Toxigenicity of *Fusarium* species causing wilt of chickpea

S. Gopalakrishnan

Department of Biology
Darwin Building
University College London
Gower Street
London
WC1E 6BT

Presented for the degree of Doctor of Philosophy, University of London

ABSTRACT

The toxigenicity of isolates of *Fusarium* for chickpea, *Cicer arietinum*, the third most important legume crop in the world was studied. Fungi were grown in liquid culture and the culture filtrates assayed on cells isolated from leaflets of the plant. One isolate, designated FOC 5, produced cultures that were predominately red (70-80% of the cultures). When the culture filtrates of all isolates over time were assayed, the red cultures of FOC 5 were much more toxic than those of the other isolates and were also about 10 times more toxic than the colourless cultures of FOC 5. Toxic titres of the red FOC 5 cultures peaked at 12 days when grown at 20°C. The toxin from these red cultures were purified by solvent partitioning, solid phase extraction (SPE), thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) using the assay to monitor the stages in purification.

Shaking of culture filtrates of FOC 5 with ethyl acetate resulted in about half the toxic activity (50-55%) partitioning into the organic phase and 25-30% remaining in the aqueous phase. The activity of the aqueous phase was lost on freeze-drying suggesting a volatile compound. When the ethyl acetate phase was dissolved in aqueous acetonitrile and applied to C_{18} SPE cartridges, about 9% was not adsorbed and 35% could be eluted with methanol. Greater affinity was shown for cyano SPE cartridges with 6% not adsorbed and 45 % recoverable by elution in acetonitrile. Attempts at purification of the toxin(s) of adsorbed and non-adsorbed fractions from these reversed phase cartridges by HPLC did not yield pure products.

Recovery of activity of the ethyl acetate phase from flash chromatography on silica gel was 61-110%. However, HPLC demonstrated that several compounds were present in the active fractions

Separation of components of the ethyl acetate phase or the fraction adsorbed by cyano cartridges of culture filtrates by TLC on silica gel rather than using SPE, flash or reversed phase HPLC was more successful. Red bands corresponding to the active compound were scraped from TLC plates and eluted in acetonitrile. HPLC of the eluents on a cyano column with 10% acetonitrile as the mobile phase demonstrated a single homogeneous peak with absorption maxima of 224 and 281 nm. The purified fraction is, at the time of writing, being studied by Professor Mike Beale at Rothamsted Research using nuclear magnetic resonance techniques in order to determine its structure.

Four other isolates, identified by the International Crops Research Institute for the Semi-Arid Tropics as *F. oxysporum* f. sp. *ciceri* did not produce the red, toxic compound, throwing doubt on the correct identification of the isolates. When the sequences of ribosomal DNA of all five isolates were determined, the isolate that produced the red toxic compound most closely matched *Fusarium acutatum* (99%), in a BLAST search and this accorded with its morphology. A BLAST search showed that three of the other isolates matched the sequence of cotton pathogen, *F. oxysporum* f. sp. *vasinfectum* (100%, 100% and 97%) and one closely matched *F. oxysporum* f. sp. *vanillae* (99%) These results suggest that a re-evaluation of the taxonomy of *Fusarium* species causing wilt of chickpea is required.

ACKNOWLEDGEMENTS

I would like to thank Dr Richard Strange for his advice and guidance during the course of this research and also for his constructive suggestions, criticisms and help in preparation of the thesis. I would also thank Dr Richard Strange for helping to pay my tuition fees.

I am very grateful to the European Union Project (contract number ICA4-CT-2000-30003), with out whose funding this Ph.D. would not have been possible. I would also like to thank the Department of Biology, the Graduate School of University College London and various charities, including The South Down Trust, The Family Welfare Association, The Leche Trust and The Gilchrist Educational Trust for partially defraying the cost of my tuition fees.

I would also like to thank Dr Astrid Wingler for her guidance and help in the course of research from time to time. Many thanks are also due to Professor Mike Beale and Dr Jane Ward of Rothamstead for their help in efforts to obtain the structure of the toxin by Nuclear Magnetic Spectroscopy. I should also like to thank all my past and present friends in the Plant Pathology Laboratory at UCL, particularly Estelle Gewiss for helping me with several techniques.

I am also very grateful to my father and my late mother for their love and support. Finally I am very much indebted to my beloved wife Srilatha for her continuous patience and support and without which this thesis would not have been possible.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

Introduction

CHAPTER II

Toxigenicity of five isolates of *Fusarium oxysporum* f. sp. *ciceri*

CHAPTER III

Scale up and liquid/liquid extraction of toxin(s) from culture filtrates of FOC

CHAPTER IV

Toxin purification by solid-phase extraction and high performance liquid chromatography

CHAPTER V

Purification of FOC toxin by flash chromatography and HPLC

CHAPTER VI

Purification of the toxin by thin layer chromatography and characteristics of the purified toxin

CHAPTER VII

Genotypic comparison of the five isolates of *Fusarium oxysporum* f. sp. *ciceri*

CHAPTER VIII

Overall discussion

ABBREVIATIONS

ACN	Acetonitrile
CDCLM	Czapek Dox Cation Liquid Medium
CDLM	Czapek Dox Liquid Medium
CN	Cyano
DAS	Diacetoxyscirpenol
DCM	Dichloromethane
DNA	Deoxyribonucleic Acid
DON	Deoxynivalenol
EC	End Capped
ELISA	Enzyme Linked Immuno-Sorbent Assay
EtOAc	Ethyl acetate
FAO	Food and Agriculture Organisation
FAOSTAT	Food and Agriculture Organisation Statistics
FDA	Fluorescein diacetate
FOC	*Fusarium oxysporum* f. sp. *ciceri*
GC	Gas Chromatography
HB	Holding Buffer
HPLC	High Performance Liquid Chromatography
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
IGS	Inter-Genic Transcribed Spacer
ITS	Internal Transcribed Spacer
LSU	Large Sub-Unit

MeOH	Methanol
MS	Mass Spectrometry
NAF	Non-Adsorbed Fraction
NMR	Nuclear Magnetic Resonance
PCA	Phenazine-1-carboxamide
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
Phe	Phenyl
PO	*Penicillium oxalicum*
Rf	Relative to front
RNA	Ribonucleic Acid
SM	Starting Material
SPE	Solid Phase Extraction
SSU	Smaller Sub-Unit
TAE	Tris-acetate-EDTA
TEA	Triethylamine
TLC	Thin Layer Chromatography
UV	Ultra Violet
WANA	West Asia and North Africa
ZEN	Zearalenone

CHAPTER I

Introduction

Pulse crops, also called grain legumes, are grown for food as well as fodder and play crucial roles in world agriculture because of several important features. These include the ability to fix atmospheric nitrogen in symbiotic association with root nodule bacteria (*Rhizobium* spp.), their deep root systems that loosen the soil and allow efficient use of water and their key role in human diet in many countries as a source of protein. In particular, chickpea is an important part of the diet of people living on the Indian Subcontinent.

1.1 The chickpea, *Cicer arietinum*

Chickpea (*Cicer arietinum* L), also known as Gram or Bengal gram, is the third most important pulse crop after bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*) on a world basis but of first importance in the Mediterranean basin and South Asia, with India accounting for between 60 and 75% of the world's chickpea production (Barve *et al*., 2001; Singh and Ocampo, 1997; Tekeoglu *et al*., 2000; Table 1.1). It is known by a variety of names according to the local language including pois chiche (French), homos (Arabic), chickpea (English), grao-de-bico (Portugese), garbanzo (Spanish), chana (Hindi, Punjabi, and Urdu) and kondai kadalai (Tamil).

Country	Area harvested ('000 ha)	Production ('000 Mt)	Productivity (Kg ha^{-1})
Bangladesh	16.6	12	723.2
India	5200	3870	744.2
Nepal	14.6	12.2	832.6
Pakistan	901	397.1	440.7
World	8762.4	6445.7	735.6

Table 1.1 Chickpea area, production and productivity (FAOSTAT, 2001)

Chickpea is grown in 33 countries on an area that has been estimated at between 6.4 and 10.8 million hectares (FAO, 1988; FAOSTAT, 2001; Jodha and Rao, 1987). In the European Union, chickpea production is concentrated in the Mediterranean Basin, with Spain being the principal producer (Cachinero *et al.*, 2002). Chickpea is also gaining importance as a pulse crop in South-Western Australia, particularly on the neutral-to- alkaline fine-textured soils of the drier, eastern side of the agricultural region (Behboudian *et al*., 2001) and it is the most widely produced pulse crop in Turkey (Kanter, 2003).

The origin of chickpea is thought to have been in the area of present-day South Eastern Turkey and neighbouring Northern Syria (Van der Maesen, 1972). It has since spread to many other geographical regions of the world because of its ability to grow in diverse environments.

There are two main commercial types of chickpea, the “Desi” type with smaller and darker coloured seeds which may vary from yellow to black and the “Kabuli” type with large, smooth and light coloured seeds (Cubero, 1987; Singh, 1985). "Desi" types usually have anthocyanin pigmentation and predominate in the Indian subcontinent, East Asia, Iran and Afghanistan and account for 85-90% of world production (Van der Maesen, 1987). "Kabuli" types have white flowers, which are devoid of anthocyanin pigmentation and stems and seeds also lack anthocyanins. They are grown in the Mediterranean area, and Central and South America and account for 10 to 15% of the world's chickpea production. The Kabuli type of chickpea is important in Turkey as it is grown on 625,000 ha land in dry areas and the annual production is about 560,000 tonnes (Kantar, 2003).

Chickpea seed is mainly used as food because of its high protein (12.4 -31.5 %) and carbohydrate (52.4-70.9 %) content (Awasthi *et al*., 1991; Dhawan *et al*., 1991; Dutta *et al*.,

1985; Singh, 1985) and for the people of the Indian subcontinent, West Asia and North Africa (WANA), the high protein content is of particular value (Saxena, 1989). Chickpea is eaten raw, boiled or as dhal. This last consists of the cotyledons separated from their seed coat that are boiled in water and to which spices are added. Young leaves of the plant are also used as green vegetables. When eaten with cereals, chickpea gives a balanced diet. It is also one of the major constituents of various sweets. The dry stalks and husks, containing small broken pieces of grain obtained during milling, are fed to animals (Malik and Tufail, 1981). Medicinal properties have been claimed in glandular hairs of the plant which contain 94% malic acid and 6% oxalic acid and they are also used as a vinegar (Alam, 1989). It has been claimed that chickpea proteins are helpful in reducing cholesterol levels in blood serum owing to their beneficial effect on lipid metabolism (Zulet and Martinez, 1985).

