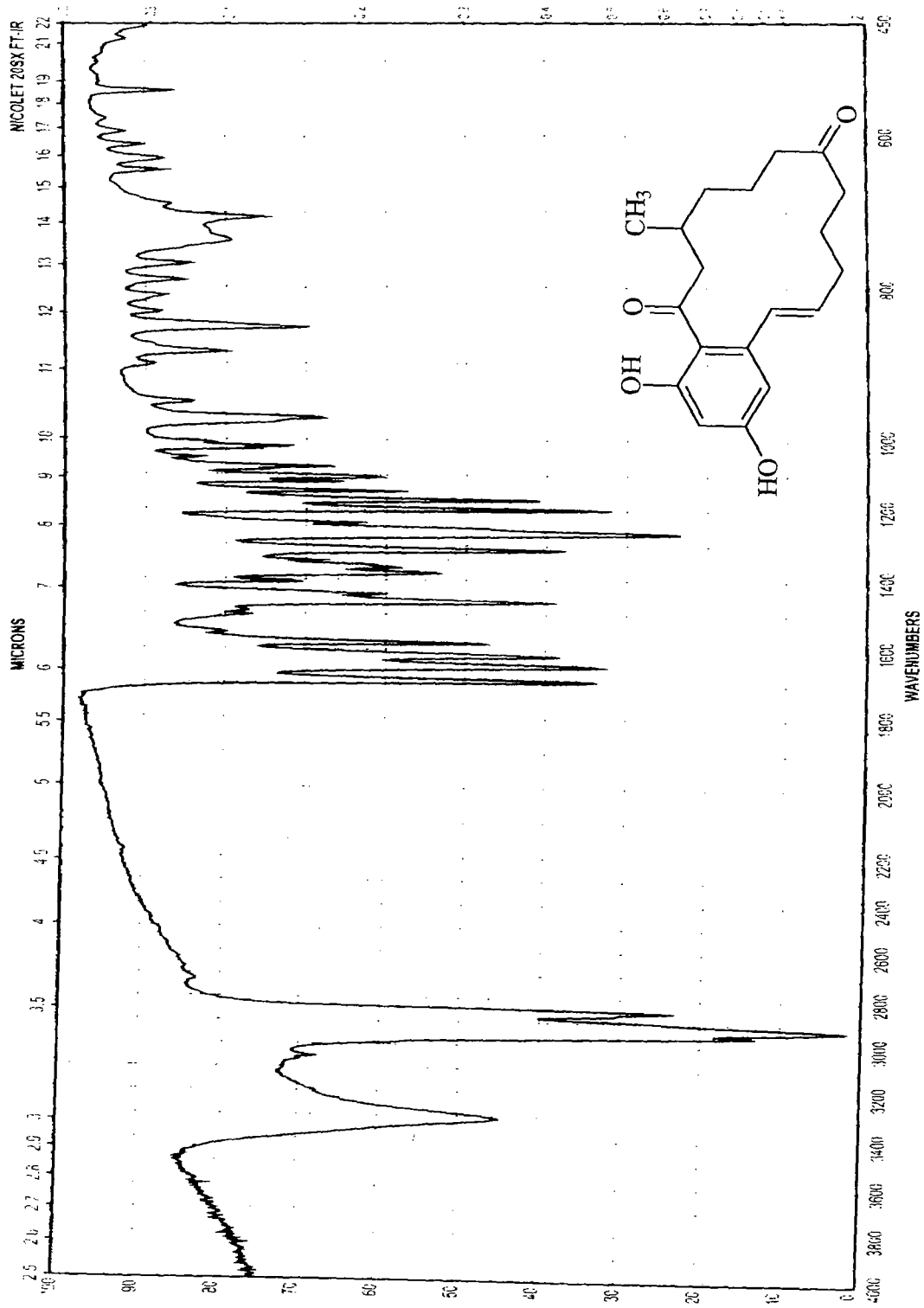


Fig 25a . FT-IR spectrum of Zerealenone

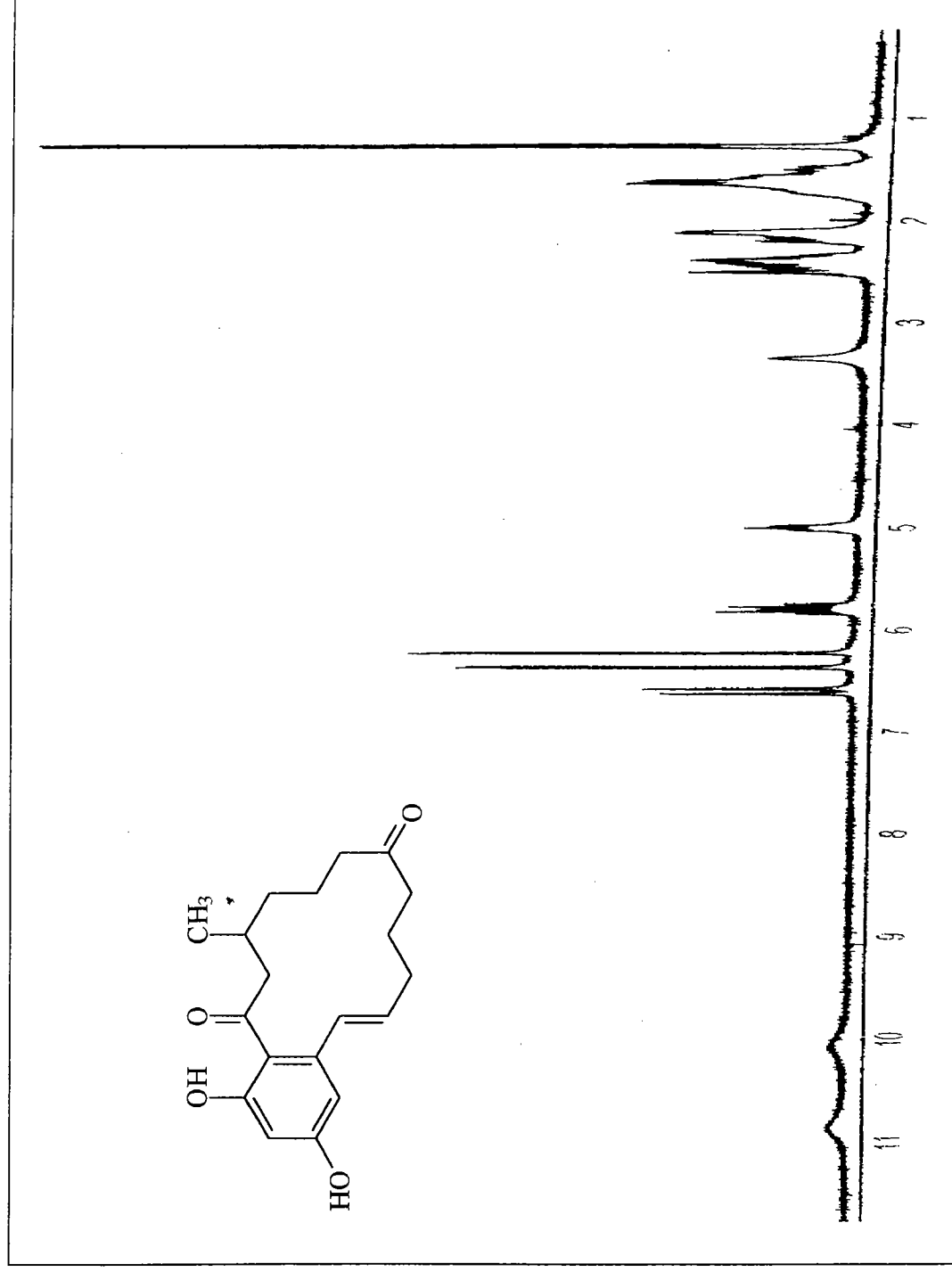
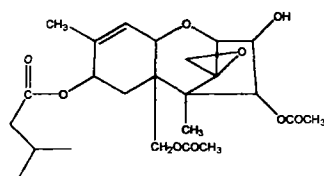
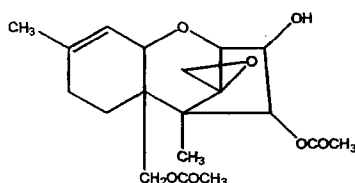


Fig 25b . ¹H-NMR spectrum of Zerealenone



(15)

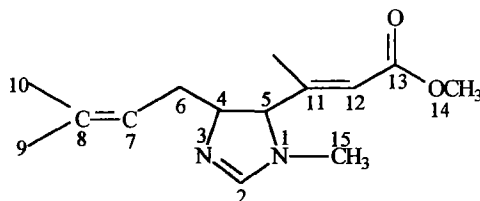
Further elution of column with dichloromethane and acetone (4:6, v/v) gave colorless solid, which was further purified by crystallization from hexane (20mg). Mass spectrum showed a molecular ion peak at m/z 366. (M^+). Its NMR spectrum was closely related to spectrum of T-2 toxin except missing of 3-methylbutanoate substituent at R_5 position. Thus based on different spectrum it was identified as diacetoxyscirpenol (trichothec-9-ene-3, 4,15-triol), 12, 13-epoxy-4,15-diacetate (16).



(16)

Elution of column with dichloromethane and acetone (4:6, v/v) gave colourless solid, which was further purified by crystallization from hexane (25mg) mp. 90-93°C. Elemental analysis showed C, 66.79% (calcd. 66.64%); H, 7.67% (calcd. 7.74); N, 11.91% (calcd. 11.96); O, 13.80 (Calcd. 13.66). UV max 300nm. FT-IR showed bands at 3097 (trans –trans conjugated alkene), 2980-2820, 1704, 1633 (ester C=O and the conjugated double bonds) and 1433 cm^{-1} . Mass spectrum showed molecular ion peak at m/z 234 (M^+ , 43%), with fragment ion

peaks at m/z 219 ($M^+ - 15$), 187 (21% $M^+ - CH_3OH$), 173 (64%), 159 (100%) and m/z 133(52%). ^1H and ^{13}C -NMR are reported in Table 22, Figure 26. In the ^1H -NMR spectrum the high coupling constant of C-11 and C-12 protons indicated a trans configuration at the relevant double bond. On the basis of the spectroscopic data and comparison with the reported data, the structure visolstricin (17) was established as 3-[1-methyl-493-methyl-2-butenyl]-imidazol-5-yl]-2-propenoic acid methyl ester (Visconti and Solfrizzo, 1994)

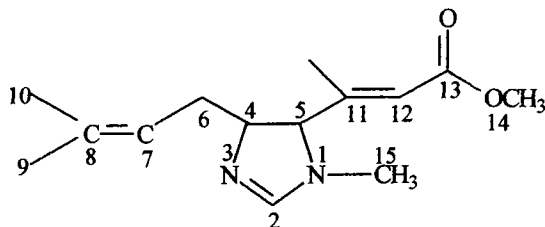


(17)

Elution of column with dichloromethane and acetone (4:6, v/v) gave colorless solid, which was further purified by crystallization from hexane (12mg). It was separated into two isomers by preparative TLC using chloroform: acetone (9:1). The mass spectrum of the compounds showed molecular ion peaks at m/z 266 (M^+), with fragment ion peaks at m/z 182 (3%), 161(6%), 109(100%) as base peak and m/z 95 (42%). ^{13}C -NMR showed the presence of unsaturation (138.8 and 121.9). Based on spectral data and literature values, it was identified as 8-hydroxy-isotrichodermol (5)

Further elution of column with dichloromethane and acetone (4:6, v/v) gave colourless oil, which was purified by preparative TLC (chloroform: acetone 9:1) into two compounds. Both the metabolites showed a molecular ion peak at

Table 22. Spectral details of Visolstricin



m.p. 92⁰C - 94⁰C

IR $V_{\max}$ (KBr) (cm⁻¹) = 3097, 2980-2820, 1704

UV $\lambda_{\max}$ (Methanol) = 297 nm

Prominent mass fragments

m/z = 234(43, M⁺), 219(2), 202 (20), 187 (210), 173 (64), 159 (100),
133(52)

NMR data:

C	¹ H	¹³ C
1		
2	7.37, 1H, s	138.4
3		
4		134.3
5		134.3
6	3.4, 2H, d, J= 6.8	22.3
7	4.99, 1H, m, J = 6.8	119.3
8		134.1
9	1.68, 3H, s, J= 1.2	17.9
10	1.71, 3H, s, J= 1.2	25.5
11	7.6, 1H, d, J= 15	1335.2
12	6.5, 1H, d, J= 15.5	114.2
13		168.5
14	3.73, s, 3H	51.4
15	3.50, s, 3H	31.6

Current Data Parameters
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PROCNO 1

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SOLVENT CDC13
NS 16
DS 0
SWH 6410.256 F
FIDRES 0.195625 F
AQ 2.5559540 S
RG 90.5
DW 78.000 U
DE 4.50 U
TE 300.0 F
D1 5.00000000 S

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NUC1 1H
P1 5.80 U
PL1 3.00 C
SFO1 400.1328009 M

F2 - Processing parameters
SI 16384
SF 400.1300086 M
WDW EM
SSB 0
LB 1.00 F
GB 0
PC 1.00

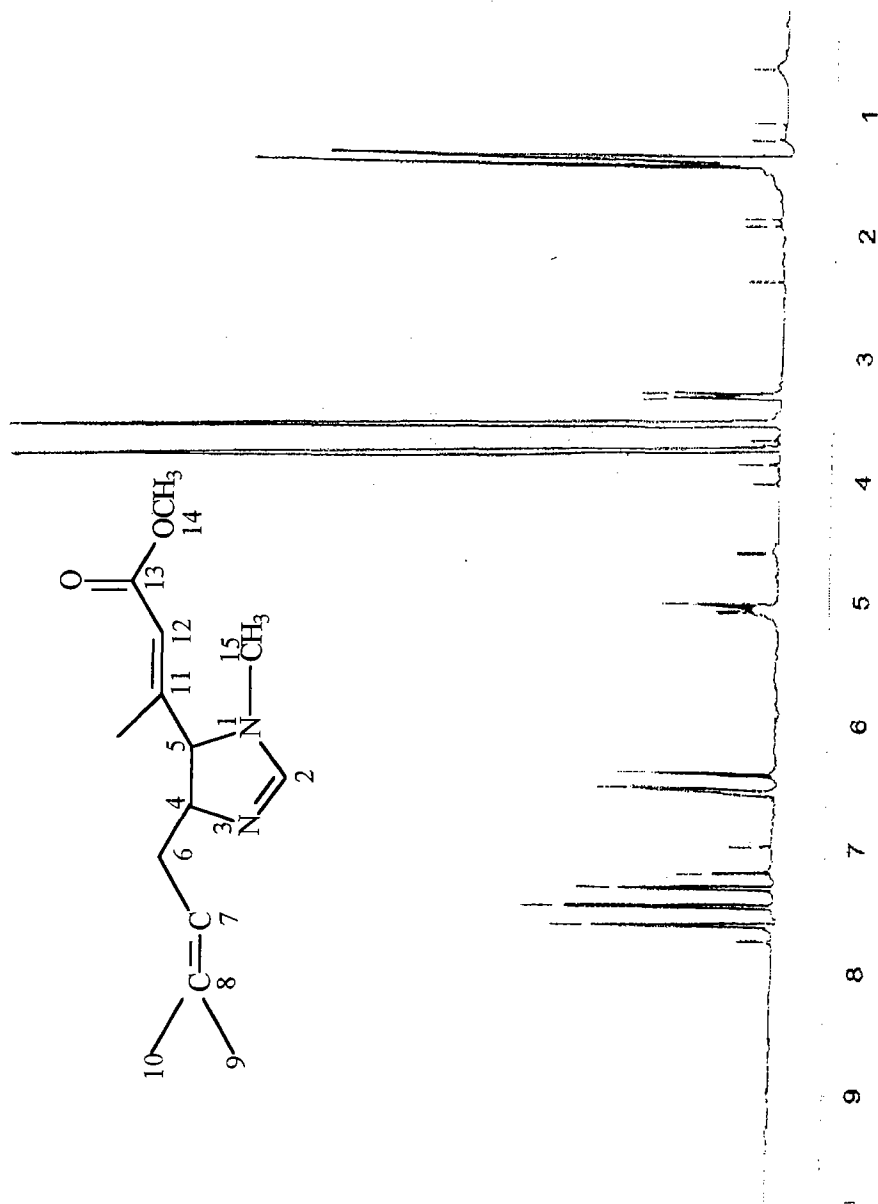
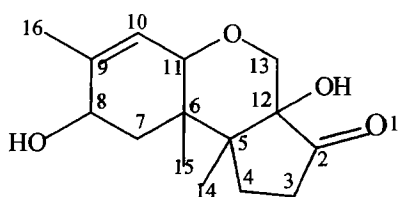


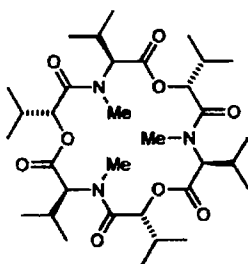
Fig 26. ¹H-NMR spectrum of Visolstricin

M^+ 266 with fragment ion peaks at m/z 242, (10), 180(30), 136(25), 109(45%). The FT-IR showed the presence of hydroxyl groups (3389cm^{-1}) and a carbonyl group (1738 cm^{-1}). The ^1H and ^{13}C -NMR are recorded in Table 23, Figure 27-28. Both the metabolites differ in the position of hydroxyl group at C_8 (δ 4.13 bm/ 4.08 m). Similar metabolites have also been reported from *F. sporotrichioides* MC-72083 (Corley et. al, 1987). These metabolites were thus identified as 8 β -hydroxysambucoin(18) and 8 α - hydroxysambucoin (19).



(19)

Elution of column with dichloromethane and acetone (4:6, v/v) gave a colourless viscous liquid (3mg), which was identified as enniatin B (20). By comparison of its NMR and mass spectral data with those in the literature (Tomoda et al, 1992).



(20)

Elution of column with dichloromethane and acetone (4:6, v/v) gave a colourless amorphous solid was purified by crystallization from methanol as

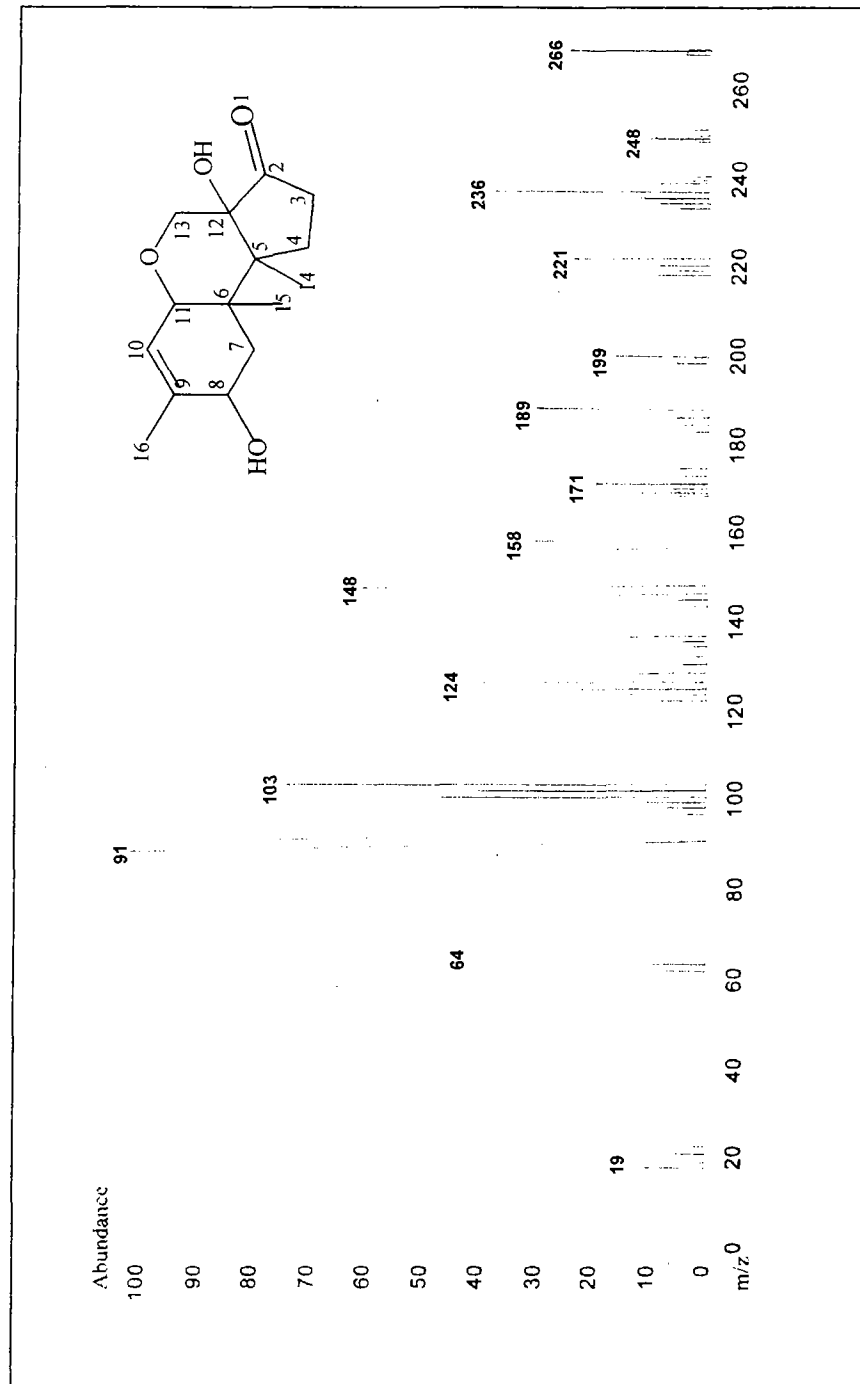


Fig 27a. Mass spectrum of 8-Hydroxy sambucoin

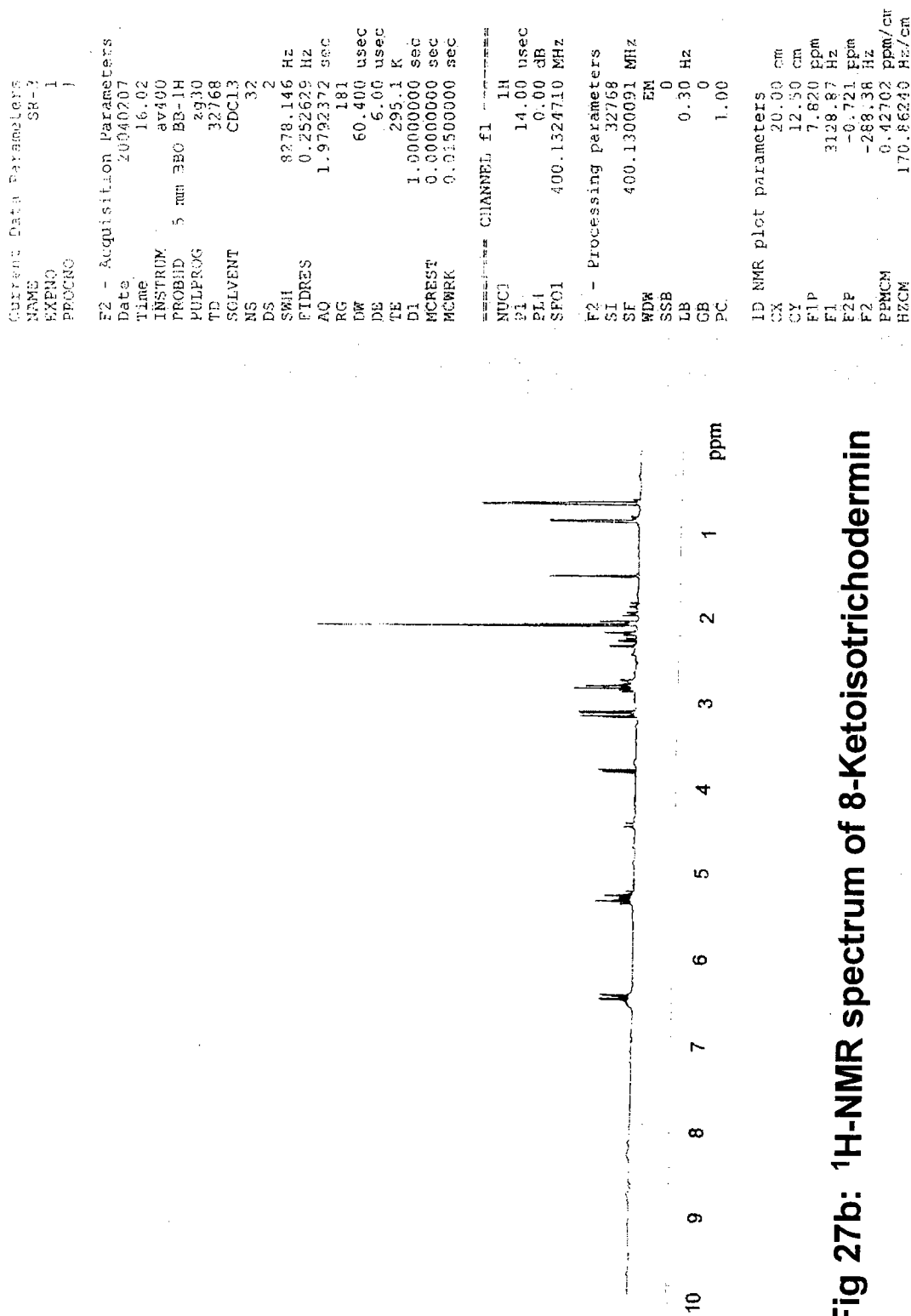
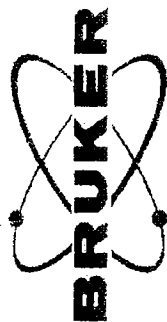
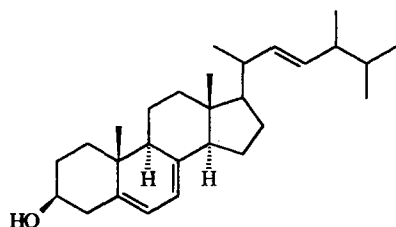


Fig 27b: ¹H-NMR spectrum of 8-Ketoisotrichodermin

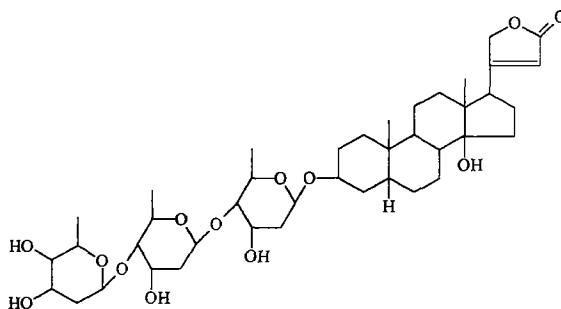
colourless prisms (100mg). FT-IR spectrum showed the presence of a broad band at $3500 - 3400\text{ cm}^{-1}$ due to hydroxyl group. The NMR (CDCl_3) showed the presence of two quaternary methyl groups (δ 0.72 and 0.87 as singlets), one isopropyl group (δ 1.5, doublet $J=6.0\text{ Hz}$), two superimposed secondary methyl groups (δ 1.20, doublet, $J=7\text{ Hz}$), one CH of the isopropyl group and a secondary carbinol methine (δ 3.55 as multiplet), and two ethylene protons (δ 5.38, doublet and δ 5.57 as doublet). Other signals were at δ 0.9, 1.0, 1.3 and between δ 1.5-2.5 as doublets and triplets. The mass spectrum showed a molecular ion peak at m/z 396, with a base peak at m/z 363 (100%). The mass spectrum showed fragment ion peaks at m/z 378 ($M^+ - \text{H}_2\text{O}$, 10%), m/z 363 ($M^+ - \text{H}_2\text{O}-\text{CH}_3$), m/z 337 ($M^+ - \text{C}_3\text{H}_7$), m/z 271, m/z 253, m/z 211, m/z 199 and m/z 183. On the basis of FT-IR, ^1H -NMR and mass fragmentation pattern (Figure 29), the metabolite was identified as ergosterol (21).



(21)

Elution of column with dichloromethane and acetone (3:7, v/v) gave colorless solid (5mg), which was further purified preparative TLC. FT-IR spectrum showed the presence of a broad band at $3500 - 3400\text{ cm}^{-1}$ due to hydroxyl group and a band at 1765 cm^{-1} due to $\text{C}=\text{O}$ stretching. ^1H -NMR and

mass spectrum were closely related to a product isolated from other fungal sources. On the basis of mass spectrum, which showed a molecular ion peak at m/z 764 (M^+), with fragment ion peaks at m/z 401, 339, 203 and 113 it was identified as digitoxin (22), Figure 30.

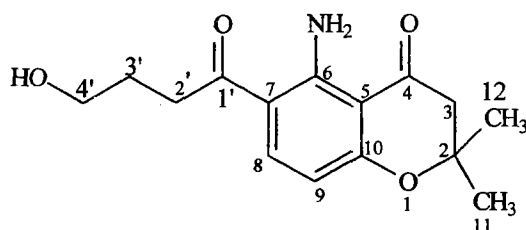


(22)

Elution of column with dichloromethane and acetone (3:7, v/v) gave a mixture, which was separated by repeated preparative TLC into two compounds. The FT-IR of the first compound showed bands at 3669, 3389 cm^{-1} for hydroxyl group, and a band at 1660 for $\text{C}=\text{O}$ group. UV max was found to be 370 nm. . The mass spectrum showed a molecular ion peak at m/z 275 (100%) as base peak with fragment ion peaks at m/z 260, 257, 244, 220 and 162. The ^1H -NMR showed the presence of an aromatic ring (δ 7.93, d, J = 9Hz) and gem dimethyl at δ 1.45 (6h, S). Based on NMR and mass spectroscopy it was tentatively identified as 2,2-dimethyl-5-amino-6- (2'ene-4'hydroxybutyl)-4-chromone (Table 24). The FT-IR of the second compound (2mg), a pale yellow solid showed band similar to first compound, but its mass spectrum showed a molecular ion peak at m/z 277 (M^+) with fragment ion peaks at m/z 259, 244, 233, 218 (100%, base peak). NMR spectrum was similar to first compound except it showed a multiplet

at d 1-95-2.2 corresponding for six protons. On the basis of these spectral data, it was tentatively identified as 2,2-dimethyl-5-amino-6- (4'-hydroxybutyryl)-4-chromene (23) (Table 25)

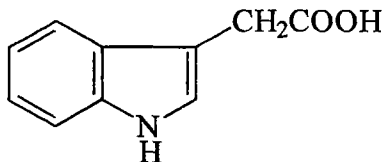
2,2- dimethyl-5-amino-6- (4'- hydroxybutyryl)-4-chromone



(23)

Beside a small amount of indole acetic acid (24) was also isolated.

(Figure 31)



(24)

4.7 Bioactivity of isolated metabolites

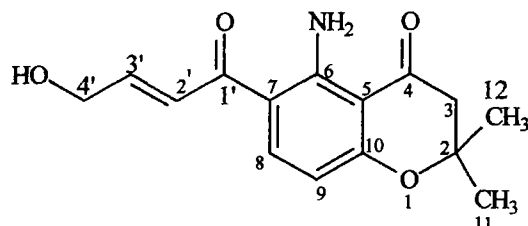
4.7.1 Fungicidal activity

4.7.1.1 Mycelia extract

4.7.1.1.1 Dichloromethane extract

Five secondary metabolites separated by column chromatography were tested for fungicidal activity against *R. bataticola*, *M. phaseolina*, *S. rolfsii*, *P. aphanidermatum* and *P. debaryanum*.

Table 23. Spectral details of 2,2- dimethyl-5-amino-6-(2' ene-4'-hydroxybutyryl)-4-chromone



Yellow Coloured liquid

IR $V_{\max}$ (KBr) (cm^{-1}) = 3669, 3389, 3277, 2957, 2852, 1660, 1651,

UV $\lambda_{\max}$ (MeOH) = 370 nm

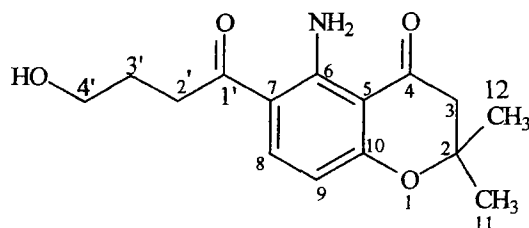
Prominent mass fragments

m/z = 275(100, M^+), 260(10), 257 (23), 244 (49), 242 (28), 220 (19),
218(30), 201(10), 188(37), 162(15), 106(17)

NMR data:

C	^1H
1	
2	
3	2.69, 2H, s
4	
5	
6	
7	
8	7.93, 1H, d, J= 9 Hz
9	6.07, 1H, d, J= 9 Hz
10	
11	1.45, 6H, s
12	1.45, 6H, s
1'	
2'	7.17, 1H, d, J= 15.2
3'	6.97, 1H, J= 15.2
4'	4.43, 2H, m
Ar-NH ₂	9.4-9.7, 2H

Table 24. Spectral details of Sporotrichol 2,2- dimethyl-5-amino-6-(4'- hydroxybutyryl)-4-chromene



pale Yellow semisolid

IR $V_{\max}$ (KBr) (cm^{-1}) = 3397, 3285, 2934, 2872, 1740

UV $\lambda_{\max}$ (MeOH) = 276 nm

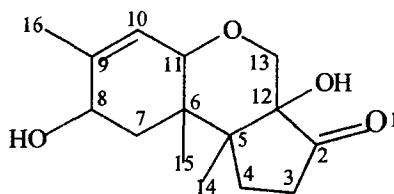
Prominent mass fragments

m/z = 277(56), 259(20), 257 (23), 244 (19), 233 (65), 218 (100), 204(16), 190(14), 176(15), 162(80), 106 (10)

NMR data:

C	^1H
1	
2	
3	2.69, 2H, s
4	
5	
6	
7	
8	7.87, 1H, d, J = 8.9 Hz
9	6.07, 1H, d, J = 8.9 Hz
10	
11	1.45, 6H, s
12	1.45, 6H, s
1'	
2'	2.99, 1H, t, J = 7
3'	1.95-2.2, 1H, m
4'	3.71, 2H, t, J = 6 Hz
Ar-NH ₂	9.37-9.6, 2H

Table 25. Spectral details of 8- hydroxysambucoin



IR $\nu_{\max}$ (KBr) = 3389 (-OH), 1738 (C=O)

UV $\lambda_{\max}$ (Methanol) = 194 nm

Prominent mass fragments

m/z = 266, 242 ($M - H_2O$)⁺(10), 180 (30), 136(25), 123(30), 109(45),
55(40), 43(100)

NMR data:

C	¹ H	¹³ C
1		
2		210.2, s
3	2.3-2.5, m	34.2, t
4	2.0, m	27.5, t
5		46.1
6		41.1, s
7	1.45, dd, J= 9.6	37.7
8	4.13	69.4, d
9		136, s
10	5.33	125, d
11	4.03	75, d
12		74.13
13	3.4, d, J = 11.3	66.4 t
14	1.8, s	16
15	0.7, s	15
16	1.76,	18.6

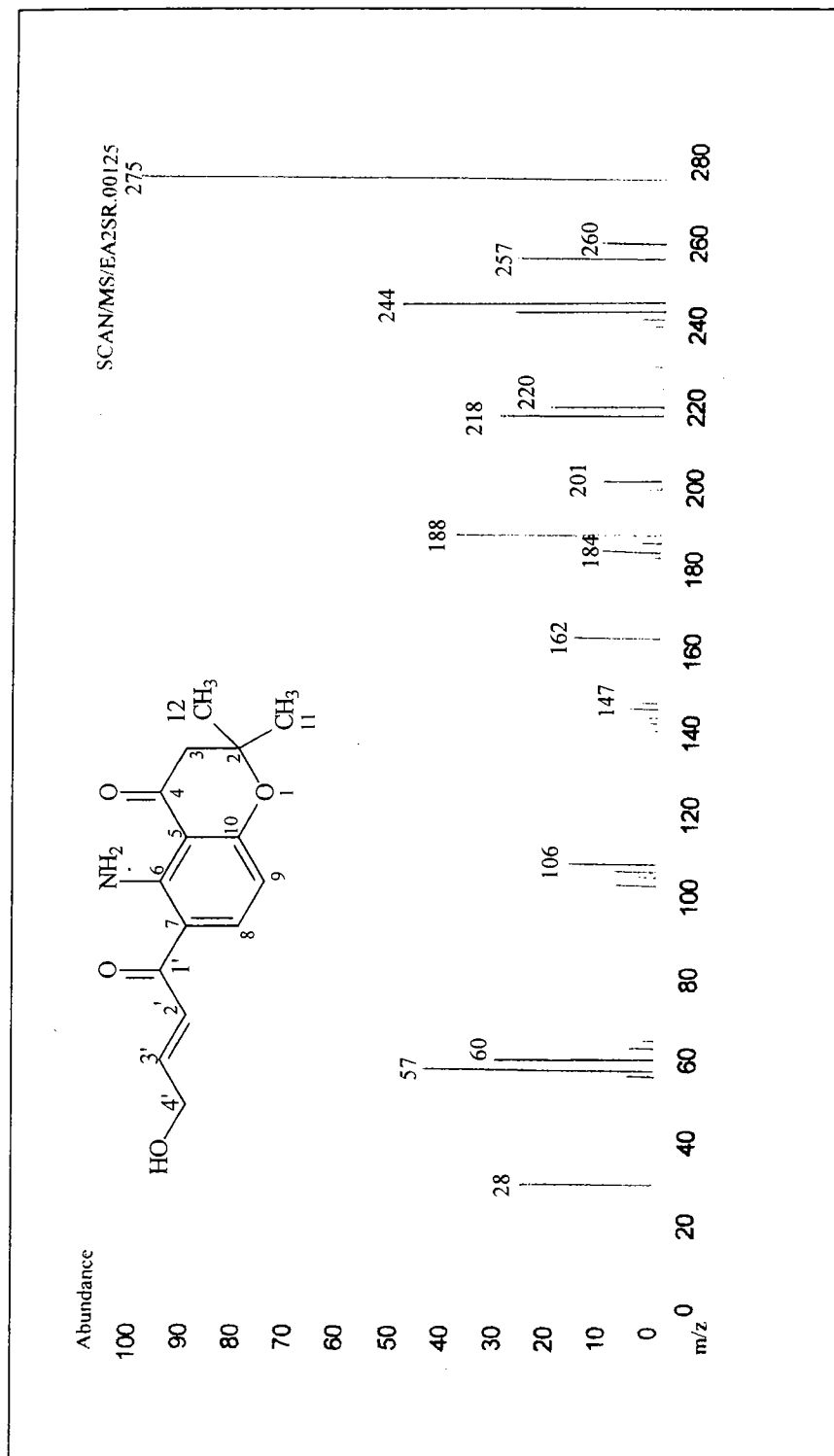


Fig 28a. Mass spectrum of 2,2- dimethyl-5-amino-6-(2' ene-4'- hydroxybutyryl)-4-chromone

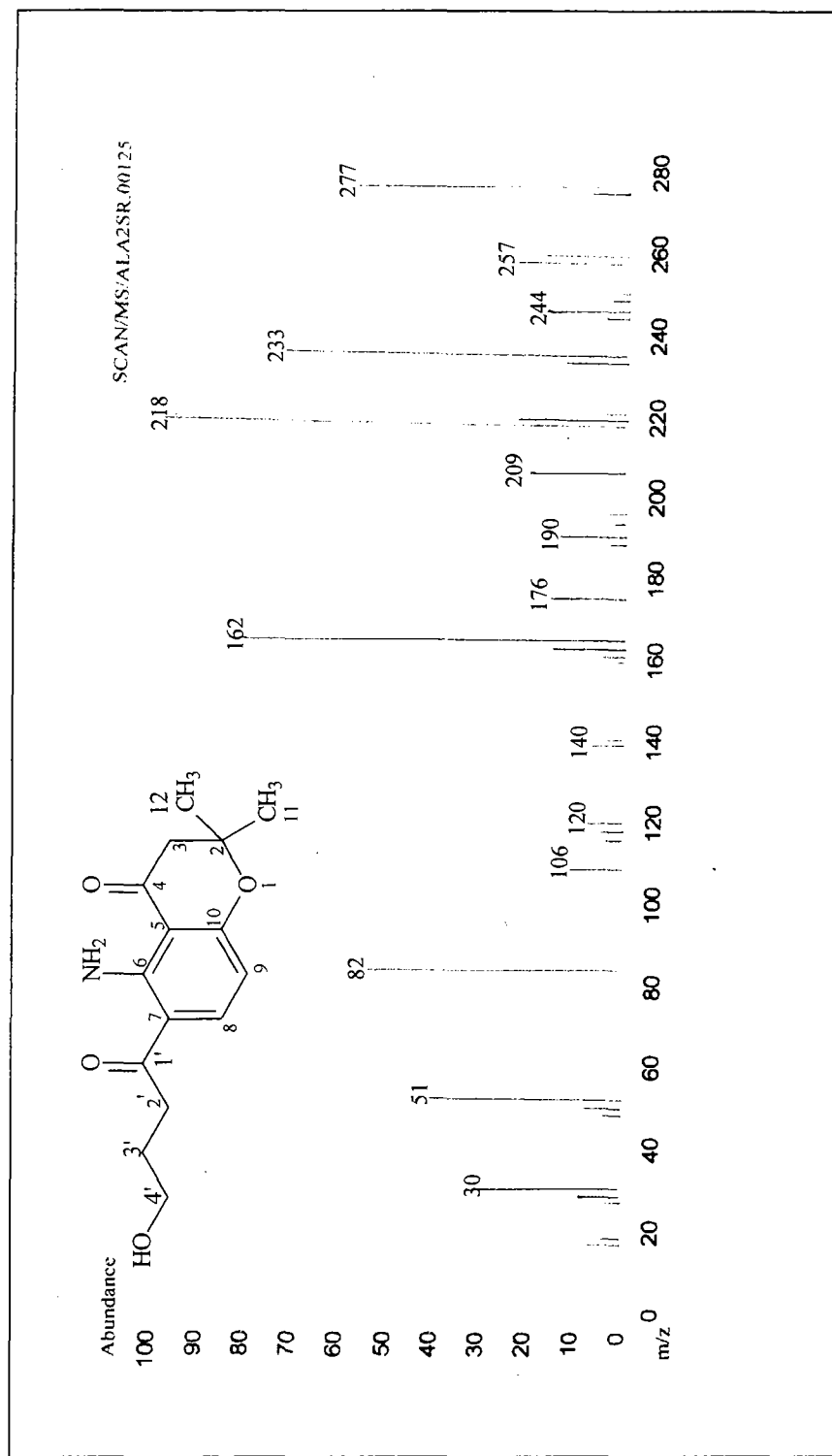


Fig 28b. Mass spectrum of. 2,2- dimethyl-5-amino-6-(4'- hydroxybutyryl)-4-chromone

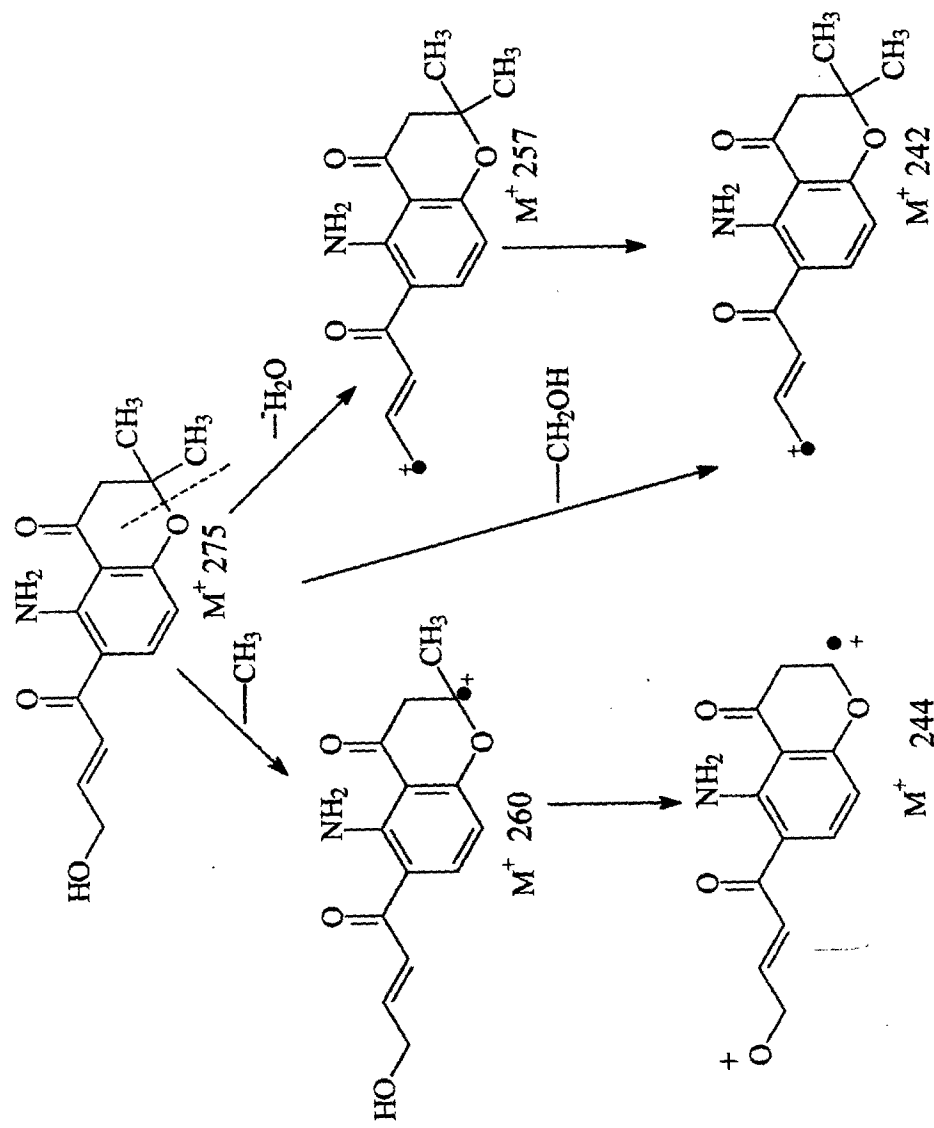


Figure 28c. Mass fragmentation pattern of 2,2-dimethyl-5-amino-6-(2' ene-4'-hydroxybutyryl)-4-chromone



Current Data Parameters
NAME: SR-1
EXCHG:
PROCNO:

F2 - Acquisition Parameters

Date_ 20040207
Time 16.02
INSTRUM av400
PROBHD 5 mm BBO BB-1H
PULPROG zgpg30
PC 32768
SOLVENT CDCl3
NS 32
DS 2
SWH 8276.146 Hz
FIDRES 0.252629 Hz
AQ 1.9792372 sec
RG 181
LW 60.400 usec
DE 6.00 usec
TE 295.1 K
D1 1.00000000 sec
MCREST 0.00000000 sec
MCMRK 0.01500000 sec

===== CHANNEL F1 =====

NUC1 1H
P1 14.00 usec
PL1 0.00 dB
SFO1 400.1324710 MHz

F2 - Processing parameters

SF 32768
SF 400.1300091 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

ID NMR plot parameters

CX 20.00 cm
CY 12.50 cm
FID 7.826 ppm
F1 3128.87 Hz
F2 -0.721 ppm
F3 -288.36 Hz
PPMCM 0.42702 ppm/cm
HZCM 170.86240 Hz/cm

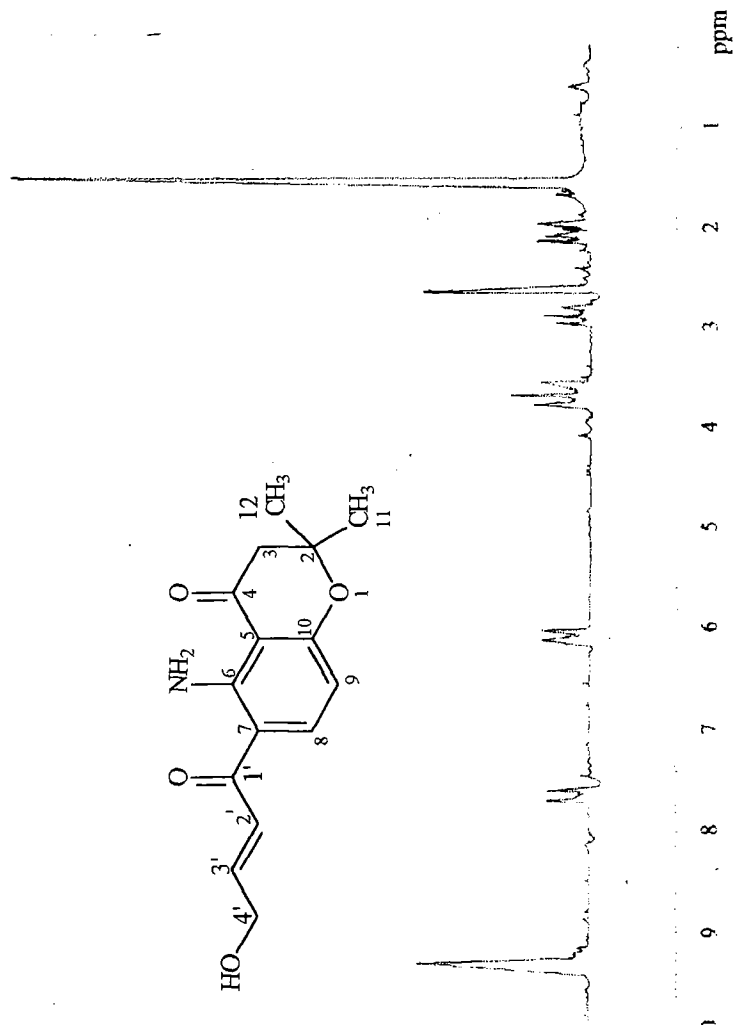


Fig 28d . ¹H-NMR spectrum of 2,2- dimethyl-5-amino-6-(2' ene-4'- hydroxybutyryl)-4-chromone