1.2 Constraints to chickpea production

Productivity may be considerably improved if the adverse effects of abiotic (climate and soil) and biotic (living organisms) stresses are reduced. The major climatic stresses are cold, heat and drought. Of these, drought is the major limiting factor, particularly as chickpea is usually grown as a post-rainy season crop. Drought stress can be managed with irrigation, but this is not available to many chickpea growers. Advancing sowing dates in certain regions can alleviate the effect of water stress and increase seed yield but the best results are achieved through growing drought-tolerant cultivars that are generally early maturing. With regard to soils, chickpea is particularly sensitive to salinity and this is an important problem in India and Pakistan. Scientists in both nations and at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in India have identified salt tolerant

genotypes but these lines do not generally yield well. Lowering the water table through improved drainage can be effective in areas where it is high, otherwise areas of high salinity should be avoided for cultivating chickpea (ICRISAT, 1997).

The crop is vulnerable to a number of airborne and soil-borne diseases, some of which may be devastating. Chickpea suffers from about 172 pathogens consisting of fungi, bacteria, viruses, and nematodes. Thirty-eight of these pathogens, belonging to 19 genera of fungi, are soil-borne. *Rhizoctonia solani* Taub, *Sclerotium rolfsi* Sacc. *Fusarium oxysporum* f. sp. *ciceri* Padwick Snyd. and Hans are the most serious and they are responsible for wet root rot, collar rot and mature plant wilt, respectively. Together they may cause losses as high as 60-70% when conditions favour disease (Nene, 1985). There are also some minor soil-borne diseases such as black root rot, dry root rot and root and seed rot caused by *Fusarium solani*, *Rhizoctonia bataticola* and *Pythium ultimum,* respectively. The foliar diseases, which may seriously damage chickpea under favourable conditions, are blight caused by *Ascochyta rabiei* (Pass.) Labr. and grey mould caused by *Botrytis cinerea* Pers. Ex Fr. Ascochyta blight and Fusarium wilt are considered to be the two most devastating diseases of chickpea (Hamid *et al*., 2001).

Bacterial blight caused by *Xanthomonas cassiae* was also found to be damaging to chickpea in India (Nene, 1980; Rangaswamy and Prasad, 1960). Important viral diseases include stunt caused by Pea Leaf Roll Virus, chlorosis and dwarfing caused by Chickpea Chlorotic Dwarf Virus, mosaic caused by Alfalfa Mosaic Virus, proliferation caused by Cucumber Mosaic Virus, narrow leaf caused by Bean Mosaic Virus and necrosis caused by Lettuce Necrotic Yellow Virus. Other viral diseases such as Pea Enation Mosaic Virus and

Pea Streak Virus have also been reported in the USA but their importance is not known (Horn and Reddy, 1996; Nene and Reddy, 1987).

Among the nematodes infecting chickpea, *Meloidogyne* spp., *Heterodera* spp. and *Pratylenchulus* spp, cause heavy losses of the crop in several countries. *M. incognita* and *M. javanica* are of economic importance in India and *M. artiellia* is important in the Mediterranean region (Ansari *et al*., 2002; Di-Vito *et al*.,1996; Greco, 1987).

1.3 Fusarium wilt of chickpea

Fusarium oxysporum is the causal agent of wilt of many plant species and is well represented among rhizosphere microflora. All strains may exist saprophytically and some are considered to be non-pathogenic but many are well known for inducing wilt or root rots on a variety of plants (Fravel *et al*., 2003). Often isolates are specific to particular hosts and this is generally recognised by the addition of a *forma specialis* name. For example, *F. oxysporum* f. sp. *lycopersici* infects tomato and *F. oxysporum* f. sp. *cubense* infects banana. In some formae speciales, such as *F. oxysporum* f. sp. *lycopersici* there are races of the pathogen which are specific to certain cultivars of hosts in a gene-for-gene specific manner (Armstrong and Armstrong, 1981; Fravel *et al*., 2003).

Wilt of chickpea is normally considered to be caused by *F. oxysporum* f. sp. *ciceri* (Padwick) Snyd. and Hans, hereafter designated as FOC. The disease is important between the latitudes of 30°N to 30°S of the equator, where the chickpea-growing season is dry and warm, and has been reported from 23 countries (Nene *et al*., 1989). Although the fungus is primarily pathogenic to chickpea, it can also invade several other crops including lentil, pea,

pigeonpea, alfalfa and broad bean but without causing overt symptoms (Haware and Nene, 1982a; Trapero-Casas and Jimenez-Diaz, 1985b).

Losses of chickpea from Fusarium wilt have been reported to vary from 10 to15% (Jalali and Chand, 1991; Trapero-Casas and Jimenez-Diaz, 1985b) but losses of up to 70% have been reported in some years in Northern India and Pakistan (Grewal and Pal, 1970). In Tunisia the disease can completely destroy the crop under specific conditions (Halila *et al*., 1984; Halila and Harrabi, 1990). As a facultative saprophyte, FOC can survive in soil and on crop residues as chlamydospores for up to six years (ICRISAT, 1985). The pathogen is also seed-borne and may therefore be spread by means of infected seed (Haware *et al*., 1978). FOC is considered to be a major threat to chickpea production in India, Iran, Pakistan, Nepal, Burma, Spain and Tunisia (Jalali and Chand, 1991).

1.4 The fungus, *Fusarium oxysporum* f. sp. *ciceri*

Fusarium taxonomy has been based on morphological characteristics of the anamorph, including the size and shape of macroconidia, the presence or absence of microconidia and chlamydospores, colony colour and conidiophore structure (Windels, 1992). When grown in culture, *F. oxysporum* initially produces colourless to pale yellow mycelium that turns pink or purple with age and it has no known sexual stage (Di Pietro *et al*., 2003). Van der Maesen (1972) described *Fusarium oxysporum* as having a whitish mycelium with a red-pigmented plectenchymatous layer in some isolates, ovoid microconidia and spindle-shaped, septate macroconidia. Sometimes solitary one- or two-celled chlamydospores occur either in terminal or inter-calary positions.

FOC was classified in the dictionary of fungi (www.indexfungorum.org/Names/namesrecord.asp?RecordID=416240) as follows; Genus: Fusarium; Family: Nectriaceae; Order: Hypocreales; Class: Sordariomycetidae; Subclass: Ascomycetes; Phylum: Ascomycota; Kingdom: Fungi. Nelson (1981) described macroconidia of FOC as most often found on branched conidiophores but may also be produced singly on aerial mycelium in culture. They are thin-walled and 2-5 septate while microconidia are kidney shaped and occur on short microconidiophores. Chlamydospores are thick-walled and are produced in hyphae or conidia and are formed both in culture and in dead host plant tissue in the final stages of wilt.

Fusarium oxysporum includes non-pathogenic, plant pathogenic and human pathogenic strains. Plant pathogenic strains are very host specific attacking only one or a few species of plants. *Fusarium oxysporum* causes wilting in many plant species and in order to differentiate them Snyder and Hansen (1945), Snyder and Tousson (1965) and Tousson and Nelson (1968, 1975 and 1976) classified them as formae speciales (f. sp.). The f. sp. name was often taken from the name of the species of plant infected. Initially it was believed that formae speciales were specific to one host and hence the name was taken from the host (see Table 1.1 for a small selection of formae speciales and their hosts; Nelson, 1981).

Formae speciales (f. sp) of *Fusarium oxysporum*	**Crops**
f. sp. *ciceri*	Chickpea
f. sp. *lycopersici*	Tomato
f. sp. *chrysanthemi*	Chrysanthemum
f. sp. *cubense*	Banana
f. sp. *dianthi*	Carnation
f. sp. *perniciosum*	Mimosa, a woody tree
f. sp. *batatas*	Sweet potato
f. sp. *coffeae*	Coffee
f. sp. *melonis*	Muskmelon
f. sp. *lini*	Flax
f. sp. *nicotianae*	Tobacco

Table 1.2 A small selection of the 120 formae speciales of *F. oxysporum* that have been identified

1.5 Races of *Fusarium oxysporum* f. sp. *ciceri*

Seven races of the pathogen (FOC) designated 0–6 have been reported worldwide on the basis of the differential disease reaction of ten chickpea lines to isolates of the pathogen (Haware *et al.,* 1990). FOC races 1, 2, 3 and 4 have been reported to be present in India (Haware and Nene, 1982). These races are geographically distinct. Race 1 is widespread in Central and Peninsular India and race 2 in Northern India. Both appear to be more virulent than races 3 and 4, which are found in Punjab and Haryana states (Barve *et al*., 2001; Haware *et al*., 1992). Races 0 and 5 are found in Spain (Jimenez-Diaz *et al*., 1989) and race 6 in California, USA (Phillips, 1988). Race 0 has also been reported in California (USA), Israel, Lebanon, Syria, Tunisia and Turkey and race 6 has been reported in Israel, Morocco and Spain while race 5, the most virulent of the three races occurring in Spain, also occurs in California (Cachinero *et al*., 2002; Halila and Strange, 1996).

1.6 Symptoms of the disease

Symptoms consist of epinasty and chlorosis of leaves, discoloration of vascular tissue and ultimately collapse of the whole plant (Figs. 1.1, 1.2, 1.3 and 1.4; Hamid *et al*., 2001; Haware, 1990). The disease may be diagnosed by sudden drooping of leaves and petioles, which may turn yellow, and browning of the xylem and its colonisation by fungal hyphae, which are apparent when the stem is cut (ICRISAT, 1995; Nene *et al*., 1978; Van der Maesen, 1972).

Westerlund *et al.* (1974), in experiments under controlled conditions, observed that the first symptom was yellowing of lower leaves of young plants and this chlorosis progressed uniformly upwards. He also observed that yellowing occurred on only one side of the plant i.e. only some of the main branches showed symptoms when older chickpea plants were inoculated. This has been termed "partial wilting" (Nene *et al*., 1978).