Current Data Parameters
 NAME 58115-2004
 EXPNO 1
 PROCNO 1

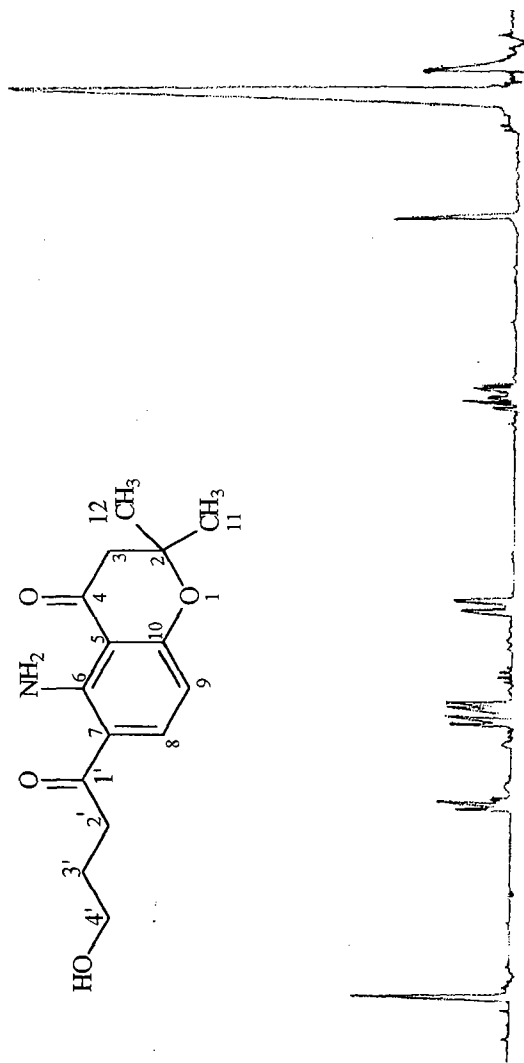
F2 - Acquisition Parameters

Date_ 20040415
 Time_ 5.51
 INSTRUM spect
 PROBHD 5 mm BBO BB-1H
 PULPROG zgpg30
 TD 65535
 SOLVENT CDCl3
 NS 26
 DS 0
 SWH 10330.578
 FIDRES 0.157632
 AQ 3.1720407
 RG 362
 DW 48.400
 DE 6.00
 TR 292.0
 D1 2.00000000
 MCREST 0.00000000
 MCWRR 0.01500000

===== CHANNEL f1 =====
 NUC1 1H
 P1 9.00
 PL1 -2.00
 SFO1 500.1330855

F2 - Processing parameters

SI 32768
 SF 500.1330123
 WDW EM
 SSB 0
 LB 0.30
 GB 0
 PC 1.00



10 9 8 7 6 5 4 3 2 1

Fig 28e . ¹H-NMR spectrum of 2,2- dimethyl-5-amino
 -6-(4'- hydroxybutyl)-4-chromone

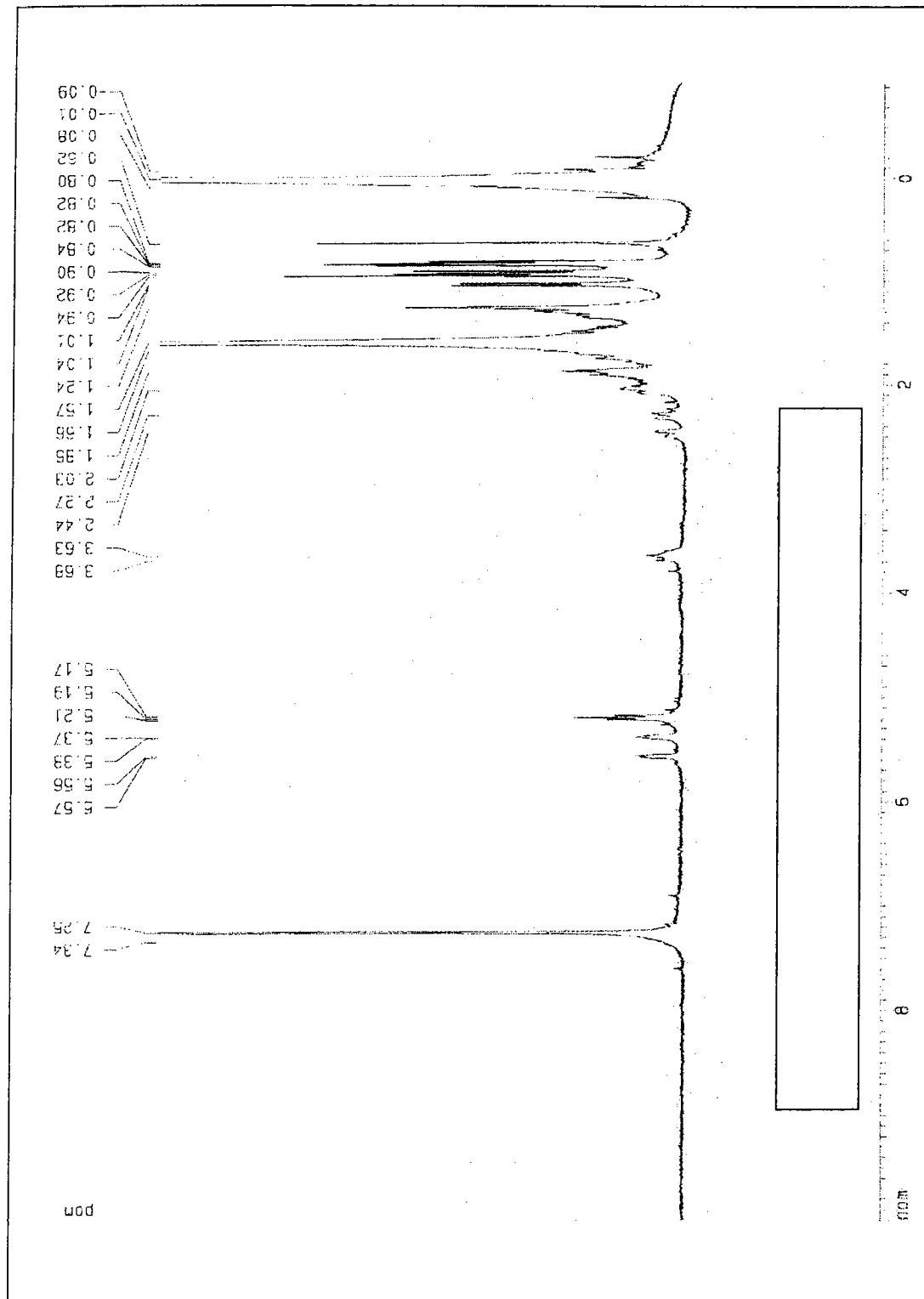


Fig 29a: ^1H -NMR spectrum of Ergosterol

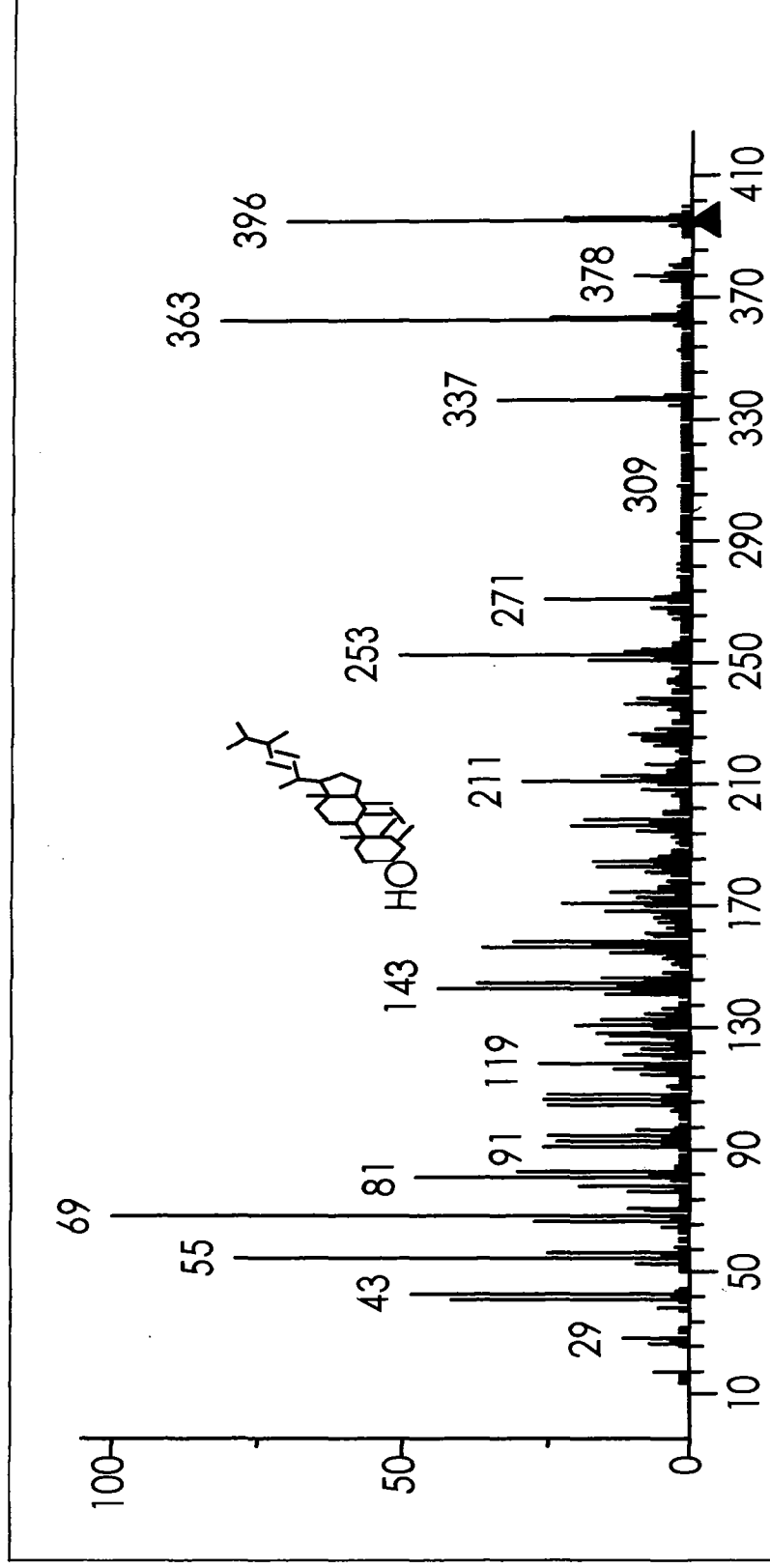


Fig 29 a. Mass spectrum of Ergosterol

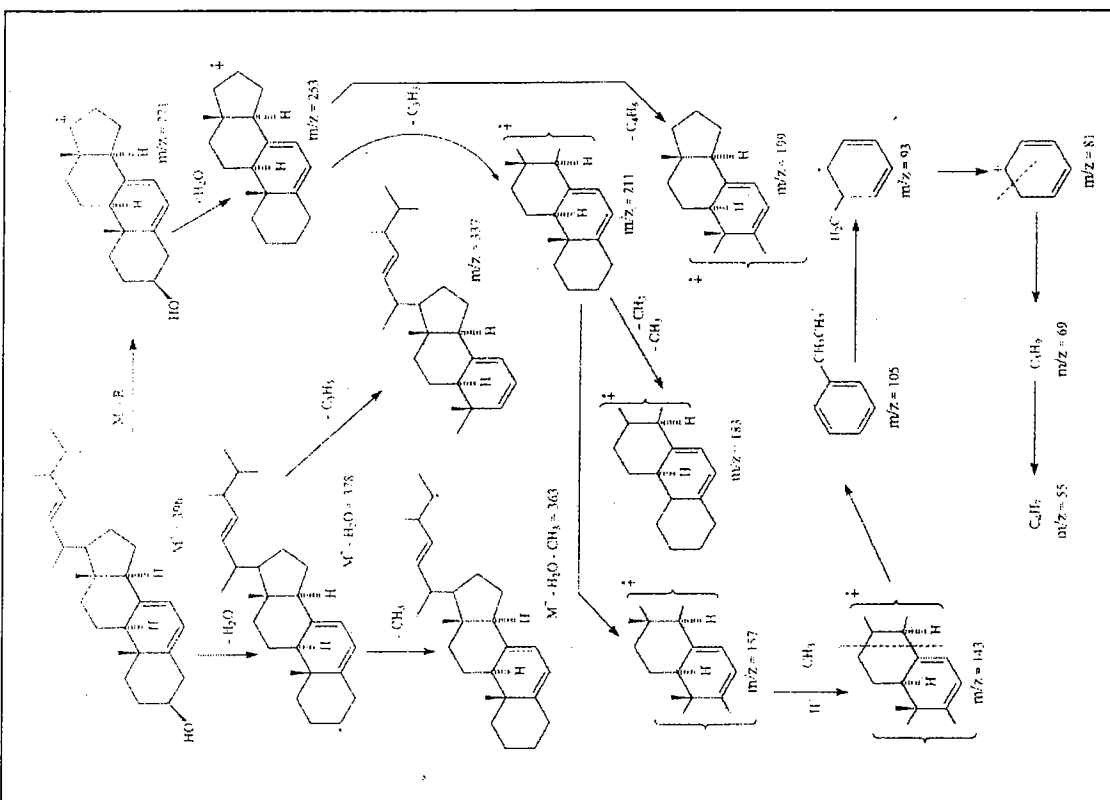


Figure 29c. Mass fragmentation pattern of Ergosterol

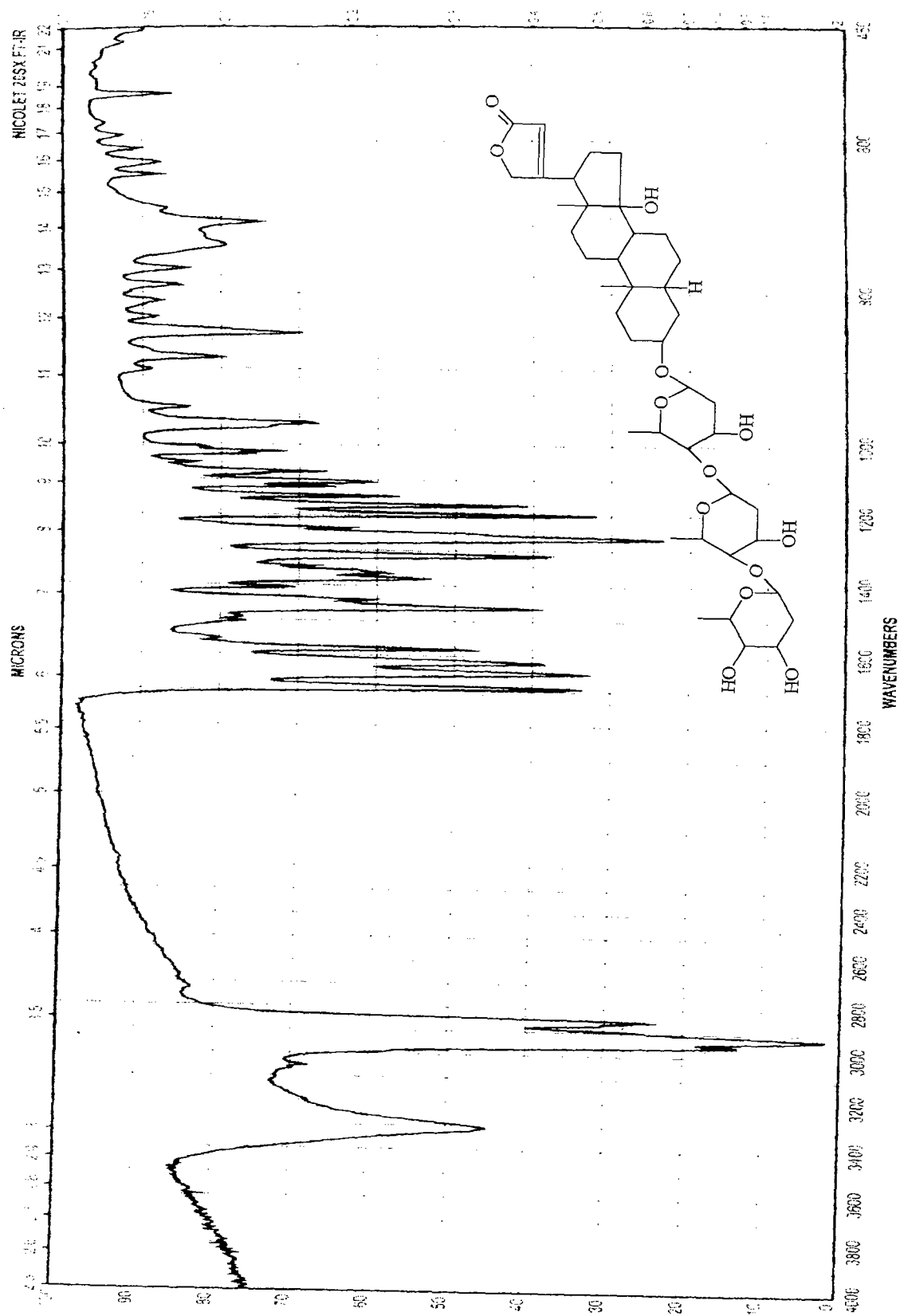


Fig 30a . FT-IR spectrum of Digitoxin

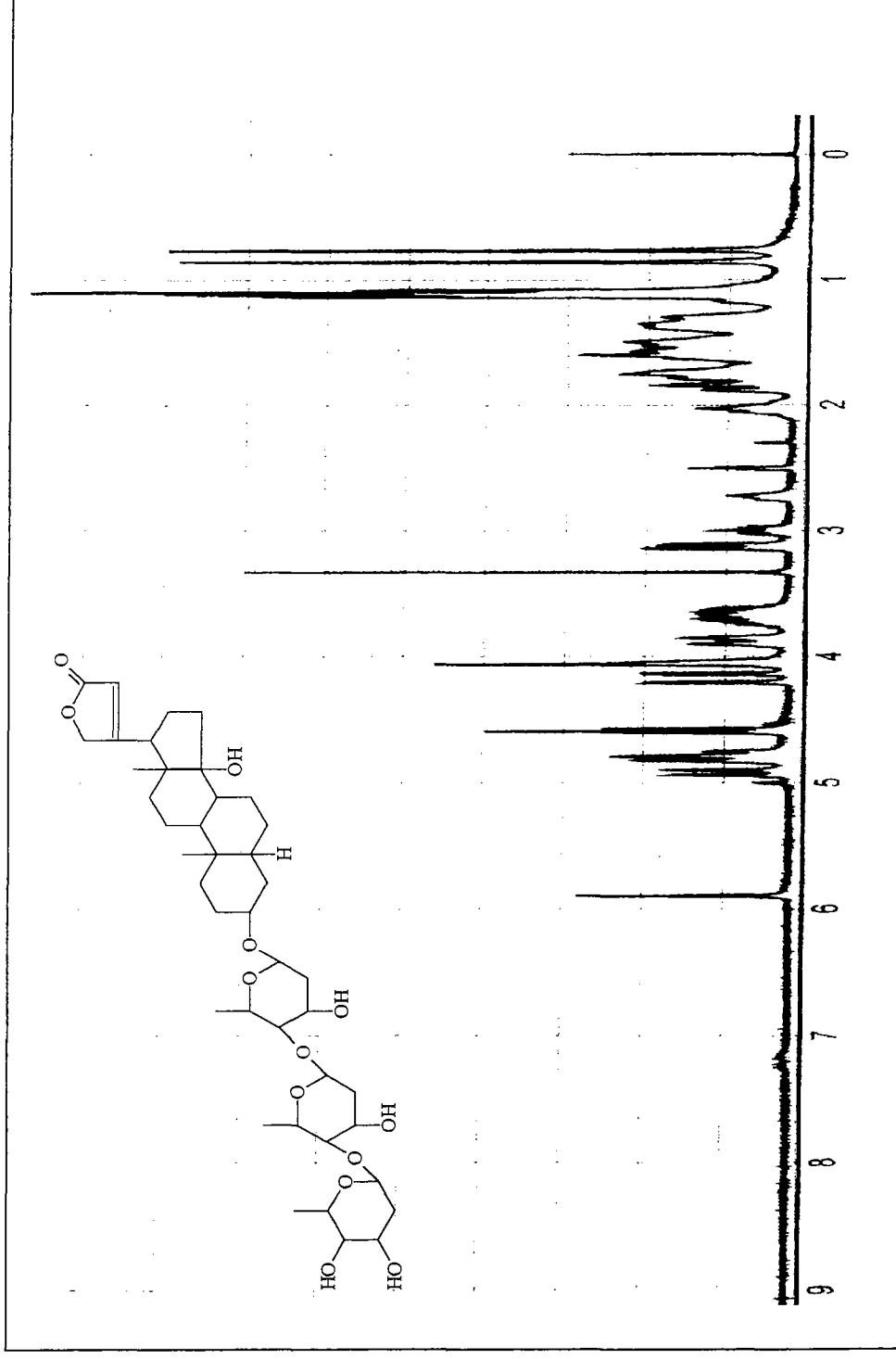


Fig 30b: ^1H -NMR spectrum of Digitoxin

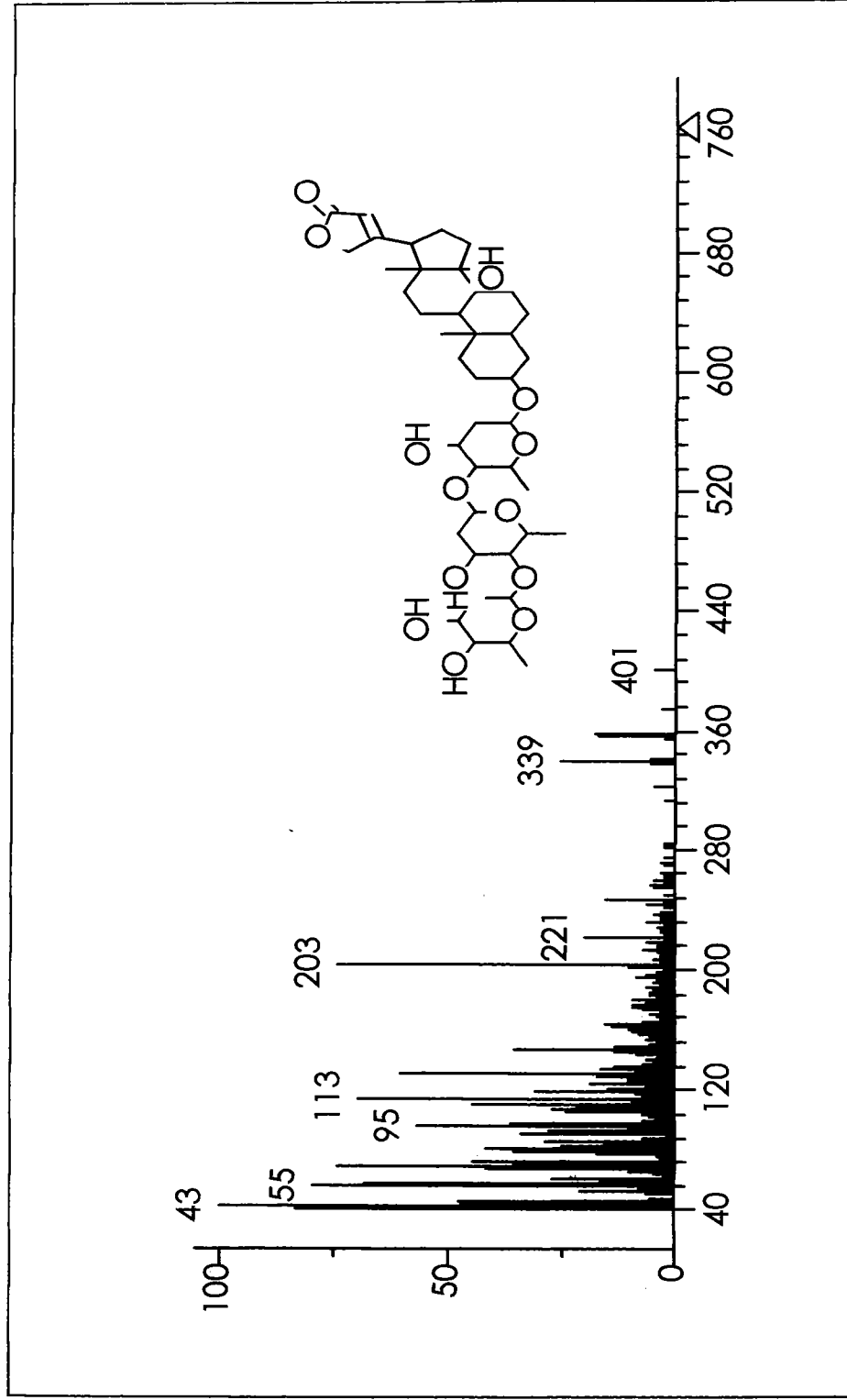


Fig 30c Mass spectrum of Digitoxin

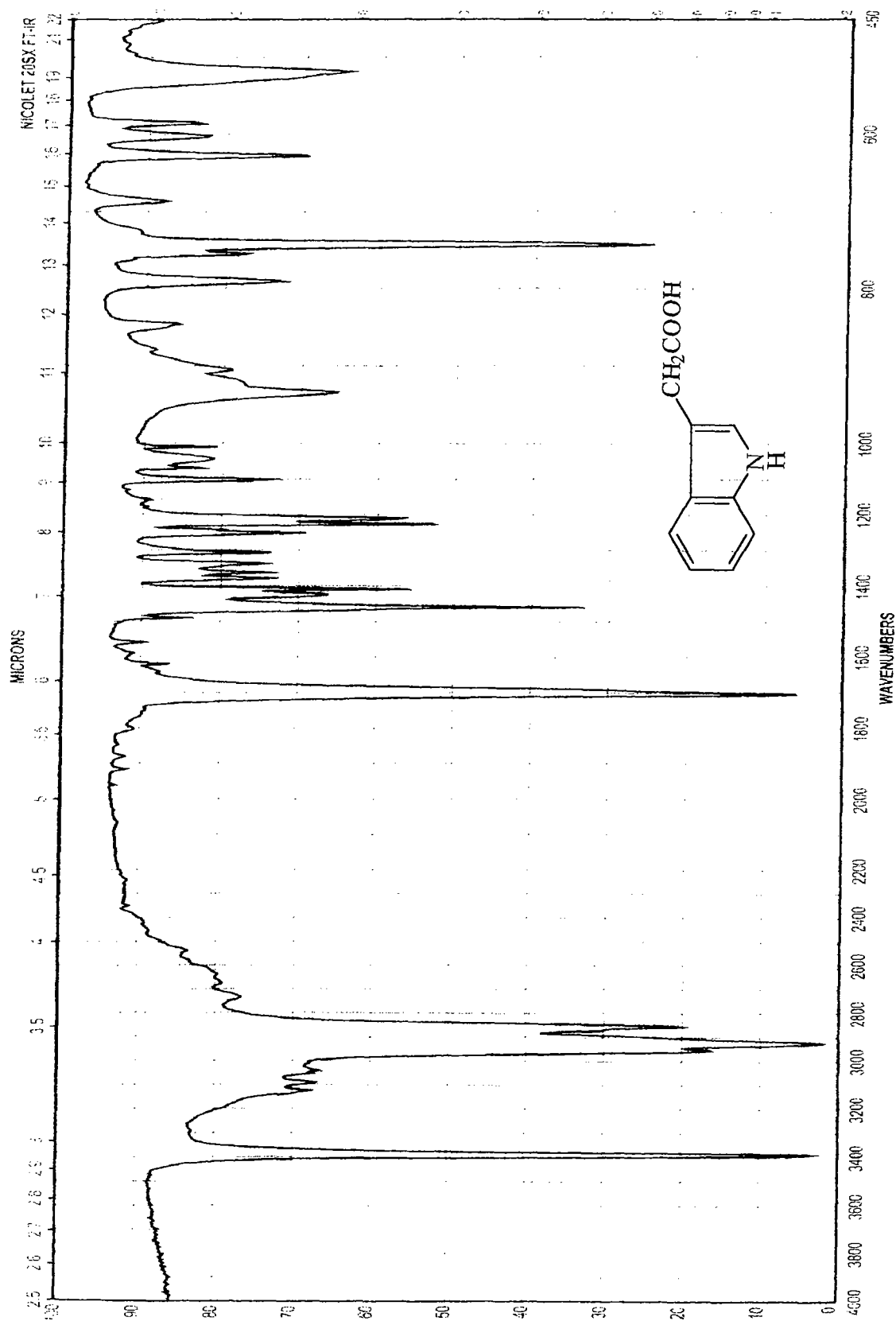


Fig 31a . FT-IR spectrum of 3-indole acetic acid

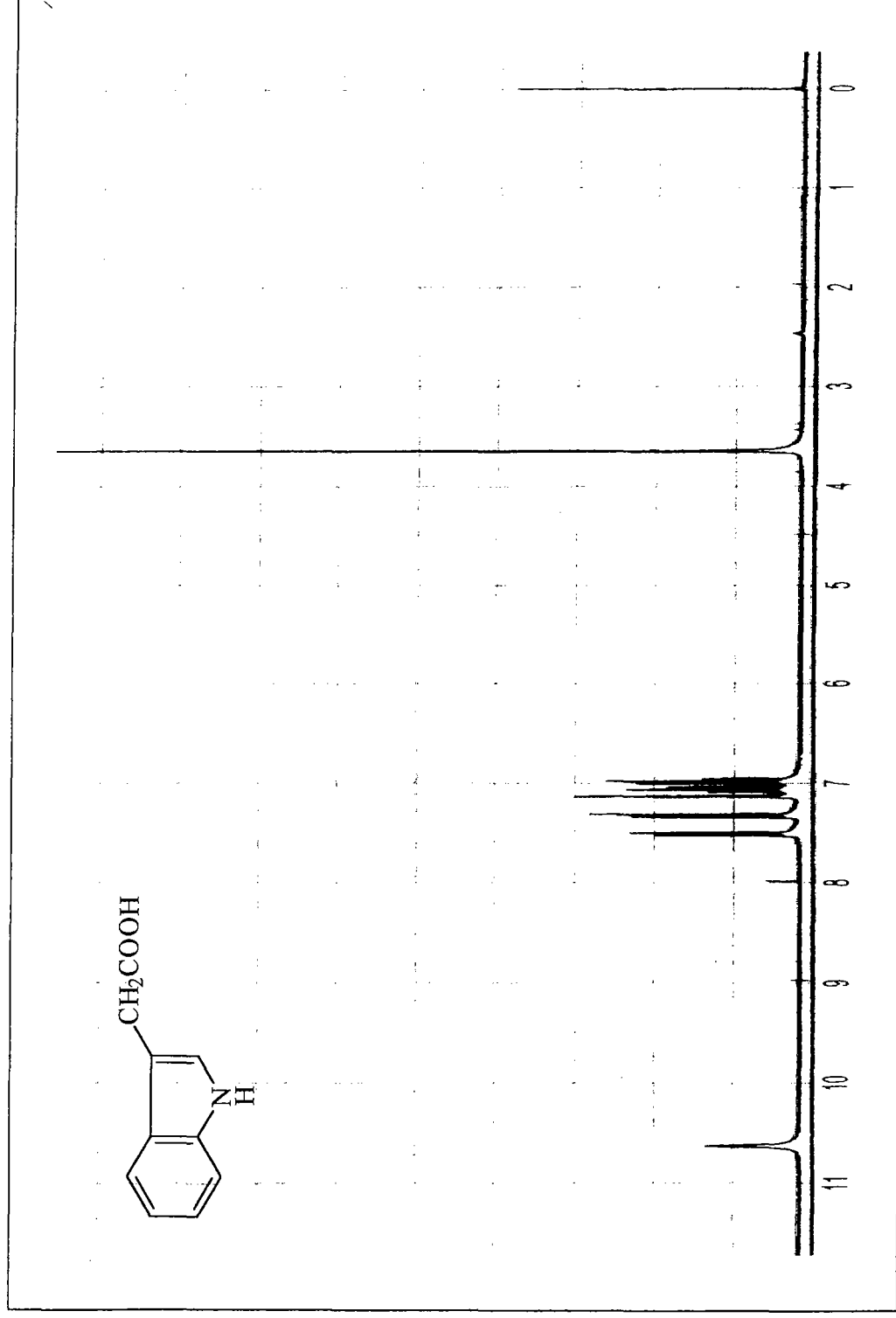


Fig 31b . ¹H-NMR spectrum of 3-indole acetic acid

Compound 1, identified as sporol showed 35.8, 50.9, and 41.3 percent inhibition of *P. aphanidermatum*, *P. debaryanum* and *R. bataticola*, respectively at 62.5 ppm with ED₅₀ of 102, 66 and 225.2 ppm (Table 26).

Secondary metabolite namely sporotrichiol, isolated in appreciable quantity when tested showed 48.1, 55.1, 40.5, 39.0 and 30.4 percent growth inhibition of *P. aphanidermatum*, *P. debaryanum*, *R. bataticola*, *M. phaseolina* and *S. rolfsii*, respectively at 62.5 ppm with ED₅₀ of 66.54, 54.37, 192.70, 251.8 and 313.2 ppm, respectively (Table 27).

Compound No 3, identified as 6-butyl- α -pyrone showed 57.1, 56.2, 39.0, and 39.5 percent inhibition of *P. aphanidermatum*, *P. debaryanum*, *R. bataticola* and *M. phaseolina*, respectively at 62.5 ppm with ED₅₀ of 37.2, 34.9, 293.2, and 248.7, respectively (Table 33). It was found less active against *S. rolfsii* (ED₅₀ 861 ppm) (Table 28)

Compound No 4, identified as fungerin showed 45.3, 34.3 and 40.0 percent inhibition of *P. aphanidermatum*, *P. debaryanum*, and *R. bataticola* respectively at 62.5 ppm with ED₅₀ of 78.2, 137.6 and 246.9 ppm respectively. It was found less active against *S. rolfsii* (ED₅₀ 417 ppm) and *M. phaseolina* (ED₅₀ 379 ppm) (Table 29)

Compound No 5, identified as moniliformin showed 55.5 and 46.5 percent inhibition of *P. aphanidermatum* and *P. debaryanum*, respectively at 62.5 ppm with ED₅₀ of 29.25 and 78.00 ppm respectively. It was found less active against *S. rolfsii* (ED₅₀ 662.3 ppm) and *M. phaseolina* (ED₅₀ 289.7 ppm) (Table 30) (Figure 32)

Table 26. Percent growth inhibition of various fungi caused by Sporol and its ED₅₀ value

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500	74.8±0.36	$Y = 2.77 + 1.10X$	3.19	2.01	102
	250	67.7± 0.32				
	125	60.3± 0.64				
	62.5	35.8± 0.42				
<i>Pythium deborianum</i>	500	70.9± 0.87	$Y = 3.88 + 0.63X$	0.23	1.77	66.0
	250	65.4± 0.47				
	125	59.6± 0.10				
	62.5	50.9± 0.92				
	31.25	41.7± 1.38				
<i>Rhizoctonia bataticola</i>	500	56.4± 0.31	$Y = 4.02 + 0.41$	0.06	2.35	225.2
	250	44.8± 0.65				
	125	45.6± 0.27				
	62.5	41.3± 0.35				
<i>Macrophomina phaseoli</i>	500	68.1± 0.15	$Y = 1.13 + 1.50x$	2.83	2.4	287.6
	250	39.4± 0.21				
	125	31.6± 0.36				
<i>Sclerotium rolfsii</i>	500	46.1± 0.10	$Y = 2.84 + 0.75X$	0.248	2.86	728.5
	250	34.4± 1.40				
	125	29.2± 0.32				

Table 27. Percent growth inhibition of various fungi caused by Sporotrichiol and its ED₅₀ value

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500	72.5±0.55	$Y = 3.72 + 0.69X$	0.25	1.82	66.54
	250	65.3± 0.15				
	125	59.7± 0.45				
	62.5	48.1± 0.21				
	31.25	40.8±0.56				
<i>Pythium debeyanum</i>	500	73.4± 0.44	$Y = 3.80 + 0.68X$	0.97	1.73	54.37
	250	67.4± 0.35				
	125	60.8± 0.66				
	62.5	55.1± 0.15				
	31.25	40.4± 0.44				
<i>Rhizoctonia bataticola</i>	500	58.6± 0.36	$Y = 3.80 + 0.52X$	0.08	2.28	192.7
	250	52.9± 0.20				
	125	44.9± 0.38				
	62.5	40.5± 0.49				
	500	56.5± 0.41				
<i>Macrophomina phaseoli</i>	250	49.7± 0.21	$Y = 3.79 + 0.50x$	0.113	2.4	251.8
	125	42.7± 0.72				
	62.5	39.0±0.30				
	500	56.1±0.20				
	250	46.5±0.35				
<i>Sclerotium rolfsii</i>	125	39.2±0.25	$Y = 3.19 + 0.72X$	0.036	2.49	313.2
	62.5	30.4±0.59				

Table 28. Percent growth inhibition of various fungi caused by 6-Butyl- α - pyrone and its ED₅₀ value

Fungi	Dose (ppm)	Growth (cm)	Average % Growth Inhibition $\pm$ S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500	2.9	82.7 $\pm$ 1.02	$Y = 3.77+0.78X$	1.44	1.57	37.2
	250	3.3	75.0 $\pm$ 0.10				
	125	4.1	61.1 $\pm$ 1.05				
	62.5	4.7	57.1 $\pm$ 0.40				
	31.25		49.8 $\pm$ 0.20				
<i>Pythium deborianum</i>	500	3.8	83.1 $\pm$ 0.15	$Y = 3.80+0.77X$	1.20	1.54	34.9
	250	4.6	75.1 $\pm$ 0.15				
	62.5	6.1	56.2 $\pm$ 0.62				
	31.25	6.6	51.4 $\pm$ 0.38				
	500	3.6	56.3 $\pm$ 0.32				
<i>Rhizoctonia bataticola</i>	250	4.3	47.2 $\pm$ 0.10	$Y = 3.77+0.49X$	0.59	2.46	293.2
	125	4.9	40.4 $\pm$ 0.58				
	62.5	5.6	39.0 $\pm$ 0.56				
	500	4.9	55.9 $\pm$ 0.15				
	250	5.4	49.8 $\pm$ 0.17				
<i>Macrophomina phaseoli</i>	125	6.1	44.0 $\pm$ 0.30	$Y = 3.89+0.46x$	0.024	2.39	248.7
	62.5		39.5 $\pm$ 0.35				
	500	1.2	44.2 $\pm$ 0.15				
	250	2.3	31.2 $\pm$ 0.15				
	125	2.7	27.3 $\pm$ 0.58				
<i>Sclerotium rolfsii</i>	500			$Y = 2.74+0.76X$	0.52	2.93	861.0
	250						

**Table 29. Percent growth inhibition of various fungi caused by
Fungerin and its ED₅₀ value**

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500	76.6±0.55	$Y = 3.15 \pm 0.97X$	0.83	1.89	78.2
	250	69.2± 0.10				
	125	61.5± 1.30				
	62.5	45.3± 0.15				
	31.25	33.7±3.360				
<i>Pythium deborianum</i>	500	70.3± 0.79	$Y = 2.78 + 1.03X$	0.53	2.13	137.6
	250	62.1± 0.15				
	125	50.3± 0.58				
	62.5	34.3± 0.60				
<i>Rhizoctonia bataticola</i>	500	60.0± 0.27	$Y = 3.59 + 0.58X$	1.29	2.39	246.9
	250	52.4± 0.21				
	125	45.5± 0.26				
	62.5	40.0± 0.30				
<i>Macrophomina phaseoli</i>	500	53.3± 0.27	$Y = 3.44 + 0.60x$	0.111	2.57	379.0
	250	44.5± 0.21				
	125	39.6± 0.10				
	62.5	31.5±0.46				
<i>Sclerotium rolfii</i>	500	53.4±0.44	$Y = 2.74 + 0.86X$	0.12	2.62	417.0
	250	41.0±0.70				
	125	33.3±0.32				

**Table 30. Percent growth inhibition of various fungi caused
by Moniliformin and its ED₅₀ value**

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500	70.5±0.62	$Y = 4.35 \pm 0.44X$	0.18	1.46	29.25
	250	67.3± 0.26				
	125	59.6± 0.21				
	62.5	55.5± 0.51				
	31.25	51.1±0.35				
<i>Pythium debaryanum</i>	500	70.0± 0.25	$Y = 3.74 + 0.66X$	1.00	1.89	78.0
	250	61.1± 1.04				
	125	59.7± 0.58				
	62.5	46.5± 0.32				
	31.25	38.7±0.15				
<i>Rhizoctonia bataticola</i>	500	68.3± 0.39	$Y = 1.38 + 1.48X$	2.88	2.43	275.2
	250	40.7± 0.63				
	125	33.9± 0.30				
	500	67.7± 0.15				
<i>Macrophomina phaseoli</i>	250	39.0± 0.56	$Y = 1.93 + 1.24x$	5.44	2.46	289.7
	125	29.7± 0.27				
	62.5	24.5±0.31				
	500	47.4±0.47				
<i>SSclerotium rolfsii</i>	250	35.9±0.55	$Y = 2.93 + 0.73X$	0.23	2.82	662.30
	125	30.7±0.05				

4.7.1.1.2 Methanol extract

Five secondary metabolites separated by column chromatography were tested for fungicidal activity against *R. bataticola*, *P. aphanidermatum* and *P. debaryanum*.

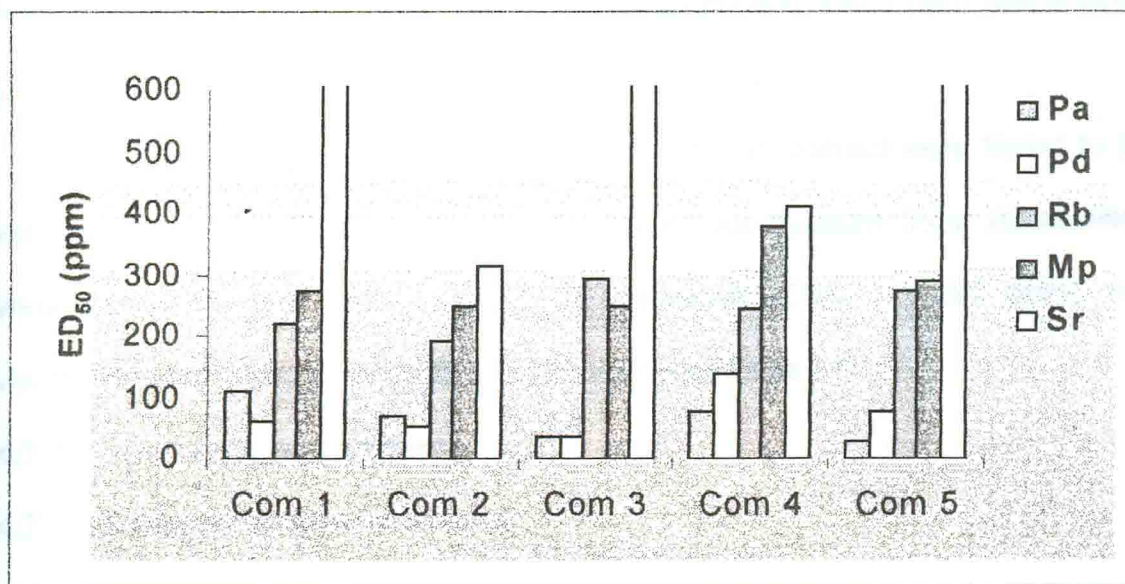
Compound No 1, identified as visolstricin showed 34.4, 46.1 and 45.8 percent inhibition of *P. aphanidermatum*, *P. debaryanum*, and *R. bataticola*, respectively at 62.5 ppm with ED₅₀ of 108.7, 72.23 and 70.49 ppm respectively (Table 31, Figure 33).

Compound No 2, identified as 8-hydroxyisotrichodermol showed 47.5, 46.3 and 34.5 percent inhibition of *P. aphanidermatum*, *P. debaryanum*, and *R. bataticola*, respectively at 62.5 ppm with ED₅₀ of 78.84, 70.39, and 111.39 (Table 32)

Compound No. 3, identified as T-2 toxin showed 47.5, 46.3 and 34.5 percent inhibition of *P. aphanidermatum*, *P. debaryanum*, and *R. bataticola*, respectively at 62.5 ppm with ED₅₀ of 78.84, 70.39, and 111.39 ppm, respectively (Table 33).

Compound No. 4, identified zerealenone showed 49.6, 35.7 and 34.9 percent inhibition of *P. aphanidermatum*, *P. debaryanum*, and *R. bataticola*, respectively at 62.5 ppm with ED₅₀ of 68.07, 103.9 and 227.67 ppm respectively (Table 34).

Compound No 5, identified as diacetoxyscirpenol showed 45.6, 44.8 and 35.4 percent inhibition of *P. aphanidermatum*, *P. debaryanum*, and *R. bataticola*,



Com1- Sporol

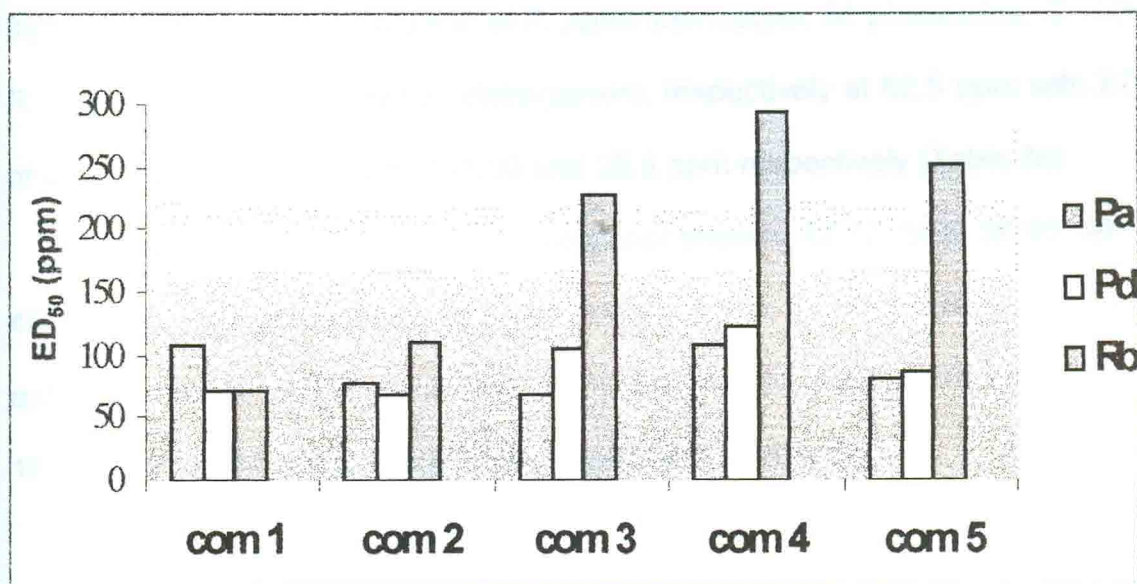
Com 2- Sporotrichiol

Com 3-Butyl pyrone

Com 4- Fungerin

Com 5- Moniliformin

Fig 32. Fungicidal activity of various isolated compounds from DCM mycelia extract of *Fusarium oxysporum*



Com1- Visolstricin

Com 2- isotrichoderminol

Com 3-T-2 toxin

Com 4- Zerealenone

Com 5- Diacetoxyscirpenol

Fig 34. Fungicidal activity of various isolated compounds from Methanol mycelial extract of *Fusarium oxysporum*

respectively at 62.5 ppm with ED₅₀ of 76.80, 81.90 and 250.1 ppm respectively (Table 35).

All the five compounds isolated from methanol extract were found to be very active against *P. aphanidermatum* and *P. debaryanum*. Four metabolites were somewhat less active against *R. bataticola* (ED₅₀ 111-290 ppm), but visolstricin was found active with ED₅₀ 70.49 ppm (Table 31)

4.7.1.2 Culture filtrate extract

4.7.1.2.1 Dichloromethane extract

Four secondary metabolites separated by column chromatography were tested for fungicidal activity against *R. bataticola*, *M. phaseolina*, *S. rolfsii*, *P. aphanidermatum* and *P. debaryanum*.

Compound No 1, identified as JM-47, showed 55.64, 10.6, 30.5, 35.56, 39.91 and 60.3 percent inhibition of *P. aphanidermatum*, *M. phaseolina*, *S. rolfsii*, *R. solani*, *R. bataticola*, and *P. debaryanum*, respectively at 62.5 ppm with ED₅₀ of 44.1, 179.9, 208.3, 102.9, 101.00 and 26.8 ppm respectively (Table 36).

Compound No 2, identified as nivalenol showed 42.17, 38.8, 24.95, 40.17, 40.13 and 21.63 percent inhibition of *P. debaryanum*, *M. phaseolina*, *S. rolfsii*, *P. aphanidermatum* and *R. bataticola*, respectively at 62.5 ppm with 122.3, 140.7, 181.3, 152.6, 132.4 and 155.20 ppm respectively (Table 37).