A) Fig. 1.1 Transverse sections of chickpea stems infected with *Fusarium oxysporum* f. sp. *ciceri* showing discoloration of internal tissues (courtesy: International Crops Research Institute for the Semi Arid Tropics; ICRISAT)

Fig. 1.2 A longitudinal section of a stem from a plant infected with *Fusarium oxysporum* f. sp. *ciceri* showing blackening of xylem tissue (courtesy: ICRISAT)

Fig. 1.3 Chickpea plants devastated by wilt caused by *Fusarium oxysporum* f. sp. *ciceri* (courtesy: ICRISAT)

Fig. 1.4 A chickpea plant killed by *Fusarium oxysporum* f. sp. *ciceri* (courtesy: Dr R N Strange)

1.7 Chemical and cultural control

The chickpea crop has a long life span - 120-150 days in cold weather and 120 to as little as 80 days in the warmer winter environments - and several pathogens are involved in taking a heavy toll of the crop at different stages. It has not been possible to manage all these disease by use of chemicals or by resistant varieties. FOC may be eliminated from the seed using the fungicide Benlate T (30% Benomyl + 30% Thiram) at 0.25% (Haware *et al*., 1978; Haware *et al*., 1986a; Mandeel, 1996). Use of other protectant or systemic fungicides as seed dressings improved seedling emergence in moderately susceptible chickpea cultivars and delayed development of symptoms but did not provide satisfactory control (Jimenez-Diaz and Trapero-Casas, 1985).

FOC can survive in the soil for more than 6 years (ICRISAT, 1985) and also in symptomless carriers (Haware and Nene, 1982a; Trapero-Casas and Jimenez-Diaz, 1985). Therefore it is not possible to control the disease by normal crop rotation. Soil solarization reduced the pathogen population and incidence of wilt with the result that plant growth and yield were improved (Chauhan *et al*., 1988). However, cost considerations would limit the use of this technique for the control of wilt in the commercial farming of chickpea. Sterilisation of the soil by methyl bromide is also not an option as it is both costly and environmentally damaging (Fravel *et al.,* 2003). Date of sowing seems to have an effect on the incidence of wilt. In India, it has been reported that delayed sowing usually lowers fungal attack but also yield (Singh and Singh, 1984), while in contrast in Spain, incidence and severity of wilt disease was reduced significantly in moderately susceptible cultivars, but not in susceptible ones, when planting was advanced from spring to winter (Trapero-Casas and Jimenez-Diaz, 1985).

1.8 Biological control

With the increasing concern over environmental pollution by pesticides, major efforts are being made to develop environmentally friendly methods of plant disease control. Biological control is one possible solution to the problem of managing FOC. This is the use of predator, pathogen, antagonist or competitor populations of a third organism to suppress a pest population, making it less abundant and thus less damaging than it would be otherwise. Apart from a broad spectrum of activity and long-term protection, biological control has several advantages over chemical control. These include a) reduced health hazard and reduced pollution, b) little chance of the pathogen developing resistance to the microbial agent, c) no phytotoxicity, in fact may have plant growth promoting effects, d) compatibility with most agricultural practices, e) restoration of the ecological balance and f) favourable economics. Biological control of plant diseases usually occurs by one or more of several distinct mechanisms. These include competition for nutrients, parasitism, the production of antibiotics and induced systemic resistance (Van Driesche and Bellows, 1996; van Loon *et al.* 1998).

Microorganisms recognised as suppressing fungal diseases include species and strains of *Agrobacterium, Bacillus* and *Pseudomonas*. The first commercial biological control agent was probably strain K84 of *Agrobacterium* that has been used successfully to control crown gall disease caused by *Agrobacterium tumefaciens* in many countries of the world (Penyalver *et al*., 2000). This strain harbours a plasmid, pAGK84, which encodes the production of a bacteriocin and immunity to it. In order to avoid the fear of the plasmid being transferred to pathogenic strains of *Agrobacterium tumefaciens*, a strain K 1026 has been genetically

engineered to remove the transfer region of the plasmid (Penyalver *et al*., 2000; Strange, 2003).

Loeffler *et al*. (1986) found that *Bacillus subtilis* gave good control of *Rhizoctonia solani* in many crops. The bacterium produces bacilysin and fengymycin. Bacilysin inhibits yeasts and bacteria while fengymycin inhibits filamentous fungi (Strange, 2003). Strains of *Pseudomonas* produce several antibiotics. These include phenazine-1-carboxamide (PCA), phenazine-1-carboxylic acid, anthranilic acid, diacetyl-phloroglucinol, pyoluteorin, pyrrolnitrin and viscosinamide. Thomashow and Weller (1990) showed the importance of PCA, produced by strains of 2-79 of *Pseudomonas fluorescens*, which control damping-off disease of pea seedlings.

Fungi may also give biological control. Lifschitz and co-workers (1986) found that *Trichoderma harzianum* and *T. koningii* both controlled damping-off of pea by *Pythium* when they were used to coat the seed. Woo *et al*. (2002) used mixtures of organisms, *Trichoderma atroviride* (strain P1) in combination with *Pseudomonas syringae* pv. *syringae* (strain B359), in the postharvest control of infection of apple fruit by *Botrytis cinerea*. *Gliocladium virens* is an antagonist of a number of important soil-borne plant pathogens, which include *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Pythium ultimum* (Strange, 1993).

De Cal *et al.* (1999) reported that watering tomato seedlings in seedbeds 7 days before transplanting with conidial suspension of *Penicillium oxalicum* (PO) Thom. controlled wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici*. De Cal and Melgarejo (2001) also reported that repeated application of PO prolonged the duration of control of Fusarium wilt especially when disease pressure was high. Saprophytic Fusarium fungi are

able to suppress populations of pathogenic *Fusarium* sp. by competing for nutrients in the soil, affecting the rate of chlamydospore germination of the pathogen, competing for infection sites on the root and inducing systemic resistance (Fravel *et al*., 2003). Harvas *et al*. (1995) showed that prior inoculation of germinated chickpea seeds with either incompatible FOC races or isolates of non host *F. oxysporum* isolates can suppress Fusarium wilt caused by the highly virulent race 5 of FOC. Larkin and Fravel (2002) demonstrated a non-pathogenic *Fusarium oxysporum* isolate, CS-20, and non a pathogenic *Fusarium solani* isolate, CS-1, effectively reduced Fusarium wilt of tomato under a variety of environmental conditions and suggested that they have potential for further development. Freeman *et al*. (2002) generated nonpathogenic mutants from wild-type isolates of *Fusarium oxysporum* f. sp. *melonis*, the causal organism for the Fusarium wilt of muskmelon by continuous dip inoculation and UV mutagenesis techniques. He also showed that disease incidence was reduced significantly upon inoculating these nonpathogenic isolates along with pathogenic isolates on the crop.

Although biological control often appears promising in specialised environments, disappointing results frequently are obtained in the field as several factors determine the survival and delivery of the antagonist. These remain the greatest limitation in practical application. For example, Harman *et al.* (1981) found that *Trichoderma* spp., applied as a seed treatment, were ineffective in controlling damping-off pathogens if the soil temperature was below 18°C.

Therefore, the strategy to combat the disease should be to integrate different methods of control including disease resistant cultivars, biological control, fungicides and cultural practices. For instance, Singh *et al*., (2003) showed that two strains of *Pseudomonas*

fluorescens in combination with thiram @ 1.5 g kg^{-1} effectively controlled collar rot of chickpea caused by *Sclerotium rolfsi* in both glass house and field experiments.

1.9 Breeding for resistance

Disease resistance is another way to control plant disease if satisfactory levels of long-lasting resistance can be incorporated into culturally desirable crop plants. Resistant varieties save time, effort, and money otherwise spent fighting plant diseases. Furthermore, adequate resistance allows the plant to remain healthy and yield well in the presence of the pathogen. Moreover, resistant cultivars obviate the need for costly pesticides, not only saving money but also protecting the crop and the environment from contamination with pesticide residues.

Resistance is the inherent ability of plants to remain relatively unaffected by disease. Moderately resistant (i.e. slightly or moderately susceptible) plants have recognisable amounts of disease but better yields than susceptible plants. Tolerance is the ability of plants to become diseased and possibly show it but not have yields appreciably or proportionately affected while immunity is absolute freedom from infection or disease, the plants remaining disease free under all circumstances. This is the ideal and ultimate control, but unfortunately it is extremely rare within a given plant species (Maloy, 1993).

Maintenance of high levels of resistance to disease is normally achieved by selection and hybridisation (Maloy, 1993). Selection involves exposing plant populations to high disease pressure and selecting individuals that survive. These plants are further tested, often by artificial inoculation, to ensure they have not simply escaped infection. Those plants that are resistant are used as parents in hybridisation in order to improve disease resistance.

In breeding for resistance, hybridisation involves combining disease resistance with desirable agronomic or horticultural features. Plants with good horticultural properties but poor disease resistance are crossed with plants that are less desirable horticulturally but have good disease resistance. From the progeny plants with both good agronomic or horticultural features and good disease resistance are selected.

Resistance can be also developed by mutagenesis using chemicals such as methyl or ethyl-methanesulphonate, diethyl sulphate or ionising radiation such as X or gamma rays. These agents have been used to obtain growth characteristics such as reduced plant size, altered or improved flower and fruit colours, larger and more uniform fruit size and earlier fruiting and ripening. However, most resistant mutants have been generated by gamma radiation. After selection under controlled conditions, mutants are screened in the field. Generation of resistance by gamma radiation has been reported for several diseases including Ascochyta blight, wilt, stunt and root rot of chickpea; potato late blight, leaf roll and wart; sugarcane red rot and smut; grape downy mildew; citrus canker; apple powdery mildew and scab but none of these has yet reached commercial application (Maloy, 1993).

Although varieties of plants that are resistant to some fusarial diseases are known, e.g., all the varieties of tomato grown in glasshouses for fresh fruit production are resistant to common races of *F. oxysporum* f. sp. *lycopersici* (Fravel *et al*., 2003), there are several plants for which for no dominant gene for disease resistance to *Fusarium* is known e.g. carnation, cyclamen, flax. Despite the presence of races of the fungus, chickpea in relation to FOC appears to fall into this category.