Compound No. 3 identified as sambucoidin showed 34.63, 18.20, 30.00, 31.6, 9.12 and 36.9 percent inhibition of *P. debaryanum*, *M. phaseolina*, *S. rolfsii*, *P. aphanidermatum* and *R. bataticola* respectively at 62.5 ppm with ED₅₀ of 112.4, 211.5, 352.4, 287.1, 161.8 and 159.3 ppm, respectively (Table 38).

**Table 31. Percent growth inhibition of various fungi caused
by Visolstricin and its ED₅₀ value**

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500	74.1±0.25	$Y = 2.70 \pm 1.12X$	3.41	2.03	108.7
	250	67.5±0.35				
	125	59.3±0.51				
	62.5	34.4±0.29				
<i>Pythium deberianum</i>	500	70.2±0.74	$Y = 3.70 \pm 0.69X$	1.15	1.85	72.23
	250	65.2±0.15				
	125	60.9±0.23				
	62.5	46.1±0.20				
	31.25	39.3±0.67				
<i>Rhizoctonia bataticola</i>	500	70.3±0.38	$Y = 3.68 \pm 0.71X$	1.46	1.85	70.49
	250	66.5±0.15				
	125	61.4±0.42				
	62.5	45.8±0.17				
	31.25	39.6±0.06				

**Table 32. Percent growth inhibition of various fungi caused
by 8-Hydroxy isotrichodermol and its ED₅₀ value**

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500	69.1±0.35	Y = 3.69±0.68X	0.811	1.89	78.84
	250	63.4± 0.29				
	125	59.0± 0.27				
	62.5	47.5± 0.10				
	31.25	37.2±0.36				
<i>Pythium debierianum</i>	500	71.6± 0.30	Y = 3.66±0.72X	0.98	1.84	70.39
	250	65.4± 0.40				
	125	61.2± 0.27				
	62.5	46.3± 0.38				
	31.25	39.6±0.10				
<i>Rhizoctonia bataticola</i>	500	72.9± 0.115	Y = 2.82±1.06X	3.52	2.04	111.35
	250	65.3± 0.32				
	125	59.3± 0.27				
	62.5	34.5±0.40				

*Avg. Mortality is the average of three replicates.

**Table 33. Percent growth inhibition of various fungi caused
by T-2 Toxin and its ED₅₀ value**

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500	71.2±0.45	Y = 3.73±0.69X	0.56	1.83	68.07
	250	64.7± 0.20				
	125	60.1± 0.20				
	62.5	49.6± 0.30				
	31.25	39.0±0.10				
<i>Pythium debeyanum</i>	500	75.2±0.36	Y = 2.72+1.12X	2.80	2.01	103.9
	250	68.1± 0.20				
	125	59.6± 0.35				
	62.5	35.7± 0.15				
	500	59.5± 0.36				
<i>Rhizoctonia bataticola</i>	250	49.6± 0.1	Y = 3.42+0.67X	0.19	2.36	227.67
	125	44.6± 0.10				
	62.5	34.9±0.25				

*Avg. Mortality is the average of three replicates.

Table 34. Percent growth inhibition of various fungi caused by Zerealenone and its ED₅₀ value

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500	74.3±0.38	Y = 2.66±1.14X	2.83	2.03	109.48
	250	68.3± 0.38				
	125	58.1± 0.57				
	62.5	34.6± 0.44				
<i>Pythium debierianum</i>	500	71.6±0.46	Y = 2.64±1.12X	3.04	2.09	124.2
	250	66.5± 0.35				
	125	55.3± 0.40				
	62.5	32.4± 0.44				
<i>Rhizoctonia bataticola</i>	500	56.5± 0.35	Y = 3.46±0.62X	0.13	2.46	294.7
	250	46.7± 0.15				
	125	41.4± 0.38				
	62.5	33.9±0.11				

*Avg. Mortality is the average of three replicates.

Table 35. Percent growth inhibition of various fungi caused by Diacetoxyscirpenol and its ED₅₀ value

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500	70.6±0.78	Y = 3.64±0.72X	0.85	1.88	76.80
	250	64.6± 0.25				
	125	59.9± 0.11				
	62.5	45.6± 0.05				
	31.25	38.3±0.53				
<i>Pythium debierianum</i>	500	69.8±0.21	Y = 3.63±0.72X	0.97	1.91	81.90
	250	63.6± 0.44				
	125	59.4± 0.32				
	62.5	44.8± 0.27				
	31.25	37.5±0.36				
<i>Rhizoctonia bataticola</i>	500	58.4± 0.42	Y = 3.49±0.62X	0.19	2.39	250.1
	250	48.2± 0.42				
	125	43.3± 0.15				
	62.5	35.4±0.44				

*Avg. Mortality is the average of three replicates.

Compound No 4, identified as 8-hydroxyisotrichoderminol showed 41.0, 29.60, 33.30, 39.2, 36.2 and 39.2 percent inhibition of *R. bataticola* , , *M. phaseolina*, *S. rolfsii*, *P. aphanidermatum* and *P. debaryanum*, respectively at 62.5 ppm with ED₅₀ of 59.9, 147.6, 214.20, 168.8, 90.16 and 71.04 ppm respectively (Table 39).

All the four compounds isolated from dichloromethane extract of culture filtrate were found to be very active against *P. aphanidermatum* and *P. debaryanum*.

4.7.2 Plant growth regulatory activity

Plant growth regulatory activity of four isolated compounds namely fusaic acid, indole acetic acid, isotrichodermin and nivalenol was evaluated against wheat seed germination by recording germination percent, shoot length, root length, per cent phytotoxicity and vigour index at different concentration. (Figure 34)

Fusaric acid showed 93 and 89 percent germination at 100 and 250 ppm concentrations, respectively as compared to control (88 percent). There was not much increase in the shoot length (14.7 – 13.0 cm) at all the tested concentrations as compared (13.1 cm), whereas slight increase in root length (6.3 – 5.2 cm) was recorded at all concentrations as compared to control (4.6 cm) At lower concentrations (500-100 ppm) no phytotoxicity was observed, whereas at 1500 ppm slight phytotoxicity was observed (Table 40). Vigour index varied from 1954 to 1166 for 100 – 1500 ppm concentration as compared to control (1557).

Table 36. Percent growth inhibition of various fungi caused by JM-47 Toxin and its ED₅₀ value

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500	79.45±0.06	$Y = 3.71 + 0.78X$	0.234	1.64	44.10
	250	73.32±2.21				
	125	61.98±0.18				
	62.5	54.64±0.37				
	31.25					
<i>Pythium debaryanum</i>	500	82.59±0.19	$Y = 3.94 + 0.74X$	0.02	1.43	26.80
	250	76.32±0.27				
	125	69.50±0.01				
	62.5	60.30±1.23				
	31.25					
<i>Rhizoctonia bataticola</i>	500	73.90±0.27	$Y = 3.02 + 0.98X$	0.52	2.005	101.00
	250	65.90±0.21				
	125	56.05±0.39				
	62.5	39.91±0.22				
	31.25					
<i>Macrophomina phaseoli</i>	500	81.76±0.22	$Y = -0.05 + 2.24X$	4.39	2.54	179.90
	250	62.60±0.29				
	125	43.60±1.72				
	62.5	10.60±2.64				
	31.25					
<i>Sclerotium rolfsii</i>	500	67.30±0.11	$Y = 2.49 + 1.08X$	0.54	2.31	208.30
	250	52.4±0.03				
	125	38.10±1.23				
	62.5	30.50±0.79				
	500	74.40±1.10				
<i>Rhizoctonia solani</i>	250	67.78±1.02	$Y = 2.78 + 1.09X$	3.57	2.01	102.9
	125	60.00±0.01				
	62.5	35.56±0.31				
	31.25					

**Table 37. Percent growth inhibition of various fungi caused
by Nivalenol and its ED₅₀ value**

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500	69.50±1.21	$Y = 3.29 + 0.80X$	0.58	2.12	132.40
	250	55.65±1.19				
	125	50.12±1.03				
	62.5	40.13±0.04				
	31.25					
<i>Pythium deborianum</i>	500	79.43±1.01	$Y = 1.277 + 1.69X$	2.27	2.19	155.20
	250	62.66±0.77				
	125	49.72±0.63				
	62.5	21.63±0.39				
	31.25					
<i>Rhizoctonia bataticola</i>	500	73.00±1.27	$Y = 3.19 + 0.86X$	1.28	2.08	122.30
	250	56.67±2.11				
	125	49.11±0.03				
	62.5	42.17±1.29				
	31.25					
<i>Macrophomina phaseoli</i>	500	67.70±1.12	$Y = -3.12 + 0.87X$	0.61	2.14	140.70
	250	61.10±1.19				
	125	45.50±0.17				
	62.5	38.80±0.33				
	31.25					
<i>Sclerotium rolfsii</i>	500	73.13±1.70	$Y = 1.94 + 1.35X$	0.59	2.25	181.30
	250	59.38±1.01				
	125	46.48±0.93				
	62.5	24.95±0.79				
	500	67.50±1.01				
<i>Rhizoctonia solani</i>	250	52.47±0.98	$Y = 3.38 + 0.74X$	1.005	2.18	152.6
	125	47.11±0.83				
	62.5	40.17±0.04				
	31.25					

**Table 38. Percent growth inhibition of various fungi caused
by Sambucion and its ED₅₀ value**

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500	96.40±0.07	$Y = -2.38+3.34X$	1.05	2.20	161.60
	250	70.57±0.19				
	125	35.69±0.23				
	62.5	9.12±0.69				
	31.25					
<i>Pythium deberianum</i>	500	73.18±0.36	$Y = 2.78+1.08X$	2.87	2.05	112.40
	250	65.92±0.29				
	125	58.21±1.02				
	62.5	34.63±0.91				
	31.25					
<i>Rhizoctonia bataticola</i>	500	66.90±0.12	$Y = 2.96+0.92X$	1.12	2.20	159.30
	250	60.20±0.02				
	125	42.30±0.19				
	62.5	36.9±0.93				
	31.25					
<i>Macrophomina phaseoli</i>	500	81.68±0.93	$Y = 0.62+1.88X$	6.10	2.32	211.50
	250	45.70±0.67				
	125	34.39±1.12				
	62.5	18.20±1.14				
	31.25					
<i>Sclerotium rolfsii</i>	500	54.20±0.67	$Y = 3.29+0.66X$	0.22	2.54	352.40
	250	44.70±0.33				
	125	39.60±0.21				
	62.5	30.00±0.19				
	500	57.80±1.03				
<i>Rhizoctonia solani</i>	250	46.60±1.19	$Y = 3.21+0.73X$	0.14	2.45	287.1
	125	40.00±0.97				
	62.5	31.6±0.02				
	31.25					

Table 39. Percent growth inhibition of various fungi caused by 8-Hydroxy isotrichoderminol and its ED₅₀ value

Fungi	Dose (ppm)	Average % Growth Inhibition± S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
<i>Pythium aphanidermatum</i>	500		$Y = 3.57 + 0.73X$	0.106	1.95	90.10
	250	62.60±0.76				
	125	53.40±0.99				
	62.5	46.70±0.22				
	31.25	36.20±0.10				
<i>Pythium debaryanum</i>	500		$Y = 3.81 + 0.64X$	0.414	1.85	71.04
	250	67.20±0.17				
	125	56.60±0.69				
	62.5	51.0±0.88				
	31.25	39.20±0.41				
<i>Rhizoctonia bataticola</i>	500		$Y = 3.67 + 0.74X$	0.36	1.77	59.90
	250	68.60±1.32				
	125	57.20±0.07				
	62.5	52.30±0.15				
	31.25	41.0±0.22				
<i>Macrophomina phaseoli</i>	500		$Y = 1.91 + 1.42X$	0.007	2.16	147.60
	250	62.50±0.29				
	125	46.30±0.10				
	62.5	29.60±0.39				
	31.25					
<i>Sclerotium rolfsii</i>	500	61.60±1.03	$Y = 3.07 + 0.82X$	0.10	2.33	214.20
	250	53.27±0.96				
	125	41.26±0.38				
	62.5	33.30±0.19				
	500					
<i>Rhizoctonia solani</i>	250	54.20±1.20	$Y = 3.59 + 0.63X$	0.019	2.22	168.8
	125	46.7±1.13				
	62.5	39.20±0.97				
	31.25					

Increase in percent seed germination (94 – 88 per cent) was recorded with second metabolite namely indole acetic acid as compared to control (88 percent) up to 1000 ppm concentration. Increase in shoot and root length was observed from 100 – 500 ppm (15.3 to 13.6 cm) to control (13.1 cm). Vigour index varied from 2122 to 1753 from 100-500 ppm as compared to control (1557).

A slight decrease in percent germination (87 -56 percent) and shoot length (12.1-11.1 cm) was recorded with compound 3 and 4 namely isotrichodermin and nivalenol as compared to control (88 percent, 13.1 cm, respectively). A significant increase in root length was observed at all the tested concentrations. Increase in phytotoxicity was recorded with isotrichodermin at higher concentrations. A slight decrease in vigour index was observed as compared to control at all concentrations. Thus it has been observed fusaric acid and indole acetic acid showed plant growth regulating activity at lower concentrations, whereas isotrichodermin and nivalenol did not show growth promoting activity.

4.7.3 Nematicidal activity

Six isolated secondary metabolites were tested *in vitro* for nematicidal activity, Fusaric acid against *R. reniformis* and *M. incognita*. (Table 41a, 41b) The calculated ED₅₀ value for compound 1 (fusaric acid) was 22.16 and 17.90 ppm after 24 h against *R. reniformis* and *M. incognita*, respectively. Whereas in the case of visolstricin ED₅₀ was more than 400 ppm against both the nematodes (Table 42a, 42b). Similarly compound No. 3 (8-hydroxyisotrichodermin) showed ED₅₀ after 24 hours 179 and 190ppm, but after 48 hours 482.1 and 540 ppm against *R. reniformis* and *M. incognita*, respectively (Table 43a, 43b). Similarly compound moniliformin also showed ED₅₀ 214.8 and 171 ppm after

Table 40. Plant growth regulatory activity of various compounds isolated from *Fusarium oxysporum* on wheat seeds

COMPOUND	Concentration (ppm)	Germination % (Mean $\pm$ S.D.)	Shoot length (cm) (Mean $\pm$ S.D.)	Root length (cm) (Mean $\pm$ S.D.)	Phytotoxicity (%)	Vigour index
Fusaric acid	Control	88 $\pm$ 1.30	13.1 $\pm$ 0.29	4.6 $\pm$ 0.43	00.00	1557.6
	100	93 $\pm$ 0.29	14.7 $\pm$ 0.31	6.3 $\pm$ 0.61	-17.39	1954.0
	250	89 $\pm$ 0.74	14.1 $\pm$ 1.10	5.6 $\pm$ 0.21	-28.26	1756.0
	500	79 $\pm$ 0.89	13.2 $\pm$ 0.96	6.2 $\pm$ 1.40	-32.60	1542.0
	1000	74 $\pm$ 1.13	13.0 $\pm$ 0.78	6.9 $\pm$ 0.02	6.521	1492.0
	1500	69 $\pm$ 1.20	11.6 $\pm$ 0.22	5.2 $\pm$ 0.09	10.86	1166.1
IAA	Control	88 $\pm$ 0.96	13.1 $\pm$ 0.24	4.6 $\pm$ 0.13	00.00	1557.6
	100	94 $\pm$ 1.12	15.3 $\pm$ 0.78	7.2 $\pm$ 0.41	-58.73	2122.0
	250	93 $\pm$ 0.65	15.1 $\pm$ 0.56	6.1 $\pm$ 0.36	-71.73	1971.0
	500	91 $\pm$ 0.06	13.6 $\pm$ 0.38	5.7 $\pm$ 0.17	-76.08	1753.0
	1000	88 $\pm$ 0.56	10.9 $\pm$ 1.04	5.9 $\pm$ 0.19	10.86	1477.3
	1500	86 $\pm$ 0.98	8.90 $\pm$ 0.93	4.3 $\pm$ 0.72	21.73	876.00
Isotrichodermin	Control	88 $\pm$ 1.15	13.1 $\pm$ 0.42	4.6 $\pm$ 0.84	00.00	1557.6
	100	87 $\pm$ 0.12	12.1 $\pm$ 0.36	5.3 $\pm$ 1.21	10.86	1512.0
	250	81 $\pm$ 0.58	11.9 $\pm$ 0.11	6.2 $\pm$ 0.17	28.26	1456.0
	500	76 $\pm$ 0.22	11.4 $\pm$ 0.17	6.0 $\pm$ 0.19	36.95	1323.0
	1000	63 $\pm$ 0.19	11.1 $\pm$ 1.20	9.4 $\pm$ 0.01	43.47	1289.0
	1500	56 $\pm$ 1.03	11.1 $\pm$ 1.19	10.3 $\pm$ 0.02	54.34	1199.0
Nivalenol	Control	88 $\pm$	13.1 $\pm$ 0.29	4.6 $\pm$ 0.43	00.00	1557.6
	100	86 $\pm$	13.3 $\pm$	5.2 $\pm$	-17.39	1591.6
	250	82 $\pm$	10.9 $\pm$	4.1 $\pm$	30.43	1256.0
	500	71 $\pm$	10.7 $\pm$	6.9 $\pm$	36.95	1256.0
	1000	66 $\pm$	9.70 $\pm$	5.8 $\pm$	50.00	1025.0
	1500	51 $\pm$	10.1 $\pm$	9.2 $\pm$	54.34	987.00

*Germination %, Shoot length and Root length are the average of three replicates.

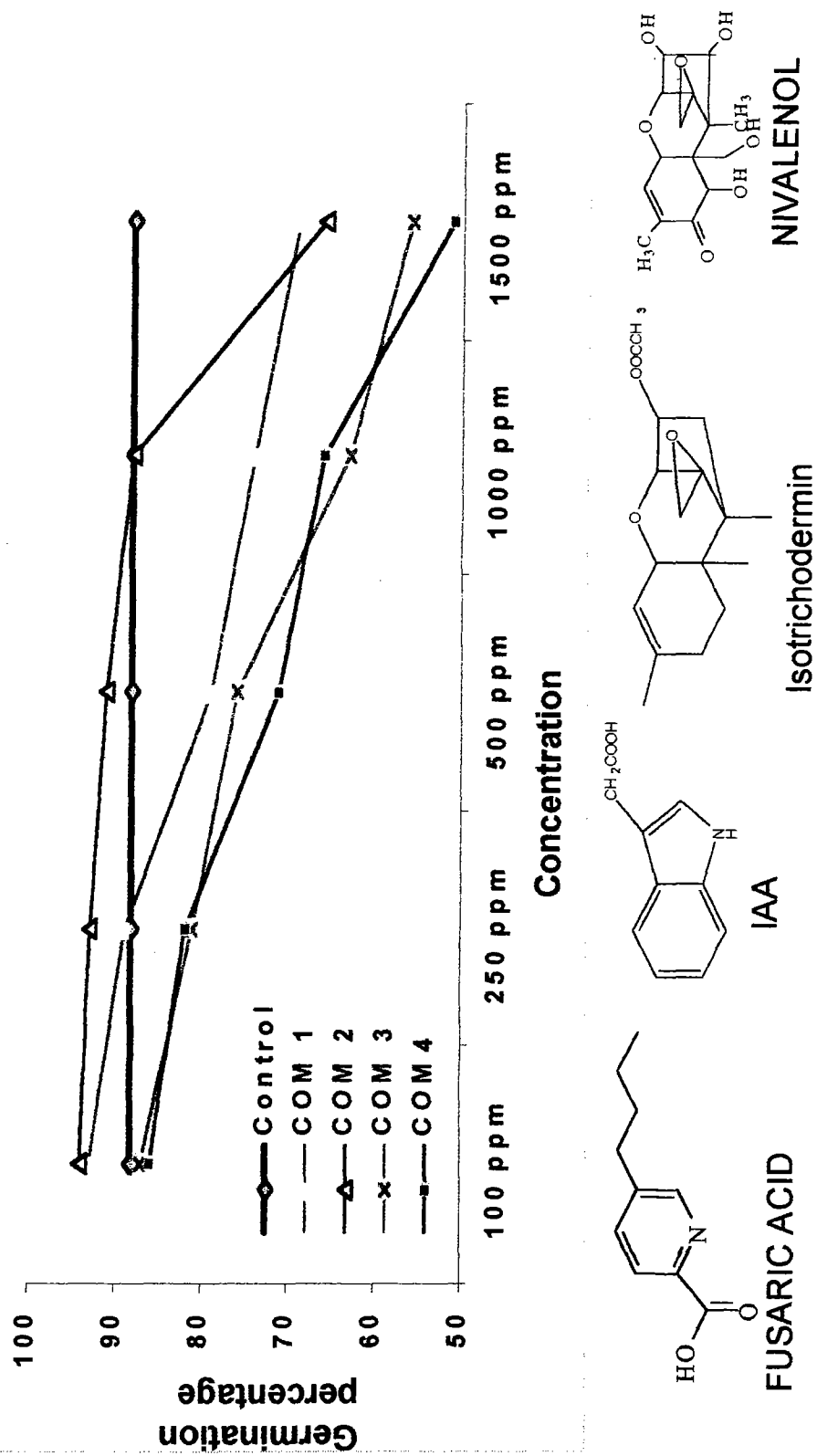


Fig 34. Effect of various isolated compounds from *Fusarium oxysporum* on Wheat germination

24 h against *R. reniformis* and *M. incognita*, respectively. (Table 44a, 44b) Compound no. 4, sambucosin was found active with ED₅₀ 24.8 and 21.23ppm after 24 h against *R. reniformis* and *M. incognita*, respectively. (45a, 45b) Among all the tested compound No.5 (2,2- dimethyl-5-amino-6- (2' ene-4'- hydroxybutyryl)-4-chromone) was found most active with ED₅₀ 15.25 and 15.29 ppm after 24 h against *R. reniformis* and *M. incognita*. (46a, 46b)

4.8 Compatibility of *F.oxysporum* and bioactive secondary metabolites with selected fungicides

Compatibility of *F. oxysporum* and isolated toxins from culture filtrate and dried mycelia with commercially available fungicides used for soil borne pathogen was studied to understand the feasibility of application of *F. oxysporum* and toxins along with the commercial fungicides to implement in the integrated pest management strategy.

4.8.1 Compatibility with *F. oxysporum*

The *F. oxysporum* spores in no fungicide treatment all germinated after 5 days of incubation in PDA media. Among the seven fungicides tested, namely captan, tridemorph, metalaxyl, thiophanate-methyl, mancozeb, sulphur dust and borax, *F. oxysporum* was least sensitive to captan, tridemorph, metalaxyl and thiophanate-methyl even at the triple of the recommended dose of these respective fungicides against all the soil borne fungal pathogens. *F. oxysporum*

Table 41 a. Nematotoxic effect of Fusaric acid against J2 of *Meloidogyne incognita*

Time	Dose (ppm)	%mortality +/- S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
24 hours	500	100	Y= 1.51 +2.78x	1.29	1.25	17.90
	250	100				
	125	99.733 ± 0.18				
	62.5	91.4 ± 0.59				
	31.25	76.3 ± 0.40				
48 hours	500	99.75 ± 0.16	Y= -1.21 +3.61	6.28	1.72	52.7
	250	99.47 ± 0.26				
	125	94.4 ± 0.54				
	62.5	53.6 ± 0.59				
	31.25	23.4 ± 0.76				

*Avg. Mortality is the average of three replicates.

Table 41 b. Nematotoxic effect of Fusaric acid against J2 of *Rotylenchulus reniformis*

Time	Dose (ppm)	%mortality +/- S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
24 hours	500	100	Y= 0.06 + 3.66x	0.083	1.34	22.16
	250	100				
	125	99.58 ± 0.21				
	62.5	95.39 ± 0.45				
	31.25	70.59 ± 0.57				
48 hours	500	99.5 ± 0.43	Y= -1.23 + 3.57x	9.69	1.74	55.62
	250	99.2 ± 0.91				
	125	73.3 ± 0.69				
	62.5	51.4 ± 0.86				
	31.25	20.36 ± 1.09				

*Avg. Mortality is the average of three replicates.

Table 42 a. Nematotoxic effect of Visolstricin against J2 of *Meloidogyne incognita*

Time	Dose (ppm)	%mortality +/- S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
24 hours	500	45.3 ± 0.35	Y= 1.70 + 1.21x	2.24	2.72	527.4
	250	37.3 ± 0.48				
	125	24.6 ± 0.65				
	62.5	14.7 ± 0.5				
	31.25	4.9 ± 0.28				
48 hours	500	76.1 ± 0.92	Y= -0.16 + 2.15x	0.640	2.38	245.13
	250	49.59 ± 0.85				
	125	24.3 ± 1.13				
	62.5	11.53 ± 1.19				

*Avg. Mortality is the average of three replicates.