Several workers have observed different patterns in the development of wilting symptoms when chickpea is infected with *Fusarium oxysporum* f. sp. *ciceri*. Halila (1994) tested 1915 varieties of chickpea for wilting reactions and suggested the following pattern.

1. Highly susceptible varieties. Those varieties that had 100% wilt incidence at 28 days after emergence of seedlings.
2. Very early wilters. Susceptible varieties that started wilting at 28 days and were completely wilted by 42 days after the emergence of seedlings.
3. Early wilters. Susceptible varieties that started wilting at 42 days and were completely wilted by 56 days after the emergence of seedlings.
4. Late wilters. Susceptible varieties that started wilting at 56 days and were completely wilted by 70 days after the emergence of seedlings.
5. Very late wilters. Susceptible varieties that started to wilt 84 days after the emergence of seedlings or later.

This differential wilting pattern, particularly the late wilters and very late wilters, could well be used in breeding programme to develop varieties of chickpea with improved wilt resistance.

1.10 Toxins and their role in the disease development

Toxins are products of microorganisms that cause obvious damage to plant tissues and are known with confidence to be involved in plant disease (Scheffer, 1983). Such damage may include wilting, water soaking, chlorosis and necrosis (Strange, 2003). Toxins have been described in a number of well-documented reports as integral factors in disease development (Scheffer, 1983 and Yoder, 1980). They have proved to be useful tools in the

selection of resistant/tolerant plants (Alam, 1989; Daub, 1986; Earle, 1978) since insensitivity to toxins can, theoretically at least, be used as the basis for selecting germplasm with resistance to the pathogen (Gengenbach *et al*., 1977).

Plant pathogens produce a variety of secondary compounds in culture that show phytotoxic activity but only a small proportion of these have a demonstrated role in plant disease. Toxins may be classified as host-selective (host specific) or non-selective (non-specific). Host-selective toxins are toxic to those plant species or cultivars that serve as hosts for the toxin-producing pathogen and lack toxicity towards non-hosts. A non-selective toxin may exhibit differential toxicity towards various plant species but toxicity is not highly correlated with the toxin-producer's host range (Knoche and Duvick, 1987). Host-selective toxins are found principally in species of *Alternaria* and *Cochliobolus* and non-selective toxins in species of *Fusarium*, *Ascochyta, Leptosphaeria* and also some species of *Pseudomonas* and *Xanthomonas* (Table 1.1 and 1.2; Strange, 2003).

Pathogen	Host/Pathotype	Toxin	Chemical class
Alternaria alternata	Japanese pear	AK-toxin	Epoxy-decatrienoic esters
	Strawberry	AF-toxin	Epoxy-decatrienoic esters
	Tangerine	ACT-toxin	Epoxy-decatrienoic esters
	Apple	AM-toxin	Cyclic tetrapeptide
	Tomato	AAL-toxin	Aminopentol esters
	Rough Lemon	ACR(L)-toxin	Terpenoid
Bipolaris sacchari	Sugarcane	HS-toxin	Glycosylated sesquiterpene
Cochliobolus carbonum	Corn	HC-toxin	Cyclic tetrapeptide
Cochliobolus heterostrosphus	Corn	T-toxin	Linear polyketols
Cochliobolus victoriae	Oats	Victorin	Cyclized chlorinated peptide
Mycosphaerella zeae-maydis	Corn	PM-toxin	Linear polyketols
Periconia circinata	Sorghum	Peritoxin	Peptidyl chlorinated polyketide
Pyrenophora tritici-repentis	Wheat	Ptr ToxA Ptr ToxB	13.2-kDa protein 6.6-kDa protein

Table 1.3 Examples of host-selective toxins (Wolpert *et al*., 2002)

Pathogen	Host	Toxin	Chemical class
Streptomyces scabies	Potato	Thaxtomins	4-nitotryptophan and phenylalanine groups linked in an L, L-configured cyclodipeptide
Pseudomonas syringae pv. *tabaci*	Tobacco	Tabtoxin	Dipeptide of either threonine or serine linked to tabtoxinine-β-lactam
Xanthomonas albilineans	Sugarcane	Albicidin	Low molecular wt. compound with several aromatic rings
Fusarium graminearum	Wheat	Trichothecene	Trichothecenes are derived from farnesyl pyrophosphate, which is cyclized to form trichodiene and trichothecenes
Fusarium moniliforme	Maize	Fumonisins	Aminopentol esters
Ascochyta rabiei	Chickpea	Solanapyrones	Polyketide

Table 1.4 Examples of non-host-selective toxins (Strange, 2003)

1.11 Bioassay

Detection of an unknown toxin can be achieved by a suitable bioassay; preferably the assay should be rapid to perform, simple, sensitive and give quantitative results (Strange, 2003). Shohet and Strange (1989) suggested a bioassay technique in which cells were isolated from leaves of pigeonpea by a combination of enzyme digestion and mechanical agitation followed by incubation with culture filtrates of *Phytophthora drechsleri* f. sp. *cajani*. Rapid cell death occurred as assessed by the vital strain phenosafranine. Csinos and Hendrix (1977) used excised tobacco leaves in a bioassay for toxins produced by *Phytophthora cryptogea* in liquid culture. Phytotoxic compounds from *P. citrophthora* were assayed with tomato (non-host) and lemon (host) seedlings (Breiman and Barash, 1981) whereas tomato cuttings were used to assay toxins produced by *P. cactorum* in culture (Pligh and Rudnicki, 1979).

1.12 Purification of toxins

Microcystins are a group of hepatotoxic, cyclic heptapeptides which are produced by bloom-forming cyanobacteria (blue-green algae) in both fresh water and marine environments. They are responsible for numerous animal fatalities and several cases of human illness. A simplified method of purification of microcystins was developed by Edwards *et al*. (1996). Cyanobacterial scum was extracted in methanol and applied to a reversed phase flash chromatography cartridge. Microcystins were eluted with 70% aqueous methanol and concentrated before checking for their purity by analytical HPLC.

Isolates of *Ascochyta rabiei* produced the toxins, solanapyrones A and C when grown in Czapek Dox liquid medium (CDLM) supplemented with chickpea seed extract (Alam *et*

al., 1989) and also solanapyrone B when grown on CDLM supplemented with metal cations, of which Zn, Ca, Cu, Co and Mn were the most important (Chen and Strange, 1994). These toxins were isolated by solvent partitioning with ethyl acetate and flash chromatography of the organic fraction on silica gel. The compounds were identified in the flash fractions by their characteristic UV spectra and those with similar spectra were combined. Purity of the compounds in the combined fractions was monitored by HPLC on an analytical C_{18} column with aqueous mixtures of methanol, acetonitrile and tetrahydrofuran as mobile phases (Hamid and Strange, 1997).

Eighty percent of fumonisin B (FB) $_1$, and 60% of FB_2, mycotoxins produced by the fungus *Fusarium moniliforme*, were recovered after extraction of cultures of the fungus on maize meal with methanol/water (3:1). The fumonisins were further purified using Amberlite XAD-2, silica gel and reversed phase (RP) C_{18} chromatography. The Amberlite XAD-2 purification step proved to be the most effective clean-up procedure, while subsequent chromatography on silica gel and RP C_{18} effectively separated the individual fumonisins to a purity of over 90% (Cawood *et al.*, 1991). Schaafsma *et al.* (1998) showed that thin layer chromatography (TLC) methods for identifying and quantifying fumonisins, deoxynivalenol (DON; a mycotoxin found in maize and other grains produced by *Fusarium graminearum* and *F. culmorum*) and zearalenone (produced by *F. graminearum* and usually found in stored grain) in grain samples were the cheapest, compared to expensive enzyme-linked immunosorbent assay (ELISA) and HPLC methods and equally reliable.

1.13 Aims of the project

Demonstration of a fundamental role of a toxin in a plant disease offers new means of control: plants can be selected for insensitivity of the toxin or they may be genetically modified with a gene encoding an enzyme for its detoxification. Therefore, the possibility that the wilting symptoms caused by FOC might be attributable to toxin production was investigated. This involved the following procedures.

1. The fungus was grown in liquid culture and the toxin purified from the culture filtrates by solvent partitioning, solid phase extraction, flash chromatography, HPLC and TLC.
2. Stages in purification were monitored by a live/dead assay employing chickpea cells.
3. The toxin was subjected to nuclear magnetic resonance studies in order to attempt the identification of the compound.
4. Toxicity of the available isolates of the fungus was compared.
5. Owing to the results obtained the identity of the isolates became questionable. This was therefore studied by molecular techniques.

CHAPTER II

Toxigenicity of five isolates of *Fusarium oxysporum* f. sp. *ciceri*

2.1 Introduction

A number of strains of *Fusarium oxysporum* cause wilting in a variety of plants. They include *Fusarium oxysporum* f. sp. *lycopersici* infecting tomato, *F. oxysporum* f. sp. *lini* infecting flax, *F. oxysporum* f. sp. *dianthi* infecting carnation, *F. oxysporum* f. sp. *chrysanthemi* infecting chrysanthemum, *F. oxysporum* f. sp. *cubense* infecting banana, *F. oxysporum* f. sp. *perniciosum* infecting mimosa, a woody tree and *F. oxysporum* f. sp. *ciceri* infecting chickpea (Nelson, 1981).

Symptoms of Fusarium wilt of chickpea consist of epinasty of the leaves, chlorosis, discoloration of vascular tissue and ultimately collapse of the whole plant (Chapter 1 and Haware, 1990). Such symptoms are consistent with the production of a toxin(s) by the pathogen, although there seems to be no reports of these symptoms at a distance from the plant or fungus, one of the criteria for the presence of a toxin. However, this may be more a case of lack of observation rather than lack of the phenomenon.