Table 42 b. Nematotoxic effect of Visolstricin against J2 of *Rotylenchulus reniformis*

Time	Dose (ppm)	%mortality +/- S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
24 hours	500	55.03 ± 0.51	Y= 1.36 + 1.37x	2.27	2.64	441.3
	250	31.4 ± 0.51				
	125	25.5 ± 0.54				
	62.5	13.5 ± 0.36				
	31.25	4.6 ± 0.71				
48 hours	500	75.34 ± 1.27	Y= 0.51 + 1.90x	1.54	2.36	229.4
	250	52.18 ± 0.95				
	125	27.27 ± 1.16				
	62.5	17.26 ± 0.84				
	31.25	4.49 ± 0.81				

*Avg. Mortality is the average of three replicates.

**Table 43 a. Nematotoxic effect of 8-hydroxy isotrichoderminol
against J2 of *Meloidogyne incognita***

Time	Dose (ppm)	%mortality +/- S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
24 hours	500	73.24 ± 0.59	Y= 1.08 + 1.72x	4.92	2.28	190.2
	250	57.3 ± 0.68				
	125	44.5 ± 0.70				
	62.5	22.6 ± 0.69				
	31.25	4.8 ± 0.50				
48 hours	500	47.5 ± 0.79	Y= 0.63 + 1.59x	1.02	2.73	540.81
	250	28.4 ± 0.93				
	125	18.38 ± 0.39				
	62.5	5.32 ± 0.08				

*Avg. Mortality is the average of three replicates.

**Table 43 b. Nematotoxic effect of 8- Hydroxyisotrichoderminol
against J2 of *Rotylenchulus reniformis***

Time	Dose (ppm)	%mortality $\pm$ S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
24 hours	500	74.12 $\pm$ 0.55	Y= 0.99 + 1.77x	3.39	2.25	179.1
	250	63.50 $\pm$ 0.70				
	125	43.0 $\pm$ 0.51				
	62.5	21.3 $\pm$ 0.58				
	31.25	6.14 $\pm$ 0.52				
48 hours	500	50.46 $\pm$ 0.48	Y= 0.639 + 1.63x	1.98	2.68	482.1
	250	30.61 $\pm$ 0.70				
	125	19.16 $\pm$ 0.087				
	62.5	9.22 $\pm$ 0.051				
	31.25	0.98 $\pm$ 0.02				

*Avg. Mortality is the average of three replicates.

Table 44 a. Nematotoxic effect of Moniliformin against J2 of *Meloidogyne incognita*

Time	Dose (ppm)	%mortality $\pm$ S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
24 hours	500	68.5 $\pm$ 0.82	Y= 1.80 + 1.45x	9.44	2.20	159.5
	250	67.87 $\pm$ 0.72				
	125	49.19 $\pm$ 1.2				
	62.5	30.64 $\pm$ 0.73				
	31.25	9.54 $\pm$ 0.74				
48 hours	500	72.13 $\pm$ 0.21	Y= 0.94 + 1.74x	1.50	2.33	214.8
	250	57.33 $\pm$ 0.40				
	125	32.23 $\pm$ 0.07				
	62.5	20.29 $\pm$ 0.13				
	31.25	5.69 $\pm$ 0.04				

*Avg. Mortality is the average of three replicates.

Table 44 b. Nematotoxic effect of Moniliformin against J2 of *Rotylenchulus reniformis*

Time	Dose (ppm)	%mortality $\pm$ S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
24 hours	500	86.37 $\pm$ 0.78	Y= 0.97 + 1.92x	9.20	2.09	123.6
	250	67.33 $\pm$ 1.03				
	125	53.42 $\pm$ 0.82				
	62.5	24.37 $\pm$ 0.75				
	31.25	10.48 $\pm$ 0.83				
48 hours	500	69.38 $\pm$ 0.31	Y= 2.23 + 1.24x	1.82	2.23	171.0
	250	52.57 $\pm$ 0.93				
	125	40.36 $\pm$ 0.28				
	62.5	31.68 $\pm$ 0.35				
	31.25	16.78 $\pm$ 0.22				

*Avg. Mortality is the average of three replicates.

Table 45 a. Nematotoxic effect of Sambucosin against J2 of *Meloidogyne incognita*

Time	Dose (ppm)	%mortality $\pm$ S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
24 hours	500	99.5 $\pm$ 0.53	Y= 2.01 + 2.02x	6.98	1.47	29.86
	250	94.6 $\pm$ 1.32				
	125	89.5 $\pm$ 0.98				
	62.5	82.7 $\pm$ 1.71				
	31.25	45.67 $\pm$ 0.89				
48 hours	125	94.09 $\pm$ 0.35	Y= 2.17 + 2.13x	1.96	1.33	21.23
	62.5	83.52 $\pm$ 0.24				
	31.25	61.07 $\pm$ 0.18				
	15.62	41.59 $\pm$ 0.38				

*Avg. Mortality is the average of three replicates.

Table 45 b. Nematotoxic effect of Sambucosin against J2 of *Rotylenchulus reniformis*

Time	Dose (ppm)	%mortality +/- S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
24 hours	500	99.54 ± 0.56	Y = 2.27 + 1.84x	7.64	1.48	30.46
	250	92.38 ± 0.65				
	125	86.35 ± 0.26				
	62.5	80.5 ± 1.18				
	31.25	45.37 ± 0.91				
48 hours	250	100	Y = 1.65 + 2.39x	1.96	1.39	24.8
	125	95.19 ± 0.24				
	62.5	80.79 ± 0.15				
	31.25	56.38 ± 0.66				
	15.62	34.39 ± 0.33				

*Avg. Mortality is the average of three replicates.

**Table 46 a. Nematotoxic effect of Dimethyl alkenyl chromone
against J2 of *Meloidogyne incognita***

ime	Dose (ppm)	%mortality +/- S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
24 hours	500	100	Y= 1.53 + 2.34x	2.79	1.48	30.13
	250	92.3 ± 0.17				
	125	89.2 ± 0.92				
	62.5	77.5 ± 0.48				
	31.25	52.7 ± 0.66				
48 hours	62.5	100	Y= -0.005 + 4.23x	0.74	1.18	15.25
	31.25	89.21 ± 0.27				
	15.62	52.63 ± 0.28				

*Avg. Mortality is the average of three replicates.

**Table 46 b. Nematotoxic effect of Dimethyl alkenyl chromone derivative
against J2 of *Rotylenchulus reniformis***

Time	Dose (ppm)	%mortality +/- S.D.	Regression equation	χ^2	Log ED ₅₀	ED ₅₀ (ppm)
24 hours	250	100	Y= 2.22 + 2.28x	5.11	1.21	16.52
	125	99.4 ± 0.37				
	62.5	85.4 ± 0.65				
	31.25	76.1 ± 0.55				
	15.62	48.4 ± 0.37				
48 hours	62.5	100	Y= -0.15 + 4.3x	0.59	1.18	15.29
	31.25	89.96 ± 0.18				
	15.62	52.3 ± 0.12				

*Avg. Mortality is the average of three replicates.

spores were most sensitive to mancozeb, sulphur dust and borex at the rates used.

Table 47 Mean percentage inhibition of *F. oxysporum* spore germination when exposed to field rates of selected fungicides

Fungicides	Inhibition of spore germination (%)
captan,	0
tridemorph,	0
metalaxyl,	7
thiophanate-methyl,	0
mancozeb,	45
sulphur dust	78
borax	100
Means values of three replicates (P<0.05)	

4.8.2 Compatibility with isolated secondary metabolites

Compatibility of isolated secondary metabolites was studied by mixing commercial fungicide in 1:1 ratio and testing *in vitro* fungicidal activity against soil borne pathogens in PDA media and storing under room condition. It was found these toxins were compatible with commercial fungicides namely captan, tridemorph, metalaxyl and thiophanate-methyl but were not able to inhibit spore

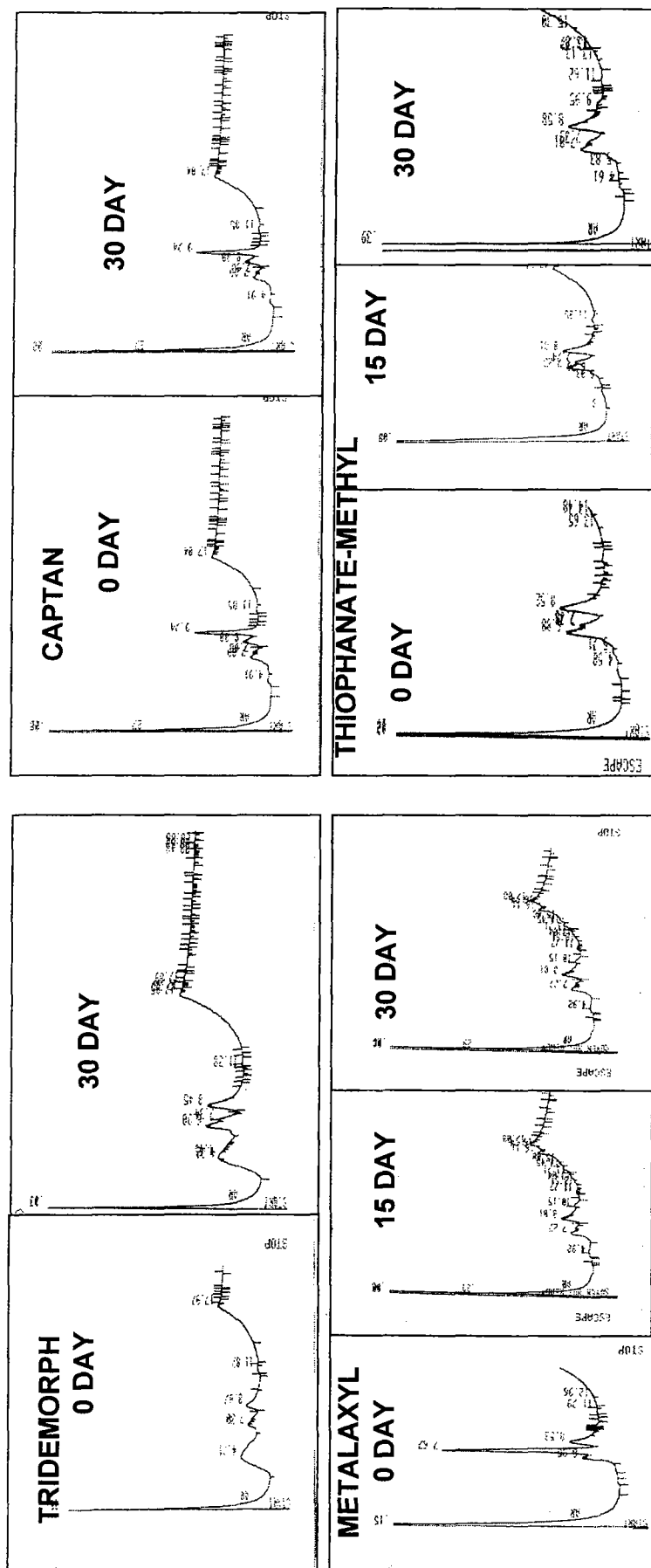


Fig 35a. Chemical compatibility of Sporol and Sporotrichiol with different commercial fungicides.

germination of pathogenic fungi when mixed with mancozeb, sulphur dust and borax.

Change in the concentration of tridemorph, captan, metalaxyl and two fungicidally active toxins when mixed together and stored for 30 days was monitored by GC and HPLC. (Figure 35) No change in the amount of tridemorph, captan and thiophanate-methyl and toxins were recorded after 30 days of storage. Whereas a decrease in the concentration of metalaxyl was observed with time when mixed with these toxins was observed. There was complete degradation of metalaxyl within fifteen days.

Thus in *vitro* germination assay showed that *F. oxysporum* and two of the fungicidal active toxins are highly sensitive to mancozeb, sulphur dust and borax, less sensitive to metalaxyl and relatively insensitive to captan, tridemorph and thiophanate-methyl. Thus we can conclude that although these two fungal toxins are physically compatible with four commercial fungicides, but, out of these four, only three are chemically compatible.

4.8.3 Potentiation of fungicidal activity of fungicides by mixing toxins

Secondary metabolites of *F.oxysporum* were found compatible with three commercial fungicides namely tridemorph, captan and thiophanate-methyl. LD₅₀(s) and potentiation ratio was calculated. Toxin namely sporotrichiol along with tridemorph and thiophanate-methyl when tested against *P. aphanidermatum* showed co-toxicity coefficient value of more than 100, thus signifying that mixing enhances the activity. It also showed prominent degree of potentiation when tested along with captan and thiophanate-methyl, against *P*

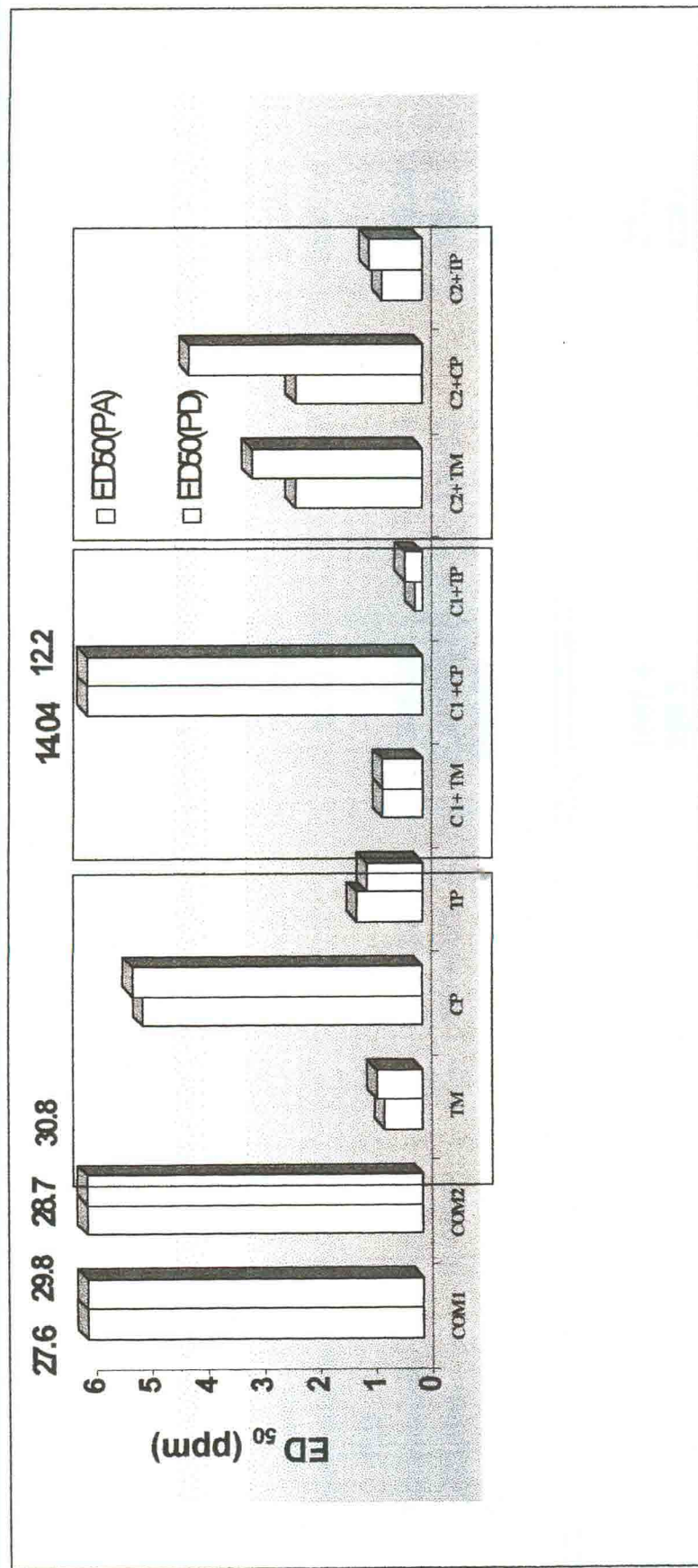


Fig 35b. Combined fungicidal activity of isolated compounds from *Fusarium oxysporum* with commercial fungicides

Table 48. SYNERGISTIC ACTIVITY OF ISOLATED COMPOUNDS WITH FUNGICIDES

Com	Toxicant ED ₅₀ (%)	Mixture ED ₅₀ (%)	Theoretical toxicity	Actual toxicity	Toxicity coefficient
Sporotrichum					
P.A					
Tridemorph	0.000071	0.000073	1993.662	3780.8	189.64
Captan	0.0005	0.001404	326.19	196.67	60.30
Thiophanate P.D	0.00012	0.000016	1200.11	17250.0	1437.50
P.D					
Tridemorph	0.00007	0.000225	348.32	1324.4	378.23
Captan	0.0005	0.000226	2178.571	1316.3	60.0
Thiophanate	0.0001	0.000072	1540.00	4138.9	268.76
Sporotrichum					
P.A					
Tridemorph	0.000091	0.000074	1687.4	4027.0	38.88
Captan	0.0006	0.00122	298.3	244.3	539.0
Thiophanate P.D	0.00016	0.000031	981.3	9612.9	232
P.D					
Tridemorph	0.00006	0.000303	2616.7	1016.5	38.5
Captan	0.00069	0.00042	273.2	733.3	268
Thiophanate	0.0003	0.000097	563.3	3175.3	563

debaryanum (co toxicity coefficient is 378 and 268, respectively). Similar observation was observed with another toxin namely sambucoid. Co toxicity coefficient has shown that, mixing sporotrichiol and sambucoid with thiophanate-methyl gives far better relative advantage of mixing as reflected by high value of co toxicity coefficient than any of the combination. Better results were observed by mixing sambucoid with fungicides for testing against *P. aphanidermatum* (co-toxicity coefficient 239) as compared to sporotrichiol (co-toxicity coefficient 189). Both captan and thiophanate-methyl when mixed with sambucoid gave promising potentiation. Effective potentiation of sporotrichiol with captan against *P. debaryanum* than sambucoid with thiophanate-methyl was observed (co-toxicity coefficient of 564 and 289, respectively). Mixing of captan with sporotrichiol and sambucoid were not much effective, when tested against *P. aphanidermatum* and *P. debaryanum* (co-toxicity coefficient less than 100). (Table 48)

Thus from these results it can be concluded that out of the seven tested fungicides, only three are both biologically and chemically compatible with isolated secondary metabolites of *F. oxysporum*.

4.9 Estimation of mycotoxins of *F. oxysporum* by gas chromatography

Most of the mycotoxins are very polar compounds and contain a number of hydroxyl groups. Therefore, they cannot be estimated by GLC using flame ionization detector. Only method for estimation of these toxins by GLC is to make it less polar by suitably derivatising the free hydroxyl groups. A method was therefore, standardized by derivatising with trimethyl silyl chloride and diazomethane prior to analysis by GC. Thus the dichloromethane extract of

culture filtrate and mycelia was treated with diazomethane and analyzed by GLC. GLC chromatogram showed the presence of a number of compounds. The structure of these compounds was further confirmed by GC-MS as different fatty acids.

4.9.1 Simultaneous analysis of *Fusarium* toxins

The five most important toxins namely diacetoxyscirpenol, fusaretin, T-2 toxin, HT-2 toxin and zearalenone-, which are usually, present as contaminants in *Fusarium* infected maize and other cereals, have to be analyzed routinely prior to export of these cereal commodities. A method was standardized for extraction of contaminated sample with a mixture of ethyl acetate and methanol (1:1) and GC estimation after derivatization with trimethyl silyl chloride using following conditions: column temperature programmed from 70 to 270 °C @ 5°C, with injector and detector temperature at 300°C. Limit of detection of the method is 0.001 ppm and limit of determination 0.01ppm.. During the entire study, presence of four out of these five most hazardous *Fusarium* toxins was observed in strain-infected sample. Neither of the samples showed the presence of HT-2 toxin. Presence of these toxins was further confirmed by GC-MS.

4.10 Formulations of *F. oxysporum*

4.10.1 Mycelia based solid and granular formulation

Solid formulations based on dried mycelia separated from 15 d old culture of *F. oxysporum* by mixing either with fly ash and charcoal / sawdust were prepared. Similarly mycelia based granular formulations were also prepared (section 3.4.6) and evaluated for bio-efficacy *in vivo* against *P. aphanidermatum*

and *P. debaryanum* using tomato as a test plant and mung bean for nematicidal activity

These prepared formulations were assayed over a six-week period to determine the effect of storage on the viability of the fungi. Serial dilutions of the dry preparations, prepared in sterile water, were placed on a semi-selective medium containing nutrient and antibiotics. After one week of incubation, colony-forming units/gram of dry preparation was determined. This was repeated each week with the stored dry preparations. The results are shown in Table. Comparable results were obtained from both preparations.

TABLE 49: Viability of prepared formulations

Storage	CFU/gm Dry Prep. (times. 10 ^{sup.5})	
	solid formulation	granular formulation
1 week	9.6 a ^{sup.z}	11.5 a
3 weeks	9.8 a	7.8 ab
6 weeks	9.6 a	5.6 a

.sup.z Duncan's multiple range test (DMRT), .sub. -- P = 0.05.

4.10.2 Fungicidal activity of the compound based formulations against *S. rolfsii* and *M. phaseolina*

Fungicidal activity of the compound based formulation of *F. oxysporum* was tested against *S. rolfsii* and *M. phaseolina* by poisoned food technique. ED₅₀ value recorded was somewhat more than that of the combined ED₅₀ of these two potent fungicidally active molecules. When the 1:1 mixture of sporotrichiol and sambucuin, isolated from dichloromethane culture filtrate showed a ED₅₀ of 167 and 237 ppm, for *M. phaseolina* and *S. rolfsii*, respectively, the formulated product containing both of these toxicant at same composition and concentration, showed a ED₅₀ of 213 and 296 ppm for *M. phaseolina* and *S. rolfsii*, respectively. This fact can be attributed to the slow release pattern of the toxicant from the encapsulation matrix. (Figure 36)

4.10.3 Antifungal activity of above formulations (pot experiment)

The biocontrol capability of the above formulations against *P. aphanidermatum* and *P. debaryanum* was evaluated in pot culture studies. Plant stand was determined after one and three weeks of growth. These results are shown in Table 50. The formulation significantly prevented tomato damping off. It allowed a stand comparable to that in the non-infested control. (Figure 37)

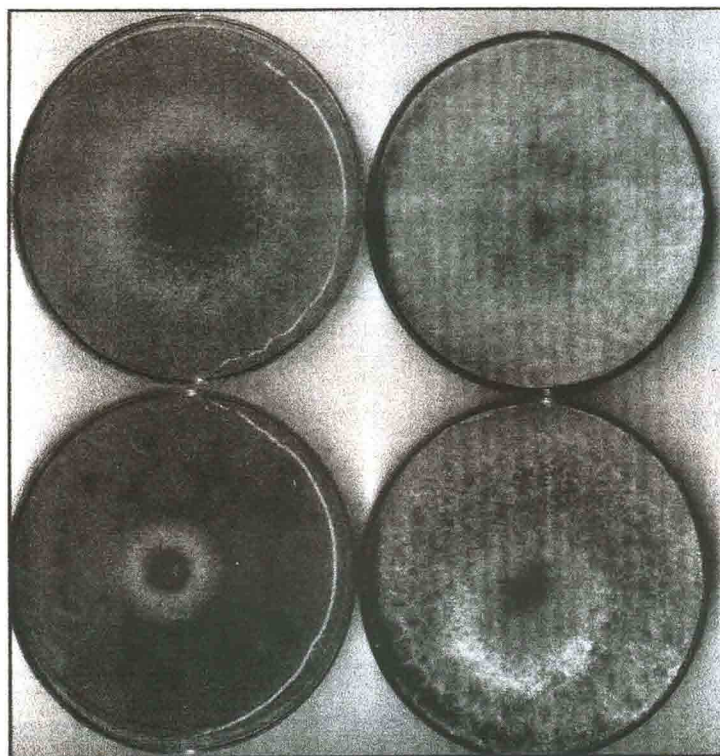
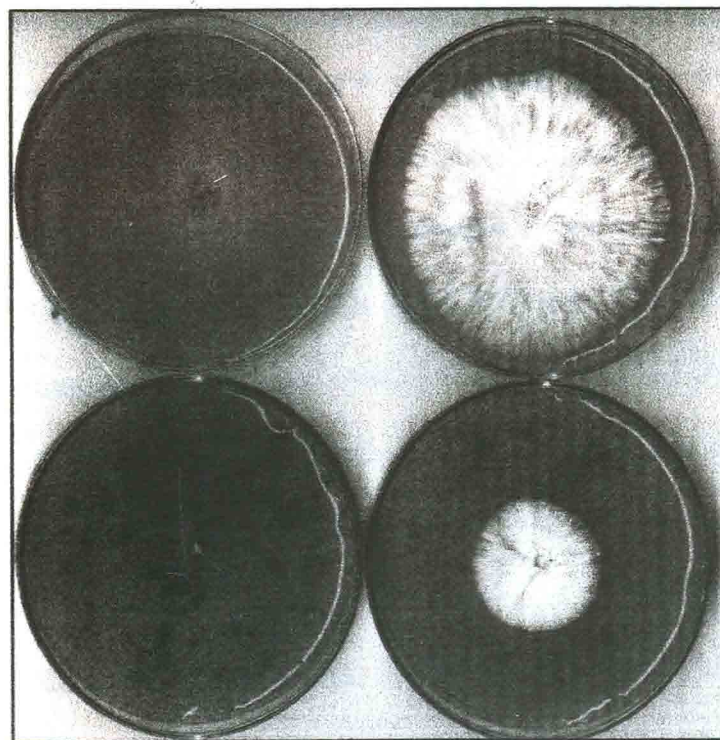


Fig 36. Antifungal activity of pellet formulation of *Fusarium oxysporum* against *Sclerotium rolfsii* and *Macrophomina phaseolina*

Table 50: Bioactivity of *F.oxysporum* formulations in green house studies

Biocontrol Formulation	Tomato Stand (%)	
	1 week	3 weeks
Control (no pathogen)	98.0 a.sup.z	98.0 a
Control (<i>P. aphanidermatum</i>)	0.0 c	0.0 d
Control (<i>P. aphanidermatum</i> /fly ash)	6.0 c	0.0 d
Fly ash + biomass formulatioonn	90.0 a	78.0 b

.sup.z DMRT, .sub.-- P = 0.05.