Extraction of a toxin from infected plant material is unlikely to be successful as the toxin may bind to a receptor or be converted to another compound. For example, the solanapyrone toxins have not been isolated from chickpea plants infected by *Ascochyta rabiei* although the fungus produces three toxic compounds in culture, solanapyrones A, B and C. Moreover, all reliably identified isolates produce at least one of the toxins, usually solanapyrone A and symptoms of the disease are reproduced by the toxins (Hamid and Strange, 2000). In this investigation therefore filtrates from *Fusarium oxysporum* f. sp. *ciceri*

grown in culture were tested for toxicity. This was done using a live/dead cell assay as, although the name of the disease suggests that wilting is the most characteristic symptom, this is difficult to quantify. Moreover, although wilting is a dramatic symptom of the disease, it may result from dysfunction of the plasma membrane since this would affect the water relations of the plant. The vital dye used was fluorescein diacetate (FDA). This is taken up by live cells but, once within the cell, non-specific esterases release fluorescein to which intact plasma membranes are impermeable. Live cells therefore fluoresce when excited at the appropriate wavelength. In contrast, dead cells do not fluoresce as they are unable to take up the dye and any fluorescein that is produced rapidly leaks from the cell.

The choice of this assay offers several advantages: use of only small amounts of toxin as the assay can be performed in the wells of a microtest plate, ease of quantification and speed, since an incubation time of only 3 h was sufficient. The cultivar ILC 482 was used in all bioassays as it is susceptible to wilt caused by *F. oxysporum* f. sp. *ciceri* and is considered to be an early wilter since it starts to wilt at 42 days after emergence and is completely wilted at 56 days (Halila, 1994 and chapter 1).

B) 2.2 Materials and methods

2.2.1 Acquisition of fungal isolates

Five isolates sent to University College London, London as *Fusarium oxysporum* f. sp. *ciceri* (Padwick) Snyd. and Hans, were used in these experiments. These were FOC 5 (isolated in Pakistan by Mr. M Hanif), race 1, race 2, V2 and Jabalpur (race 4; Haware and Nene, 1982) which were obtained from Dr S D Singh, International Crops Research Institute for the Semi Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, 502 324, India. Isolates were inoculated onto Petri plates containing potato dextrose agar (PDA; Oxoid Ltd, Basingstoke, Hampshire, England) and incubated at 20^{o}C.

2.2.2 Preservation of fungal isolates

All isolates were inoculated separately in 30 ml of a liquid medium consisting of Czapek-Dox nutrients supplemented with five cations (CDCLM; appendix 1) in 250 ml flasks. Flasks were incubated on a shaker at room temperature for 5 days in order to allow the fungus to sporulate. Spores were separated from the mycelium by filtration through four layers of sterile muslin cloth and were pelleted by centrifuging at 3000*g* for 15 min. The spores were resuspended in sterile distilled water and centrifuged. After repeating this process they were suspended in 10% sterile glycerol at 10^{7}spores/ml. Aliquots (1 ml) were placed in sterile Nunc tubes (1 ml; Nalge Nunc International, Nunc A/S, P. O. Box 280, DK-4000 Roskilde, Denmark) and stored in liquid nitrogen.

2.2.3 Acquisition of chickpea seed

The chickpea cultivar, ILC 482, which was used in all bioassays, was kindly supplied by Dr Mohamed Kharrat of the Institut de la Recherche Agronomique de Tunisie, Tunis.

2.2.4 Growing chickpea in the green house

Seed was soaked in water overnight and sown the following day in a 1:1 mixture of John Innes Compost No. 2 and silver sand (acquired from Capital Gardens, 1, Townsend Yard, Highgate High Street, London, N65JF) in plastic pots (180 mm diam.). Seven holes of 3 cm depth were made in the growth medium of each pot and two seeds were placed in each hole and covered. Pots were watered heavily soon after sowing. The plants were raised in a greenhouse (70-80% relative humidity; 20 ± 3ºC) and watered regularly. Daylight was supplemented with fluorescent light (200 $\mu E/m^{-2}.s^{-1}$.) for 16h per day during winter months.

2.2.5 Growing *F. oxysporum* f. sp. *ciceri* in liquid culture

Roux bottles (1 litre) containing 100 ml of Czapek Dox Cation Liquid Medium (CDCLM; appendix 1) were inoculated with spore suspensions of the isolates of FOC (100 µl; 10^7 spores/ ml) and incubated at 20°C and 30°C for 15 days. Cultures were sampled in triplicate (10 ml samples) at 3, 6, 9, 12 and 15 days after inoculation, all samples being taken from separate bottles. Samples were centrifuged at 3000*g* for 20 min and the supernatants were collected, freeze-dried, dissolved in 1 ml of holding buffer (HB; appendix I) and stored

in a freezer at –20°C. The mycelium of the each sample was also dried in an oven at 70°C to constant weight.

The remaining 90 ml of the samples were processed as detailed in chapter 3 section 3.2.1.

2.2.6 Bioassay of culture filtrates of *F. oxysporum* f. sp. *Ciceri*

The procedure of Chen and Strange (1994) was followed. Approximately 15-20 leaflets from well-watered and wholly intact plants were removed and allowed to float on HB. The leaflets were blotted on filter paper, cut into small pieces with sharp scissors and placed in 10 ml of digestion solution (Appendix I). A vacuum was gently drawn and released two or three times so as to fill the intercellular spaces with the enzyme solution. After stirring for 15-30 min on a magnetic stirrer at low speed (100 to 200 rpm), the resulting cell suspension was filtered through four layers of muslin into a 15 ml centrifuge tube and centrifuged at 85*g* for 5 min to pellet the cells. Cells were resuspended in 5 ml ice cold HB and the process of centrifugation and resuspension repeated twice. The resultant pellet was resuspended in HB so as to give an absorbance of 0.2 units at 620 nm on a spectrophotometer (equivalent to 2-3 X 10^4 cells/ml). Cell suspensions were kept on ice at all times when not being handled so as to prolong their viability.

HB (50 µl/well) was added to all the wells of a 96 well microtitre test plate except for those wells in the columns numbered 1 and 11. Toxin samples (100 µl), aqueous or methanolic were diluted with 900 µl of HB and were placed in duplicate wells of row 1 e.g. wells 1A and 1B, 1C and 1D (100 µl each). Serial two-fold dilutions of toxin samples were

made by transferring 50 µl of samples in well 1 to well 2, well 2 to well 3 and so on up to the wells of column 10. The extra 50 µl in these wells (those of row 10) were dispensed to well 11. Wells of column 12 served as controls. Chickpea cell suspension was added to each well (50 µl/well). After incubating for 3h, fluorescein diacetate (FDA) solution (5 mg FDA/ml in acetone, further diluted 1:49 with HB; 50 µl) was added to all wells. Cells were examined under an inverted fluorescence microscope (Olympus, Model IMT equipped with epi-fluorescence optics) after incubation for a further 5 min. For each well the number of live cells, i.e. those that fluoresced, out of 50 was counted. Results were converted to percentage viability allowing for the viability of controls not being 100% as per the following formula:

$$\frac{C - T}{C} \times 100$$

where C = Sum of viable cells in both control wells (e.g. 12A and 12B) and T = Sum of viable cells in test wells (e.g. 1A and 1B, 2A and 2B etc.). These values were converted to probits (Appendix II) and plotted against $\log_2$ of the dilution factor. One hundred percent cell death was taken as 99.5%, giving a probit value of 7.58 and viability values that were the same or greater than controls were given a value of 0.5% equivalent to a probit value of 2.67. From the graph it was then possible to ascertain the dilution factor that corresponded to the LD_{50} value (i.e. that killing 50% of the cells). This was designated as one unit of activity. The total number of units of activity in the samples could then be derived by conversion of the dilution factor giving 50% cell death to the decanery scale, multiplying by 20 to give units/ml

since only 50 µl was assayed and then by the number of ml/sample. Cell suspensions in which the viability of controls was <70% were rejected as assay material.

2.3 Results

2.3.1 Description of the five isolates of *F. oxysporum* f. sp. *ciceri*

When isolates of FOC were grown on PDA they appeared dissimilar. After incubation at 20^{o}C for 12 days, isolates designated race 1, race 2 and V2 appeared pale or yellow in colour while isolates Jabalpur and FOC 5 were mauve to red and dark red, respectively. Jabalpur was also distinguished from FOC 5 by the production of concentric rings (Fig. 2.1).

2.3.2 Preservation of fungal isolates

Over the three years of the project cultures were always recoverable from the liquid nitrogen bank.

2.3.3 Descriptions of chickpea seed

The chickpea cultivar ILC 482 is susceptible to wilt disease and was used in all bioassays. It is a typically white-seeded "Kabuli" type with a 100 seed weight of about 24.7g (Fig. 2.2).

2.3.4 Growth of chickpea plants in the green house

When chickpea seeds were sown in a 1:1 mixture of John Innes Compost No. 2 and silver sand and maintained in the greenhouse at 20 ± 3^{o}C shoots appeared above the soil surface within 1 week. Plants were thinned to seven seedlings per pot and used for bioassays when they were 3 to 4 weeks old (Fig. 2.3).

2.3.5 Demonstration of a linear relationship between probit percent cell death and $\log_2$ of the dilution of culture filtrates

When the percentage viability of chickpea cells, treated with a dilution series of toxin preparation was converted to probits and plotted against $\log_2$ of the dilution factor a straight line was obtained. The dose that killed 50% of the cells (LD_{50} value) was designated as one unit of activity (Fig. 2.4). Toxicity of preparations is therefore expressed in terms of units of activity for the remainder of the thesis.

2.3.6 Time course of toxigenicity of five isolates of *F. oxysporum* f. sp. *ciceri*

The maximum dry weight of cultures lay between 0.28 and 0.79g/100 ml culture (Fig. 2.5). However, these results mask considerable variation among the flasks for any one isolate and time interval (Appendix IV). Nevertheless, some generalisations may be made. Jabalpur was the only isolate to produce a measurable amount of mycelium at day 3. Three isolates gave dry weights that exceeded 0.6g/100 ml when grown at 20°C but at different ages, FOC 5 on day 12, V2 on day 15 and Jabalpur on days 6 and 9. At 30°C only race 2 at 15 days and V2 at 9 days after inoculation exceeded 0.6g/100 ml. FOC 5 grew at about the same rate at 30ºC as at 20^{o}C, maximum mycelial dry weights being observed at day 12.

The most striking result of toxin assays for the cultures was the high titres reached by FOC 5 at 20ºC (>70 units/ml culture filtrate) compared with the low values at 30ºC (Fig. 2.6). Among the other isolates, only V2 and Jabalpur produced 10 or more units/ml culture filtrate. With V2 this only occurred at day 15 when grown at 20^{o}C, but with Jabalpur titres of 10 units/ml were exceeded by cultures grown at 20^{o}C on days 9 and 15 and by cultures grown at 30^{o}C on days 9, 12 and 15. Toxin production at 30^{o}C by isolates FOC 5, race 1,

race 2 and V2 was less than 10 units/ml culture filtrate at all times of sampling (Fig. 2.6; appendix III).

Thus the time course showed that, in general, greater toxin titres were obtained at 20^{o}C than at 30^{o}C but this was reversed for Jabalpur and that very much greater titres were obtained for FOC 5 at the lower temperature.

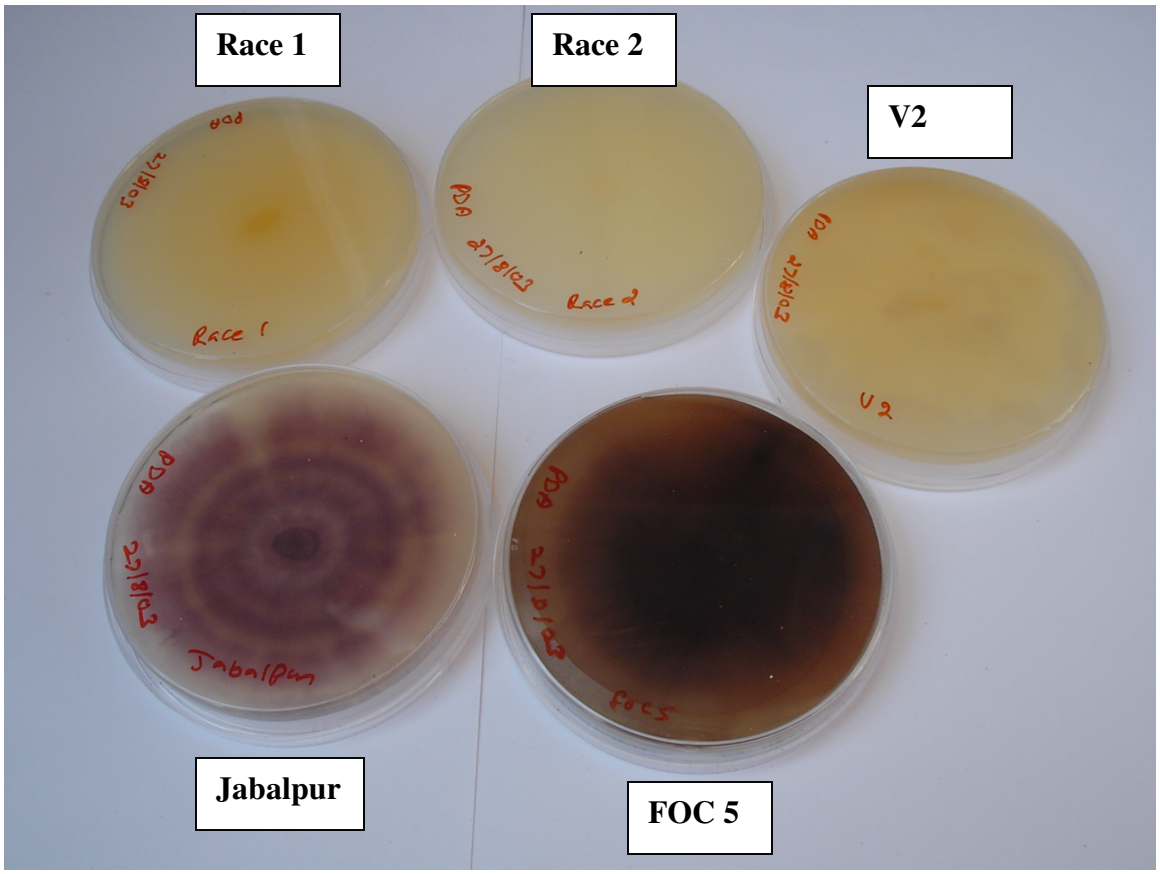

Fig. 2.1 The five isolates of *Fusarium oxysporum* f. sp. *ciceri*. FOC 5 was isolated in Pakistan by Mr M Hanif and the remaining isolates were obtained from Dr S D Singh, International Crops Research Institute for the Semi Arid Tropics, Patancheru, Andhra Pradesh, India.

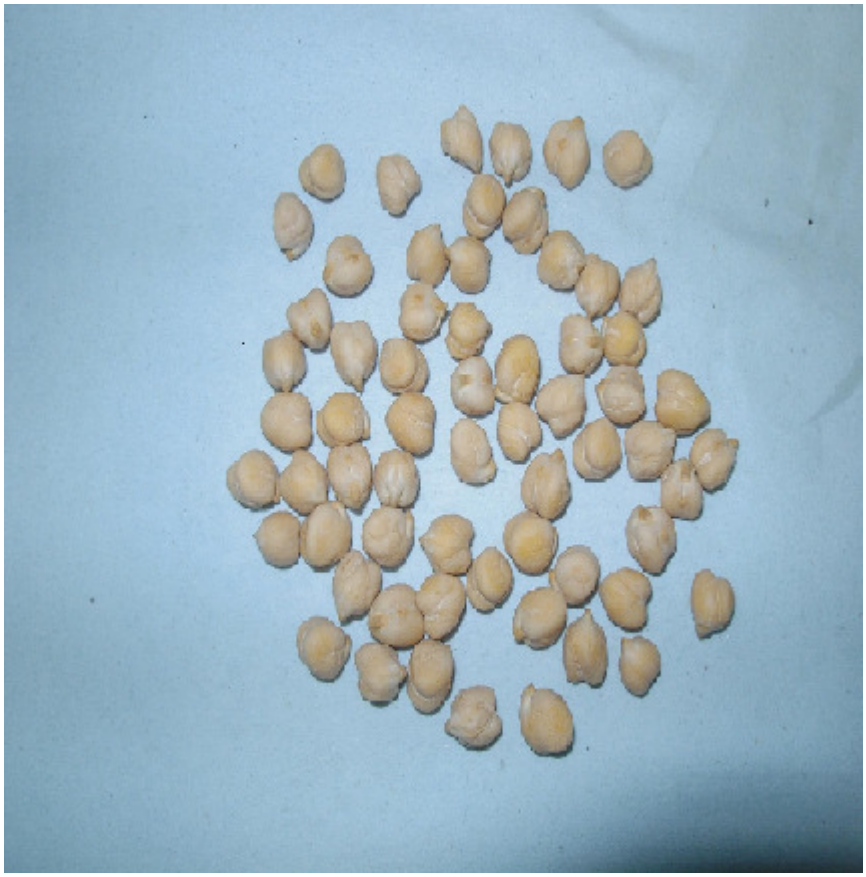

Fig. 2.2 The cultivar ILC 482, which is susceptible to wilt and which was used in this study. It is a typically white-seeded “kabuli type” with a 100 seed weight of about 24.7g. It was supplied by Dr Mohamed Kharrat of the Institut de la Recherche Agronomique de Tunisie, Tunis.

Fig. 2.3 The chickpea cultivar (ILC 482) growing in the green house. The green house was set at 20 ± 3°C with 70-80% relative humidity and daylight was supplemented with fluorescent light (200 $\mu E/m^{-2}.s^{-1}$.) for 16h per day during winter months. The plants were sown in a 1:1 mixture of John Innes Compost No.2 and silver sand in plastic pots (180 mm diam.). Plants were thinned to seven seedlings per pot and used for bioassays when they were 3 to 4 weeks old.

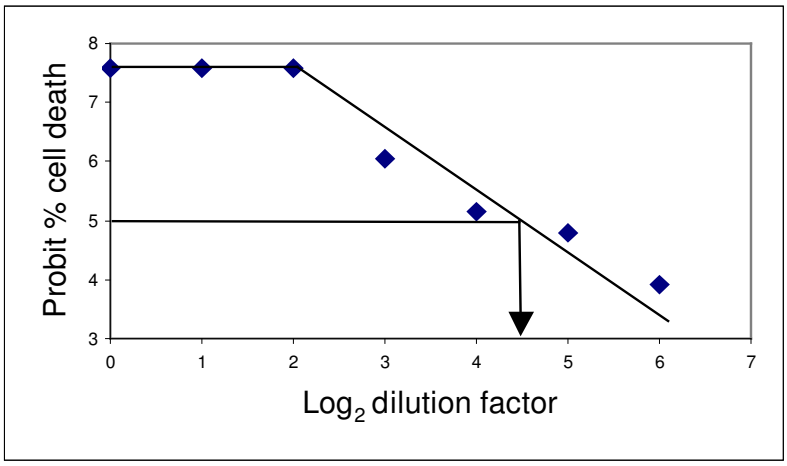

Fig. 2.4 The relationship of probit % cell death to a two-fold dilution series of a culture filtrate of *F. oxysporum* f. sp. *ciceri*. The number of units/ml in a preparation was determined by the dilution factor required to give 50% cell death (probit value of 5) and multiplying by 20 as only 50 µl of toxin preparation was used per well.