4.10. 4 Nematicidal activity of above formulations (field experiment)

Alginate based formulation of *F. oxysporum* (section 4.10.1) containing 1-5% mycelia was evaluated for their nematicidal activity @ 2-5 Kg formulation/ha by direct mixing/ spraying one week before sowing seeds of mung (*Vigna radiata*) cv. Pusa 9631. After 3 days of germination, 500 J2 of both the tested nematodes/ plant were inoculated around the growing seedling. Observations on plant growth characters and nematode gal numbers were recorded after 50 days of germination. A significant increase in plant growth parameters of mung crop was observed. However, plant growth varied and response of dosage was different for different formulations. Shoot length and plant biomass weight

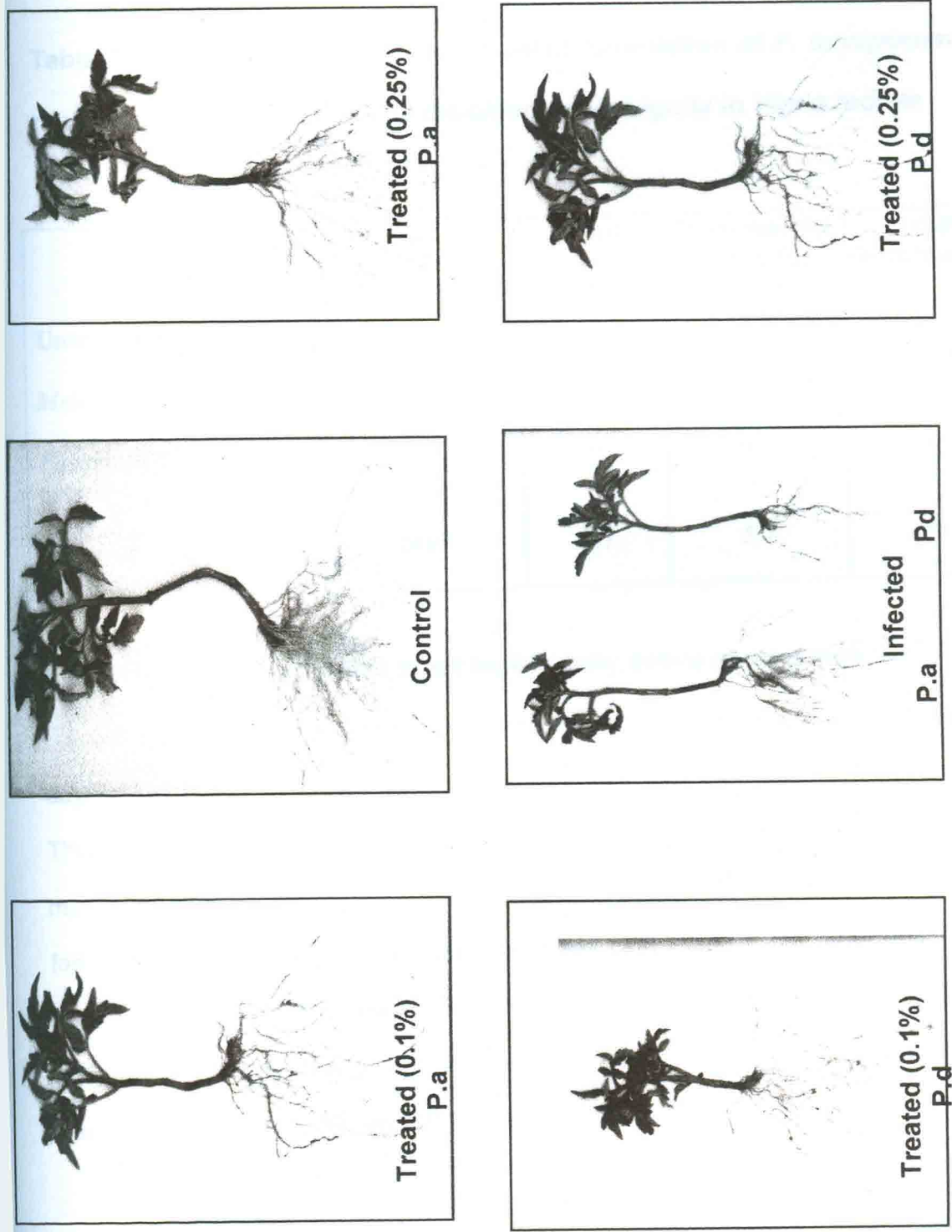


Fig37. Fungicidal activity of mycelia based formulations of *Fuarium oxysporum* - a pot culture study

increase was more in alginate-based formulation and number of galls formed was significantly less in the treated plots than in control. (Table 51) (Figure 38a, 38b)

Table 51: Effect of mycelium based pellet formulation of *F. oxysporum* on root knot disease caused by *Meloidogyne incognita* in *Vigna radiata*

Treatment	Total nematode population	Root knot index	Root weight index (g)	Total herbage (g)
Untreated	--	--	5.9	42
<i>Meloidogyne</i> only	4731	4	3.0	17
<i>Fusarium oxysporum</i> only	--	--	6.1	49
<i>Fusarium Oxysporum</i>+ <i>Meloidogyne</i>	2419	2.7	5.7	46

4.11 Release pattern of two most herbicidally active compounds

GC chromatogram of different day samples clearly reveals that with the increase in days of incubation, the concentration of both the compounds in water phase increases. This signifies that, with the progress of time, the compound gets released from the matrix of control release alginate based formulation. When the log residue remaining in formulation was plotted against time (days) we can see that the release pattern of toxicant from the matrix followed the first order kinetics for both the compounds with a significantly high correlation coefficient and the half life recorded for the both the compounds were 30.4 days and 18.7 days, respectively. (Figure 39, 40)

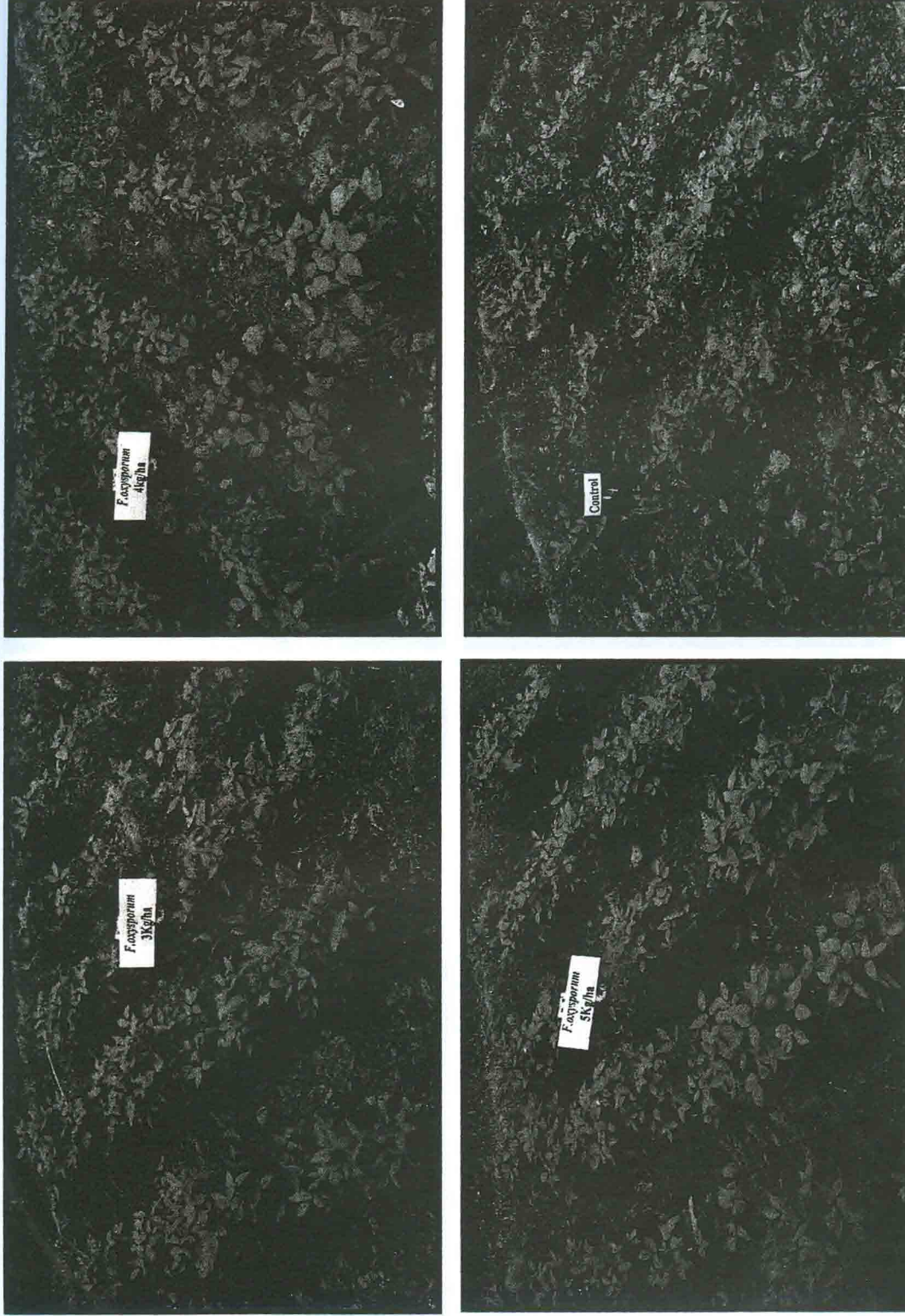


Fig 38 a. Effect of application of dried mycelia based formulation of *F. oxysporum* for *Meloidogyne incognita* preinfected mung field – *Fusarium* formulation vs. control

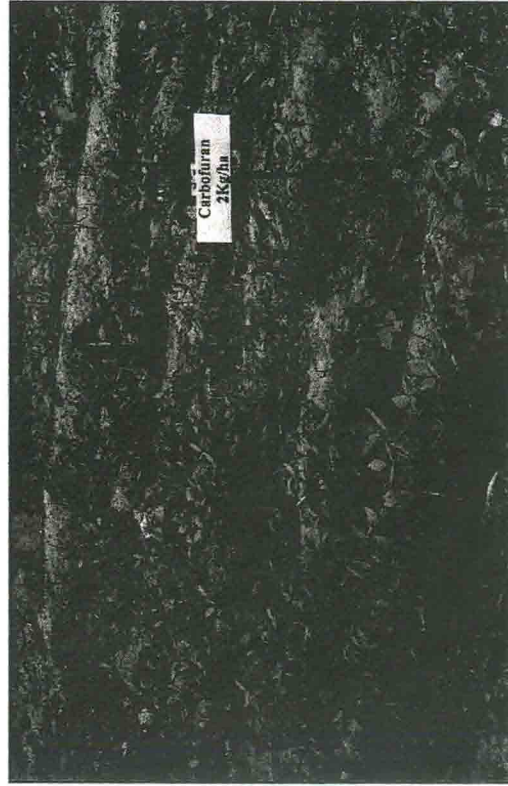
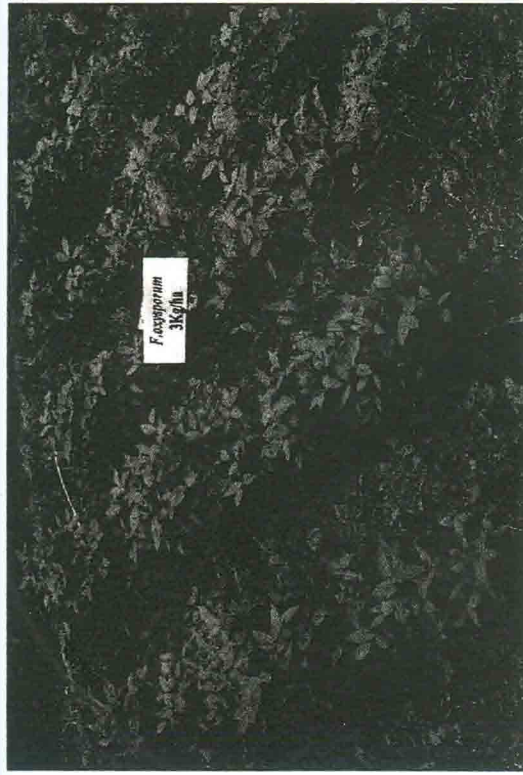


Fig 38 b. Effect of application of dried mycelia based formulation of *F. oxysporum* for *Meloidogyne incognita* preinfected mung field – *Fusarium* formulation vs. commercial nematicide, Carbofuran

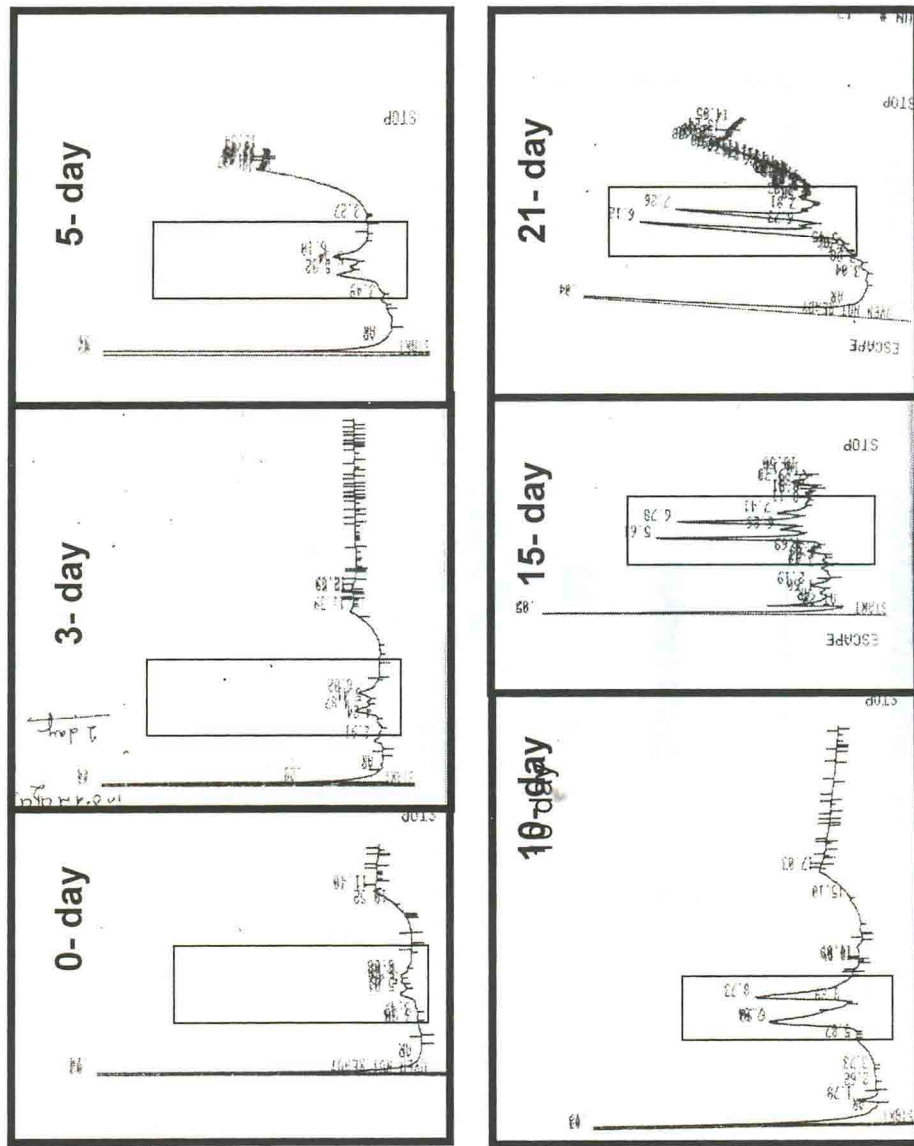


Fig 39. Release pattern of *Fusarium* toxins from matrix of Pellet formulation

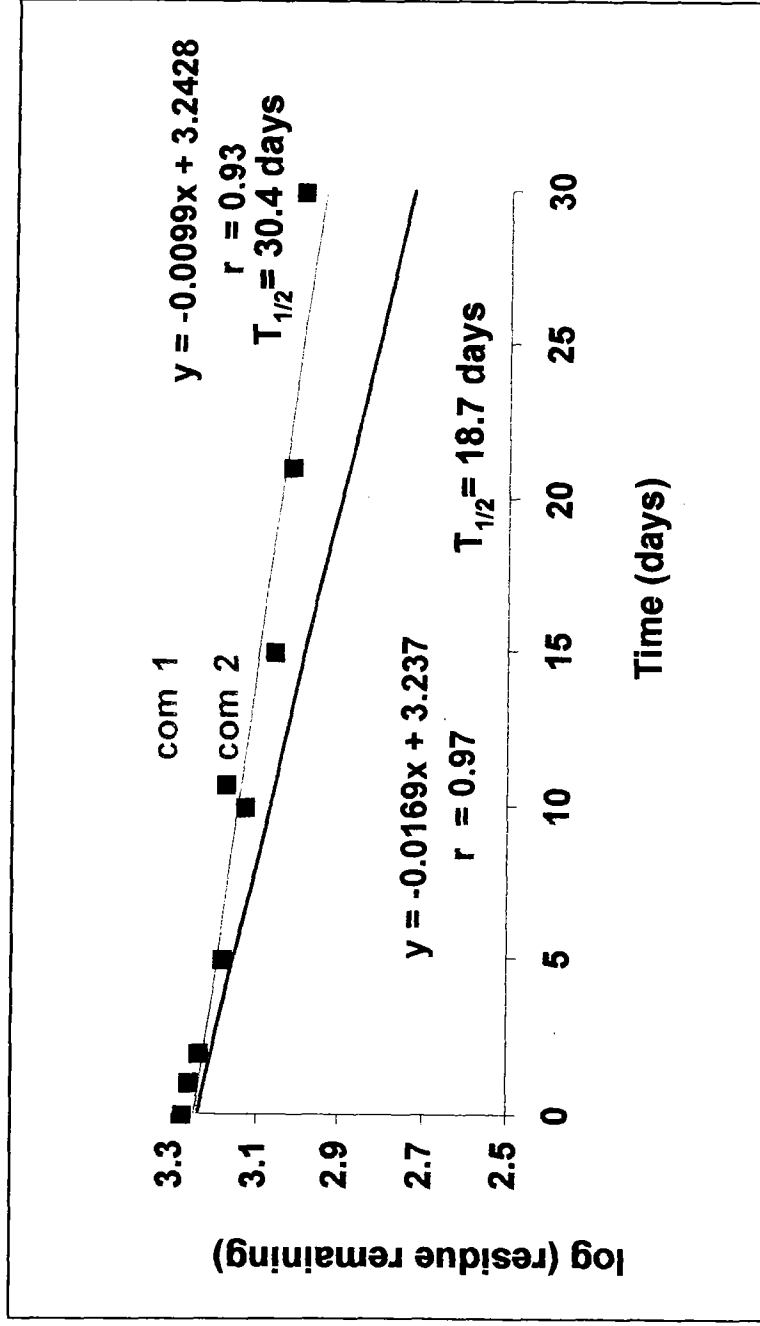


Fig 40. Rate of release of toxins from encapsulated formulation of *Fusarium oxysporum*

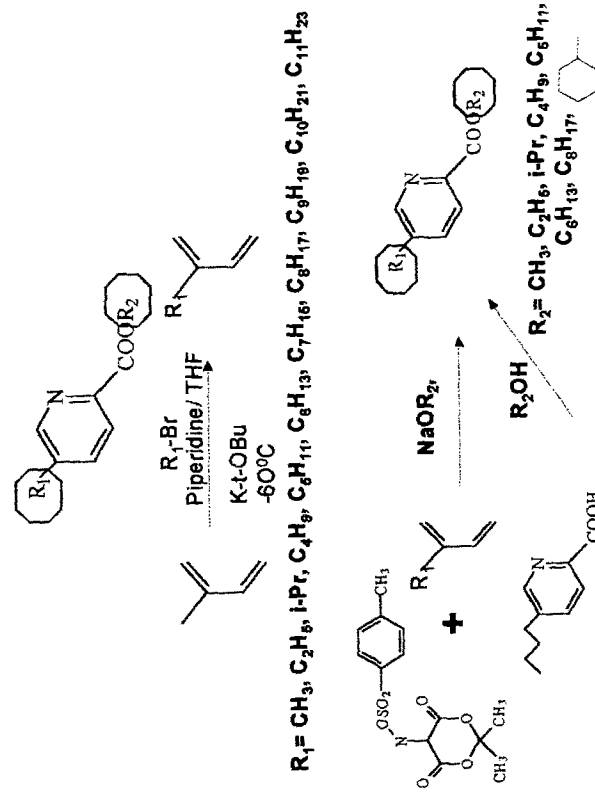
4.12 Synthesis

4.12.1. Fusaric acid analogues

Methyl-5-alkyl-picolanoate (methyl fusarate) and ethyl-5-alkyl picolanoate (ethyl fusarate) were prepared and characterized on the basis of NMR and mass spectroscopy. Different analogues of fusaric acid were synthesized by changing side chain at 5 positions (R_1). The structures were confirmed by ^1H NMR. (Table 52). These compounds were tested for antifungal activity *in vitro*. The fungicidal activity is reported in Table 53. A progressive increase in activity with observed with increasing molecular weight. Lower homologs were found to be less active. Methyl-6-hexyl-picolanoate was found to be most active compound of the series. A good correlation of the fungicidal activity with alkyl chain length was observed. Fungicidal activity was found to increase with the increase of alkyl chain length up to 6-hexyl analogue. Decrease in activity was observed with long alkyl chain. Branching in the short alkyl chains enhanced the fungicidal activity against *Rhizoctonia bataticola*, however, the activity was found to decrease six times by branching in the long alkyl chain. These compounds when tested against different fungi were found very active against *P aphanidermatum* followed by *P debaraneum*, *R. bataticola* and *S. rolfsii* (Table 53).

Ethyl-5-alkyl picolanoate were found to be less effective than corresponding methyl analogues (Table 53). The fungicidal activity of different analogues was in

Scheme 3. Synthetic route of Fusaric acid analogues



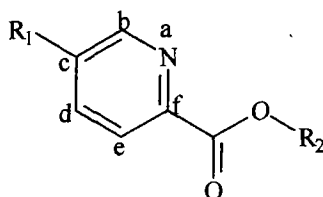
the following order. $\text{CH}_3 < \text{C}_2\text{H}_5 < \text{I-Pr} < \text{C}_4\text{H}_9 < \text{C}_5\text{H}_{11} < \text{C}_6\text{H}_{13} > \text{C}_7\text{H}_{15} = \text{C}_8\text{H}_{17} = \text{C}_9\text{H}_{19} > \text{C}_{10}\text{H}_{21} > \text{C}_{11}\text{H}_{23}$. (Table 52)

4.12.2 Palmitic acid derivatives

Nine different derivatives of palmitic acid were synthesized by esterification with different alcohols in presence of Conc H_2SO_4 . These compounds were characterized by ^1H - NMR (60 MHz) (Table 54) and tested for fungicidal activity against *M. phaseolina*, *R. bataticola* and *S. rolfsii*. They were found less active and only 50% inhibition of *Pythium spp* was observed even at 800-ppm concentration. The maximum activity was observed against *M. phaseolina* followed by *R. bataticola* and *S. rolfsii*. There was slight increase in fungicidal activity up to butyl analogue. Further increase in carbon chain reduced the antifungal activity. Fungicidal activity of these compounds were in the following order $\text{CH}_3 < \text{C}_2\text{H}_5 < \text{I-Pr} < \text{Pr} < \text{C}_4\text{H}_9 > \text{I- Bu} > \text{C}_5\text{H}_{11} > \text{C}_6\text{H}_{13} > \text{cyclohexyl} > 3\text{-methyl butyneyl}$. (Table 55)

Thus from the above study we can conclude that *F.oxysporum* is active against a number of pathogenic fungi, nematodes and also show plant growth and herbicidal activity. The mycelia based formulation is stable unto one year when stored under laboratory conditions.