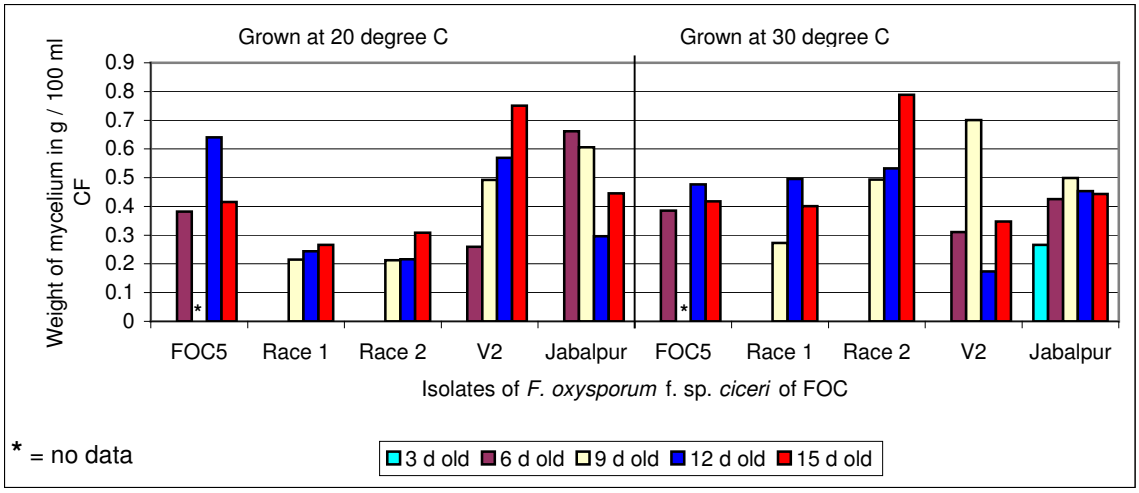

Fig. 2.5 Growth of isolates of *F. oxysporum* f. sp. *ciceri,* FOC 5, race 1, race 2, V2 and Jabalpur at 20°C and 30°C

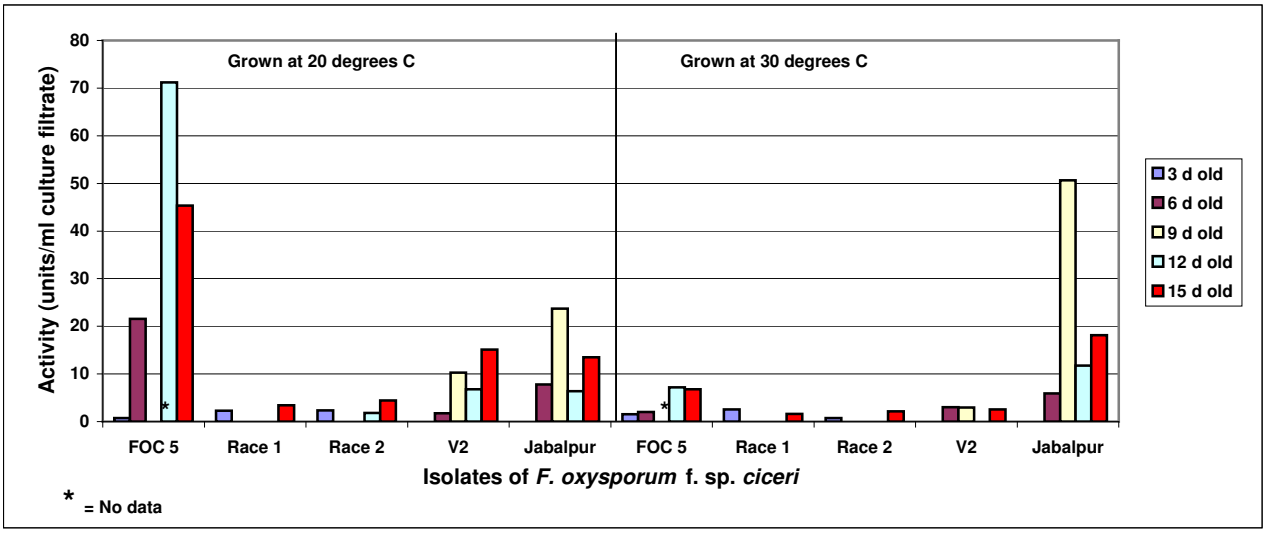

Fig. 2.6 Toxic activity of freeze-dried filtrates from cultures of isolates FOC 5, race 1, race 2, V2 and Jabalpur of *F. oxysporum* f. sp. *ciceri* grown at 20°C and 30°C

2.4 Discussion

A number of species and formae speciales of *Fusarium* have been reported to produce toxic compounds such as *Fusarium culmorum*, *F. graminearum*, *F. sporotrichioides, F. poae, F. moniliforme* and *F. oxysporum* f. sp. *medicaginis.* Some of the compounds produced such as the trichothecene toxins, deoxynivalenol (DON), T-2 toxin and diacetoxyscirpenol (DAS) as well as zearalenone (ZEN) and the fumonisins have mammalian toxicity (Cawood *et al*., 1991; Hussein and Brasel, 2001; L'vova *et al*., 2003). Additionally some, such as the trichothecene compounds are also phytotoxic (Desjardins *et al*., 1992, Desjardins *et al.,* 1995).

The symptoms of the Fusarium wilt of chickpea (section 1.6), caused by *F. oxysporum* f. sp. *ciceri* (FOC), suggest that phytotoxins may be involved (Hamid *et al*., 2001). However, there are only a few experiments reported in the literature that support this hypothesis. Kaur *et al.* (1987) found that partially purified toxin inhibited callus growth and Rao and Padmaja (2000) showed that crude culture filtrates of FOC, when diluted to 30% with water, caused wilting of 1 week old chickpea seedlings within 4-5 days. Hamid *et al.* (2001) showed that culture filtrates of FOC killed cells isolated from leaflets of chickpea plants and that the toxin could be partially purified by extraction into ethyl acetate. These data suggest that FOC is capable of producing a phytotoxic compound(s) when grown in culture. However, no further studies appear to have been made.

In the present investigation, five isolates of FOC of different geographical origin were tested; race 1, race 2, V2, Jabalpur and FOC 5. The first four isolates were isolated in India and the last, FOC 5, was isolated in Pakistan. Haware and Nene (1982) carried out pathogenicity studies on the Indian isolates using a set of 10 differential cultivars. From these

studies they proposed four races (1-4) of the pathogen. Race 1 originated from ICRISAT Centre, race 2 from Kanpur, race 3 from Gurdaspur and race 4 from Hisar and Jabalpur. In the present investigation an isolate designated V2, obtained from a wilt sick plot at ICRISAT, was also used but it is considered to be race 1 (Dr Suresh Pande, ICRISAT, personal communication). Haware and Nene (1982) and Barve *et al*. (2001) described all the isolates from Punjab and Haryana states of India as race 4. The race of FOC 5, obtained from Pakistan, is not known but, considering the apparent geographical restrictions of the races described, could be race 4 since Pakistan is situated on the border of the Indian Punjab and is also close to Haryana state.

With the exception of FOC 5 all the isolates were obtained from ICRISAT. This Institute is one of the earliest and most respected of the International Agricultural Research Institutes and, consequently, the identification of the isolates and the proof of their pathogenicity was not questioned in the earlier work of this investigations.

Detection and isolation of an unknown toxin requires a suitable bioassay. In previous work a number of plant species has been used to assay toxins from plant pathogens. For example, Brian *et al*. (1961) determined that *Fusarium equiseti* secreted phytotoxic compounds *in vitro* by spraying whole pea plants with culture filtrates of the fungus and recording the symptoms. The assay allowed them to isolate DAS, which they showed caused a just detectable inhibition of stem elongation and a noticeable scorching of the leaves at 1 µg/ml while at 10 µg/ml the toxin was lethal. Phaseolotoxin was assayed initially by its ability to cause chlorotic lesions on bean leaves (Hoitink *et al*., 1966). Subsequently, inhibition of ornithine carbamoyl transferase was found to be the primary lesion caused by the toxin and consequently inhibition of activity of this enzyme could be used as an assay for

the toxin (Mitchell, 1979; Turner and Mitchell, 1985). Csinos and Hendrix (1977) used excised tobacco leaves in a bioassay for a toxin produced by *Phytophthora cryptogea* in liquid culture and found that it caused water soaking within 12h, laminar collapse within 20h and extensive dehydration within 48h.

Once the chemistry of the toxin is known non-biological assays such as HPLC (Hayashi *et al*., 1990) or immuno assays (Phelps *et al*., 1990) or even thin layer chromatography (Schaafsma *et al*., 1998) may be used.

In the present study, the ability of *F. oxysporum* f. sp. *ciceri* to produce toxic compounds in liquid media was tested. The fungus was grown at two temperatures and both growth, determined as mycelial dry weight, and toxicity were measured. For the latter, an assay employing cells isolated from chickpea leaflets, which had previously been used successfully to monitor the isolation of the solanapyrone toxins of another chickpea pathogen, *Ascochyta rabiei* (Chen and Strange, 1994), showed that culture filtrates of FOC 5 were toxic. Cells from leaflets of the chickpea cv. ILC 482, which is highly susceptible to wilt, were used in this thesis. ILC 482 was released in Morocco under the same name (ILC 482), in France as TS 1009, in Jordan as Jubeiha-2, in Lebanon as Janta 2, in Syria as Ghab 1 and in Turkey as Guney Sarisi (Halila, 1994).

Time course experiments showed that FOC 5 produced far more toxin than any other isolate when grown at 20ºC and that maximum titres of about 70 units/ml culture filtrate were reached at 12 days. In contrast, at 30ºC this isolate produced only about 7 units/ml culture filtrate although there was no such great disparity in weight of mycelium The only other isolate that produced >20 units/ml was Jabalpur but here maxima were reached at 9 days and the titre at 30ºC was greater than at 20ºC. These results may be compared with those of Joffe

(1986) who studied 236 isolates of *Fusarium oxysporum* but for their toxicity to rabbit skin rather than plant tissue. The isolates were cultured at five temperatures, ranging from 12ºC to 35ºC. More isolates were toxic at intermediate temperatures than the extremes. Moreover, Moss (1991) and Discostanzo *et al*., (1996) found that the ideal growth conditions for *F. poae* and *F. sporotrichoides* was 21ºC but Park *et al*., (1996) found that this was not necessarily the ideal temperature for toxin elaboration. They showed that temperature optima for the production of T-2 toxin, ZEN and DON were 6-12, 19-20 and 28°C, respectively.

A notable feature of the cultures of the five isolates in the present study was that the most toxic, FOC 5 and Jabalpur secreted red pigments into the medium whereas with the other isolates the media remained a pale yellow colour (Figs. 2.1 and 2.6). Since FOC 5 produced the highest toxin titres when grown at 20°C for 12 days, this isolate and these conditions were used in subsequent experiments for the scale up and isolation of the toxin.