Table 52. ¹H- NMR details of synthesized Fusaric acid derivatives



COMP	R ₁	¹ H NMR (δ)	R ₂	¹ H NMR (δ)
1.	-CH ₃ 1	8.44 (1H, f, s), 8.13 (1H, c, d, J= 8 Hz), 7.73(1H, d, d, J= 8 Hz), 2.30(3H, 1, s)	-CH ₃ 1'	3.92 (3H, 1', s)
2.	-CH ₂ CH ₃ 1 2	7.84 (1H, f, s), 7.53 (1H, c, d, J= 8 Hz), 7.35(1H, d, d, J= 8 Hz), 2.59(3H, 1, q), 1.24(3H, 2, t)	-CH ₃ 1'	3.71 (3H, 1', s)
3	-CH(CH ₃) ₂ 1 2	8.61 (1H, f, s), 8.33 (1H, c, d, J= 8 Hz), 7.68(1H, d, d, J= 8 Hz), 3.12(1H, 1, q), 1.29(6H, 2, d)	-CH ₃ 1'	3.88 (3H, 1', s)
4		8.21 (1H, f, s), 7.96(1H, c, d, J= 8 Hz), 7.52(1H, d, d, J= 8 Hz), 2.7(2H, 1, t, J= 7.6 Hz), 1.60-1.7(2H, 2, m), 1.35-1.4(2H, 3,m), 0.95 (3H, 4, t, J= 7.3 Hz)	-CH ₃ 1'	3.73 (3H, 1', s)
5		8.71 (1H, f, s), 8.43 (1H, c, d, J= 8 Hz), 7.98(1H, d, d, J= 8 Hz), 2.9(2H, 1, t, J= 7.6 Hz), 1.48-1.5(2H, 2, m), 1.20-1.3(2H, 4, m) , 1.27(2H, 3, m) 1.08(3H, 5, t, J= 7.3 Hz)	-CH ₃ 1'	3.96 (3H, 1', s)

6		7.92 (1H, f, s), 7.76 (1H, c, d, J= 8 Hz), 7.45 (1H, d, d, J= 8 Hz), 2.83 (2H, 1, t, J= 7.6 Hz), 1.60-1.7 (2H, 2, m), 1.35-1.4 (2H, 4, m), 1.29 (4H, 3, m) 0.95 (3H, 5, t, J= 7.3 Hz)	-CH ₃ 1'	3.68 (3H, 1', s)
7		8.50 (1H, f, s), 8.10 (1H, c, d, J= 8 Hz), 7.70 (1H, d, d, J= 8 Hz), 2.7 (2H, 1, t, J= 7.6 Hz), 1.66-1.7 (2H, 2, m), 1.36-1.42 (2H, 4, m), 1.19 (6H, 3, m) 0.85 (3H, 5, t, J= 7.3 Hz)	-CH ₃ 1'	3.82 (3H, 1', s)
8		7.93 (1H, f, s), 8.68 (1H, c, d, J= 8 Hz), 7.36 (1H, d, d, J= 8 Hz), 2.5 (2H, 1, t, J= 7.6 Hz), 1.60-1.72 (2H, 2, m), 1.28-1.36 (2H, 4, m) 1.22 (8H, 3, m) 0.89 (3H, 5, t, J= 7.3 Hz)	-CH ₃ 1'	3.72 (3H, 1', s)
9		8.42 (1H, f, s), 8.12 (1H, c, d, J= 8 Hz), 7.8 (1H, d, d, J= 8 Hz), 2.65 (2H, 1, t, J= 7.6 Hz), 1.55-1.6 (2H, 2, m), 1.44-1.5 (2H, 4, m), 1.30 (10H, 3, m) 0.95 (3H, 5, t, J= 7.3 Hz)	-CH ₃ 1'	3.91 (3H, 1', s)
10		8.64 (1H, f, s), 8.30 (1H, c, d, J= 8 Hz), 7.85 (1H, d, d, J= 8 Hz), 2.8 (2H, 1, t, J= 7.6 Hz), 1.7-1.8 (2H, 2, m), 1.36-1.4 (2H, 4, m), 1.39 (12H, 3, m) 1.05 (3H, 5, t, J= 7.3 Hz)	-CH ₃ 1'	3.68 (3H, 1', s)

11		8.34 (1H, f , s), 8.05 (1H, c , d, J= 8 Hz), 7.62(1H, d , d, J= 8 Hz), 2.55(2H, 1 , t, J= 7.6 Hz), 1.58-1.62(2H, 2 , m), 1.30-1.35(2H, 4 , m) 1.24(14H, 3 , m) 0.98 (3H, 5 , t, J= 7.3 Hz)	-CH ₃ 1'	3.87(3H, 1' , s)
12		7.88 (1H, f , s), 7.64 (1H, c , d, J= 8 Hz), 7.34(1H, d , d, J= 8 Hz), 2.53(2H, 1 , t, J= 7.6 Hz), 1.38-1.45(2H, 2 , m), 1.22-1.28(2H, 3 , m) 0.82 (3H, 4 , t, J= 7.3 Hz)	-CH ₂ CH ₃ 1' 2'	4.29(2H, 1' , m), 1.22-1.28 (3H, 2' , t)
13		8.56 (1H, f , s), 8.25 (1H, c , d, J= 8 Hz), 7.85(1H, d , d, J= 8 Hz), 2.7(2H, 1 , t, J= 7.6 Hz), 1.72-1.8(2H, 2 , m), 1.43-1.5(2H, 3 , m), 1.03 (3H, 4 , t, J= 7.3 Hz)	-CH(CH ₃) ₂ 1' 2'	4.31(2H, 1' , m), 1.43-1.5 (6H, 2' , t)
14		8.24 (1H, f , s), 8.03 (1H, c , d, J= 8 Hz), 7.53(1H, d , d, J= 8 Hz), 2.55(2H, 1 , t, J= 7.6 Hz), 1.63-1.75(2H, 2 , m), 1.42-1.5(2H, 3 , m), 1.05 (3H, 4 , t, J= 7.3 Hz)		4.25(2H, 1' , t), 1.63-1.75 (2H, 2' , m), 1.29 (2H, 3' , m), 1.05(3H, 4' , t)
15		8.40 (1H, f , s), 8.09 (1H, c , d, J= 8 Hz), 7.72(1H, d , d, J= 8 Hz), 2.65(2H, 1 , t, J= 7.6 Hz), 1.62-1.7(2H, 2 , m), 1.38-1.42(2H, 3 , m), 0.95 (3H, 4 , t, J= 7.3 Hz)		4.25(2H, 1' , t), 1.62-1.7(2H, 2' , m), 1.38-1.42(2H, 4' , m), 1.25(2H, 3' , m), 0.95(3H, 5' , t)

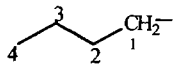
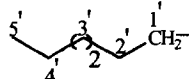
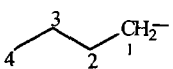
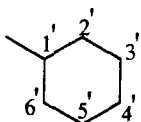
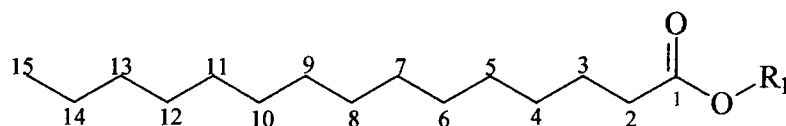
16		8.20 (1H, f ,s), 8.0 (1H, c , d, J= 8 Hz), 7.63(1H, d , d, J= 8 Hz), 2.57(2H, 1 , t, J= 7.6 Hz), 1.55-1.65(2H, 2 , m), 1.35-1.4(2H, 3 , m), 0.92 (3H, 4 , t, J= 7.3 Hz)		4.25(2H, 1' ,t), 1.55-1.65(2H, 2' ,m), 1.35-1.4(2H, 4' ,m), 1.20(4H, 3' ,m), 0.92(3H, 5' ,t)
17		7.88 (1H, f ,s), 7.63 (1H, c , d, J= 8 Hz), 7.34(1H, d , d, J= 8 Hz), 2.46(2H, 1 , t, J= 7.6 Hz), 1.51-1.56(2H, 2 , m), 1.25-1.31(2H, 3 m), 0.85 (3H, 4 , t, J= 7.3 Hz)		3.95(1H, 1' ,m), 1.79(4H, 2' and 6' ,m), 1.25-1.31(6H, 3' , 4' , and 5' ,m)

Fig 53. Antifungal activity of Fusaric acid derivatives

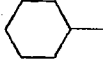
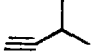
SL NO	R ₁	R ₂	ED ₅₀ (ppm)				
			P.a	P.d	M.p	S.r	R.b
1	CH ₃	CH ₃	322	423	 >700	589	415
2	C ₂ H ₅	CH ₃	197	421		471	326
3	i-Pr	CH ₃	161	362		369	281
4	C ₄ H ₉	CH ₃	159	212		329	259
5	C ₅ H ₁₁	CH ₃	97	128		289	231
6	C ₆ H ₁₃	CH ₃	62	99		215	116
7	C ₇ H ₁₅	CH ₃	89	141		361	189
8	C ₈ H ₁₇	CH ₃	91	152		342	197
9	C ₉ H ₁₉	CH ₃	99	149		369	201
10	C ₁₀ H ₂₁	CH ₃	219	221		426	285
11	C ₁₁ H ₂₃	CH ₃	432	368		587	297
12	C ₄ H ₉	CH ₃	159	212		329	259
13	C ₄ H ₉	C ₂ H ₅	127	197		221	236
14	C ₄ H ₉	i-Pr	69	111		119	93
15	C ₄ H ₉	C ₄ H ₉	186	164		167	124
16	C ₄ H ₉	C ₅ H ₁₁	289	161		218	129
17	C ₄ H ₉	C ₆ H ₁₃	287	218		341	131
18	C ₄ H ₉		521	498		381	267

Table 54. ^1H - NMR details of synthesized Palmitic acid derivatives



COMP	R ₁	^1H NMR (δ)
1.	$-\text{CH}_3$ 1'	3.67 (3H, 1',s), 2.25 (2H, 1, t), 1.68(2H, 2, m), 1.33(2H,14, m) , 1.29(20H, 4-13, m), 0.96(3H,15,t)
2.	$-\text{CH}_2\text{CH}_3$ 1' 2'	4.12 (2H, 1',m), 2.12 (2H, 1, t), 1.42(2H, 2, m), 1.18(2H,14, m) , 1.10(23H, 4-13 and 2', m), 0.83(3H,15,t)
3	$-\text{CHCH}_2\text{CH}_3$ 1' 2' 3'	4.08 (2H, 1',m), 2.29 (2H, 1, t), 1.7(4H, 2 and 2', m), 1.41(2H,14, m) , 1.35(20H, 4-13, m), 1.02(6H,15 and 3',t)
4		4.04 (2H, 1',m), 2.43 (4H, 1 and 2', t), 1.65(2H, 2, m), 1.38 (2H,14, m) , 1.28(20H, 4-13, m), 1.2-0.98(9H,15 and 3',m)
5		4.08 (2H, 1',m), 2.65 (2H, 1, t), 1.72-1.85(4H, 2 and 2', m), 1.56(4H,14 and 3', m) , 1.44(20H, 4-13, m), 1.07(6H,15 and 4',t)
6		4.28 (2H, 1',m), 2.05 (2H, 1, t), 1.58-1.69(4H, 2 and 2', m), 1.43(4H,14 and 4', m) , 1.23(22H, 4-13 and 3', m), 0.93(6H,15 and 5',t)
7		4.19 (2H, 1',m), 2.25 (2H, 1, t), 1.42-1.57(4H, 2 and 2', m), 1.23(4H,14 and 4', m) , 1.18(24H, 4-13 and 3', m), 0.89(6H,15 and 5',t)
8		3,92 (2H, 1',m), 2.35 (2H, 1, t), 1.34-1.45(6H, 2, 2' and 6', m), 1.17(2H,14, m) , 1.09(26H, 4-13 and 3', 4' and 5', m), 0.85(3H,15,t)

Fig 55. Antifungal activity of Palmitic acid derivatives

SL NO	R	ED ₅₀ (ppm) <i>P.a</i>	ED ₅₀ (ppm) <i>P.d</i>	ED ₅₀ (ppm) <i>S.r</i>	ED ₅₀ (ppm) <i>R.b</i>	ED ₅₀ (ppm) <i>M.p</i>
1	CH ₃	↑ ↓ > 800	↑ ↓ > 850	689	413	389
2	C ₂ H ₅			621	329	321
3	Pr			459	313	122
4	i-Pr			521	341	143
5	C ₄ H ₉			312	246	97
6	i-Bu			329	271	118
7	C ₅ H ₁₁	> 800	> 850	461	329	182
8	C ₆ H ₁₃			513	414	179
9				715	512	231
10		↓	↓	622	531	262

5. *Summary and Conclusions*

Biological control of insect pests has been a successful approach for decades. The biological control of plant diseases and weeds is not yet as well established in commercial agriculture, but is a rapidly developing area of research. A beneficial organism used to control a pathogen is referred as biological control agent. It interacts with plant pathogens in various ways to inhibit the disease producing ability of the pathogen. The general mechanisms of biological control of plant diseases are competition, antibiosis, predation/ parasitism and induced resistance. All known BCA's utilize one or more of these general mechanisms. Among the bio-control agents *F.oxysporum* has been reported to have high potentiality in controlling a number of soil borne diseases. The exact mechanism of action against plant pathogen is not well established. It has been reviewed that, competition with the pathogen for nutrients and space, induced resistance, and suppression of hydrolytic enzymes of pathogen, are the main mechanisms along with other such as mycoparasitism and antibiosis. The role of antibiosis to control plant disease is not well established. Therefore, the present study on chemistry and biological activity of the cultural filtrate of *F.oxysporum* was undertaken to isolate, identify and study antagonistic effect of the secondary metabolites against plant pathogens. The salient features of the research findings are summarized below.

1. The biocontrol agents, *F.oxysporum* and pathogenic fungi such as *Pythium debarynum*, *Rhizoctonia bataticola*, *Pythium aphanidermatum*, *Sclerotium*

type culture, / Division of Plant Pathology and root knot nematodes *Meloidogyne incognita* and cyst nematode *Rotylenchulus reniformis* from Division of nematology, IARI

2. Culture media such as Ala-2, Nutrient Agra, Yems media, Luria Agar, Malt Extract Agar, Rose Bengal, Sucrose yeast Peptone and Potato Dextrose Agar were used for mass culturing of *F.oxysporum* . The maximum radial growth was observed in ALA II followed by SYP, PDA and Luria broth. Based on this study it was found that the best media for mass culturing of non-pathogenic strain of *F. oxysporum*, production of secondary metabolites and inhibition against pathogenic fungi are ALA-II, PDA and SYP. Maximum fungicidal activity was exhibited by incubating *F.oxysporum* up to 15 days.
3. *F.oxysporum* was raised on potato dextrose broth at $25 \pm 1^{\circ}\text{C}$ for 15 days. The mycelia mass was separated from culture filtrate by filtering through cheesecloth. The cultural filtrate and mycelia were used separately for the further study.
4. Culture filtrate was extracted with dichloromethane and ethyl acetate. After removal of solvents the residues were tested for fungicidal, nematocidal, plant growth regulatory and herbicidal activity
5. Mycelia separated from culture filtrate was washed and dried under sterilized conditions. Viability and stability of dried mycelia mass was study for a period of one year by dual culture. The dried mycelium was extracted with hexane

followed by dichloromethane and methanol. After removal of solvent, the extracts were tested for bioactivity

6. Dichloromethane and ethyl acetate extracts of culture filtrate extract were found very activity against *P.aphanidermatum* (ED₅₀ 21.1 ppm) and *P.debaryanum* (ED₅₀ 29.9 ppm)
7. The dichloromethane extract of mycelia was found most active against *R. bataticola* (ED₅₀ 24.5 ppm) , *P. aphanidermatum* (ED₅₀ 28.40 ppm) and *P. debaryanum* (ED₅₀ 27.7 ppm) as compared to *M. phaseolina* (ED₅₀ 75.8 ppm), *S. rolfsii* (ED₅₀ 182.6ppm), and *R. solani* (ED₅₀ 104 ppm). Methanol extract was found less effective than hexane and dichloromethane extract. of mycelia mass.
8. Insecticidal activity of dichloromethane and ethyl acetate extracts of culture filtrate and hexane and dichloromethane extracts of mycelia were tested against 2nd instar larvae of *Spodoptera litura*. Culture filtrate extracts were found more effective than mycelia extract.
9. Nematicidal activity of culture filtrate (dichloromethane, ethyl acetate extracts) and mycelial extracts (dichloromethane, methanol and hexane extracts) was evaluated against J2 of *R. reniformis* and *M. incognita* after 24, 48 and 72 h of incubation period. Hexane extract was found most effective among all the extracts tested (ED₅₀ 188 and 218 ppm, against *R. reniformis* and *M. incognita*, after 24 h respectively). It showed 99.8 percent mortality even after 48 and 72 hours of incubation against both the nematodes

10. Culture filtrate and mycelia extracts were tested for their effects on wheat seed germination. Dichloromethane and ethyl acetate extracts of culture filtrate and methanol, dichloromethane and hexane extracts of mycelia showed 90 and 95 percent germination respectively at 100-ppm concentration as compared to control (88 %). Whereas at higher concentrations, there was decrease in percent germination. A slight increase in shoot length and root length was observed as compared to control. No phytotoxicity was recorded with methanol and dichloromethane extracts.
11. Different culture filtrate and mycelia extracts inhibited the seed germination of *P. hystrophorus*. Methanol extract showed the maximum germination inhibition followed by ethyl acetate and dichloromethane extracts (IC_{50} 180-600 ppm).
12. A number of secondary metabolites were isolated from cultural filtrate extract, and dried mycelial extract of *F. oxysporum* using column chromatography over silica gel and identified by spectroscopic techniques such as NMR, IR and Mass Spectroscopy.
13. Secondary metabolites such as dibutyl phthalate, bis(2-ethylhexyl)phthalate, sporol, sporotrichiol and sambucoid were identified from dichloromethane extract of culture filtrate. Metabolites such as 8-hydroxytrichothecene, JM-47, nivalenol, fusarenon X and 5-butylpicolinic acid were identified from ethyl acetate extract of culture filtrate.

14. A number of hydrocarbons namely nonadecane, 1-tetradecanol, methyl hexadecanoate,, 2- methyl pentadecane, and 4-hydroxy-4-methyl-2-pentane were identified by GC-MS of the hexane extract of mycelia along with decane, didecane, tetradecane and nonadecane
15. Metabolites such as cyclobutane and carbonitrile, sporol, 6-butyl- α -pyrone, sambucoid, fungerin and moniliformin were identified from dichloromethane of mycelia.
16. Metabolites such as zearalenone, resorcylic acid lactone, T-2 toxin, diacetoxyscirpenol, visoltricin, 8-hydroxy-isotrichodermol, 8-hydroxy sambucoid, ergosterol, digitoxin, 2-dimethyl-5-amino-6-(2'-ene-4'-hydroxybutyl)-4-chromone, 8-hydroxysambucoid, enniatin, 2,2-dimethyl-5-amino-6-(4'-hydroxybutyl)-4-chromene and a small quantity of IAA have been identified from methanol extract of mycelia.
17. Five isolated metabolites from dichloromethane extract of mycelia namely sporol, sporotrichiol, 6-butyl- α -pyrone; fungerin and moniliformin from dichloromethane extract of mycelia were tested for fungicidal activity. Maximum activity was found with moniliformin against *P. aphanidermatum* (ED₅₀ 29.25,ppm), and *P. debaryanum* (ED₅₀ 78.00 ppm) followed by 6-Butyl- α -pyrone (ED₅₀ 37.2 and 34.9 ppm, respectively) and sporotrichiol (ED₅₀ 66.54 ppm against *P. aphanidermatum*)
18. Five secondary metabolites isolated from methanol extract of mycelia namely visolstricin, 8-hydroxyisotrichodermin, T-2 toxin, zerealenone and

diacetoxyscirpenol were found active against *P. aphanidermatum* and *P. debaryanum*. Four metabolites were somewhat less active against *R. bataticola* (ED₅₀ 111-290 ppm), but visolstricin, was found active with ED₅₀ 70.49 ppm.

19. JM-47, nivalenol, sambucoid, 8-hydroxyisotrichoderminol metabolites isolated from culture filtrate were found active against *P. aphanidermatum* and *P. debaryanum* (ED₅₀ 62-112 ppm)
20. Plant growth regulatory activity of four isolated compounds namely fusaric acid, indole acetic acid, isotrichodermin and nivalenol was evaluated against wheat seed germination. It has been observed that fusaric acid and indole acetic acid showed plant growth regulating activity at lower concentrations, whereas isotrichodermin and nivalenol did not show growth promoting activity.
21. Metabolites such as fusaric acid, 8-hydroxyisotrichodermin, moniliformin and sambucoid were tested for nematicidal activity against *M. incognita* and *R. reniformis*. Sambucoid was found most active with ED₅₀ 15.25 and 15.29 ppm after 24 h against *R. reniformis* and *M. incognita*.
22. Compatibility of *F. oxysporum* and some of secondary metabolites was studied with commercial fungicides such as captan, tridemorph, metalaxyl, thiophanate-methyl, mancozeb, sulphur dust and borax. They were found compatible with captan, tridemorph, metalaxyl and thiophanate-methyl, but sensitive to mancozeb, sulphur dust and borax.

23. A GLC method was standardized for estimation of secondary metabolites of *F.oxysporum* by derivatisation with diazomethane / silylchloride.
24. Dried mycelia based solid (dust) and pellet formulations were prepared and their viability and stability at room temperature for one year were studied.
25. The biocontrol capability of the above formulations against *P. aphanidermatum* and *P. debaryanum* was evaluated in pot culture studies. Plant stand was determined after one and three weeks of growth. The formulation significantly prevented tomato damping off. It allowed a stand comparable to that in the non-infested control.
26. Alginate based formulation of *F. oxysporum* mycelia was evaluated for its nematicidal activity by direct mixing/ spraying one week before sowing seeds of mung (*Vigna radiata*) cv. Pusa 9631. After 3 days of germination, 500 J2 of both the tested nematodes/ plant were inoculated around the growing seedling. Shoot length and plant biomass weight increase was more in plants sprayed with alginate-based mycelia formulation and number of galls formed was significantly less in the treated plots than in control
27. A method was standardized for synthesis of fusaric acid one of the major and active metabolite. A series of methyl-5-alkyl-butanoate and ethyl-5-alkyl butanoate were prepared and evaluated for fungicidal activity against *P. aphanidermatum* followed by *P debaryanum*, *R. bataticola* and *S. rolfsii*. Lower analogs were found to be more active. The fungicidal activity of

different analogues was in the following order. $\text{CH}_3 < \text{C}_2\text{H}_5 < \text{I-Pr} < \text{C}_4\text{H}_9 <$

$\text{C}_5\text{H}_{11} < \text{C}_6\text{H}_{13} > \text{C}_7\text{H}_{15} = \text{C}_8\text{H}_{17} = \text{C}_9\text{H}_{19} > \text{C}_{10}\text{H}_{21} > \text{C}_{11}\text{H}_{23}$

28. Nine different derivatives of palmitic acid were synthesized by esterification with different alcohols in presence of Conc H_2SO_4 . Fungicidal activity of these compounds were in the following order $\text{CH}_3 < \text{C}_2\text{H}_5 < \text{I-Pr} < \text{Pr} < \text{C}_4\text{H}_9 > \text{I-Bu} > \text{C}_5\text{H}_{11} > \text{C}_6\text{H}_{13} > \text{cyclohexyl} > 3\text{-methyl butyryl}$.

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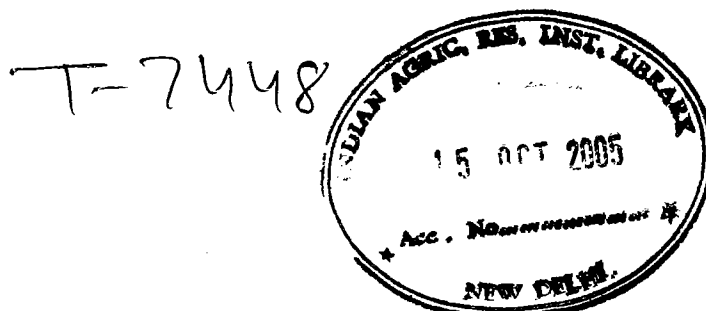
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