CHAPTER III

Scale up and liquid/liquid extraction of toxin(s) from culture filtrates of FOC

3.1 Introduction

The constituents of the culture medium used for the production of the toxin, Czapek Dox nutrients and five cations (CDCLM; appendix 1), are hydrophilic. In contrast, toxins are often lipophilic. It was therefore possible that the putative FOC toxin would partition preferentially into a solvent that is immiscible with water, which would provide a rapid and convenient method for a considerable clean-up of the compound. However, some lipophilic compounds may contain ionizable groups such as carboxylate, which unless suppressed by low pH might prevent partitioning into the organic phase. For this reason, culture filtrates were adjusted to pH 3 before partitioning against ethyl acetate (EtOAc). Moreover, since the organic phase was wet with water of the culture filtrate it was dried over anhydrous sodium sulphate before evaporation on a film evaporator. Residues were finally collected in small volumes of solvents, usually methanol or acetonitrile (Hamid and Strange, 1997). Although FOC 5 produced the highest toxin titre among the five isolates of FOC tested (race 1, race 2, V2 and Jabalpur were the other isolates; chapter 2), for the sake of completeness the four other isolates were also tested.

In order to identify a compound it is necessary to have sufficient for various physical techniques. Although little is required for mass spectrometry and proton magnetic resonance studies more is needed for ^{13}C nuclear magnetic resonance since the ^{13}C isotope is present in only low abundance (1%). Therefore, in the experiments described in this chapter litre volumes of culture filtrates of FOC 5, the isolate that produces the highest toxin titres, were

extracted and the activity in the preparations carefully monitored with the assay (section 2.2.6).

3.2 Materials and methods

3.2.1 Partitioning of the culture filtrates of FOC isolates against ethyl acetate

In the last chapter, experiments were described in which culture filtrates of FOC isolates race 1, race 2, V2, Jabalpur and FOC 5 that had been grown on CDCLM at 20°C and 30°C were harvested at 3 day intervals (section 2.2.5). In that chapter 10 ml samples from the 100 ml cultures were freeze-dried leaving 90 ml for liquid-liquid extraction (EtOAc partition experiments).

The 90 ml samples were centrifuged at 10,000*g* for 20 min and the supernatants collected. After adjusting the pH to 3.0 the supernatants were partitioned three times against EtOAc. The EtOAc phases were combined, dried over anhydrous sodium sulphate and the EtOAc removed by film evaporation on a rotary evaporator at 35°C. The residues were dissolved in 1 ml of MeOH and stored in a freezer at –20°C. Aqueous phase samples were freeze-dried and dissolved in minimal volumes of holding buffer (HB; appendix 1) and placed in the freezer until assayed.

3.2.2 Red and colourless culture filtrates of isolate FOC 5

Some of the 100 ml cultures of FOC 5 turned red during incubation and some remained colourless. These were partitioned separately against EtOAc as above and the organic phases were film evaporated at 35°C. The residues of the EtOAc fractions from each 100 ml culture were dissolved in 2.5 ml of MeOH and checked for toxic activity after dilution with nine volumes of HB. Mycelia of the red and colourless cultures were dried to constant weight at 80°C.

3.2.3 Scale up of toxin production by isolate FOC 5

A total of 78 Roux bottles, each containing 100 ml of CDCLM, was inoculated with spores of FOC 5 (100 µl per bottle of a spore suspension containing 10^7 spores/ml). After incubation for 12 days at 20°C, three samples were made of two litres each and one of 1.8 litres. Culture filtrates were harvested, filtered, centrifuged, partitioned against EtOAc and the organic phase was collected in 10 ml of methanol after evaporated as per the methods described in sections 2.2.5 and 3.2.1. Samples (400 ml) of each aqueous phase were also freeze-dried and dissolved in 10 ml of HB. The remaining aqueous phase samples were kept at 4°C after adding methanol to 20% for future use if required.

3.3 Results

3.3.1 Partitioning of culture filtrates of FOC isolates against ethyl acetate (EtOAc)

Toxic activity for all five isolates of FOC partitioned into ethyl acetate. Maximum toxic activity equivalent to over 100 units/ml culture filtrate was observed in this fraction when FOC 5 was grown for 12 days on CDCLM at 20°C but declined to 45 units/ml at day 15. Among the other isolates, only Jabalpur produced 20 or more units/ml culture filtrate but this was in cultures grown at 30°C for 15 days (Fig. 3.1; appendix V) Therefore, routinely for purification purposes, FOC 5 was grown at 20°C for 12 days and the toxin partitioned into EtOAc.

Since sufficient toxicity was present in 12 day-old cultures of FOC 5 to assay them directly without freeze-drying, it was possible to compare the activity of the crude culture filtrate with the ethyl acetate fraction and the residual aqueous fraction. Only 52% of the toxic activity was recovered in the EtOAc fraction when culture filtrates were partitioned three times against the organic solvent and about 30% remained in the aqueous phase. When these were combined the recovery was around 46%, showing, if anything, inhibition of activity rather than synergy (Fig. 3.2). In a further experiment both the aqueous and the ethyl acetate fractions were freeze-dried and assayed. This resulted in an increase of activity of the EtOAc phase and complete loss of activity of the aqueous phase, providing further evidence that the aqueous phase contained an inhibitor and that this was a volatile (Fig. 3.3).

In order to test for the completeness of partitioning of the toxin into ethyl acetate culture filtrates were partitioned 1, 3 and 10 times against the organic solvent. This resulted in 46, 85 and 115% recovery, respectively when compared with the crude non-freeze-dried

culture filtrate. Nevertheless, even after 10 partitionings, the amount of activity remaining in the aqueous phase was nearly 10% (Fig. 3.4).

3.3.2 Toxicity of red vs. colourless culture filtrates of FOC 5

Some cultures of isolate FOC 5 turned red during incubation (Fig. 3.5) at 20°C and these were more than ten times as active as colourless cultures. Mycelial growth of the fungus was also greater in the red cultures compared with the colourless ones (Fig. 3.6; appendix VI).

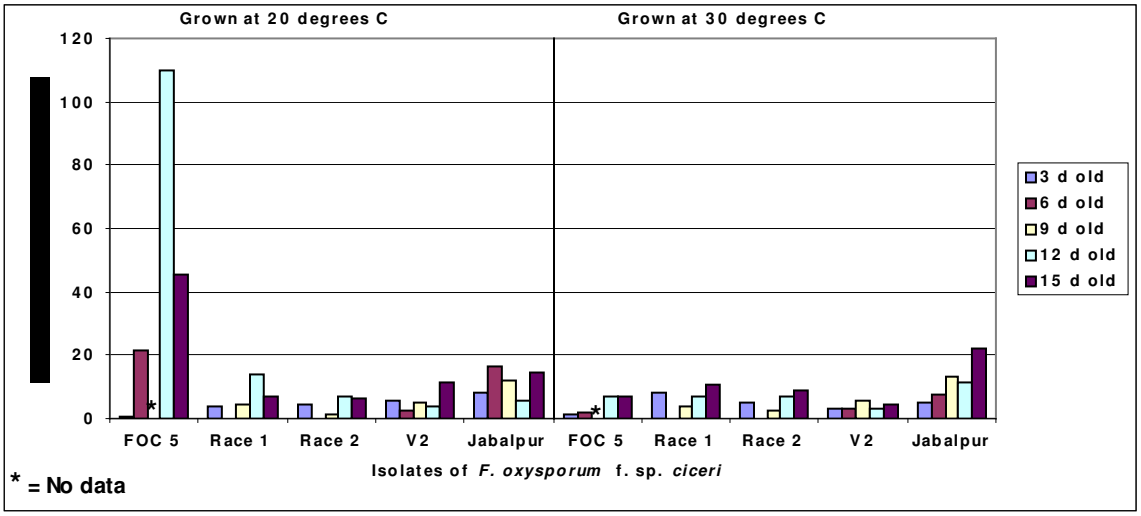

Fig. 3.1 Toxic activity of the ethyl acetate fractions from cultures of five isolates of *F. oxysporum* f. sp. *ciceri,* FOC 5, race 1, race 2, V2 and Jabalpur, grown at 20°C and 30°C for varying times

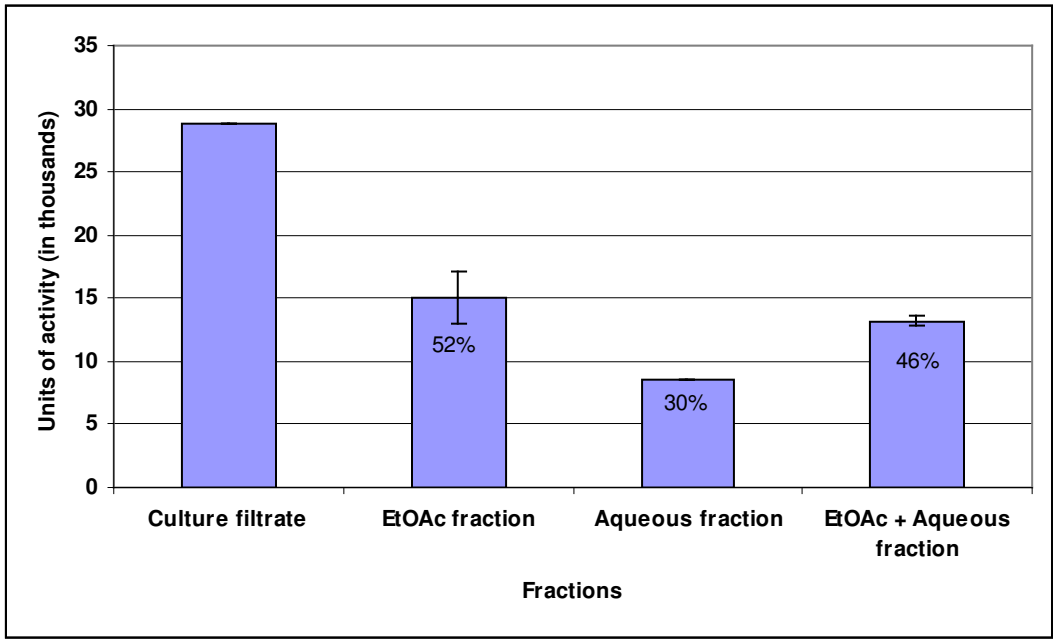

Fig. 3.2 Distribution of toxic activity between ethyl acetate and aqueous phases. No synergy was recorded when the two fractions were mixed.

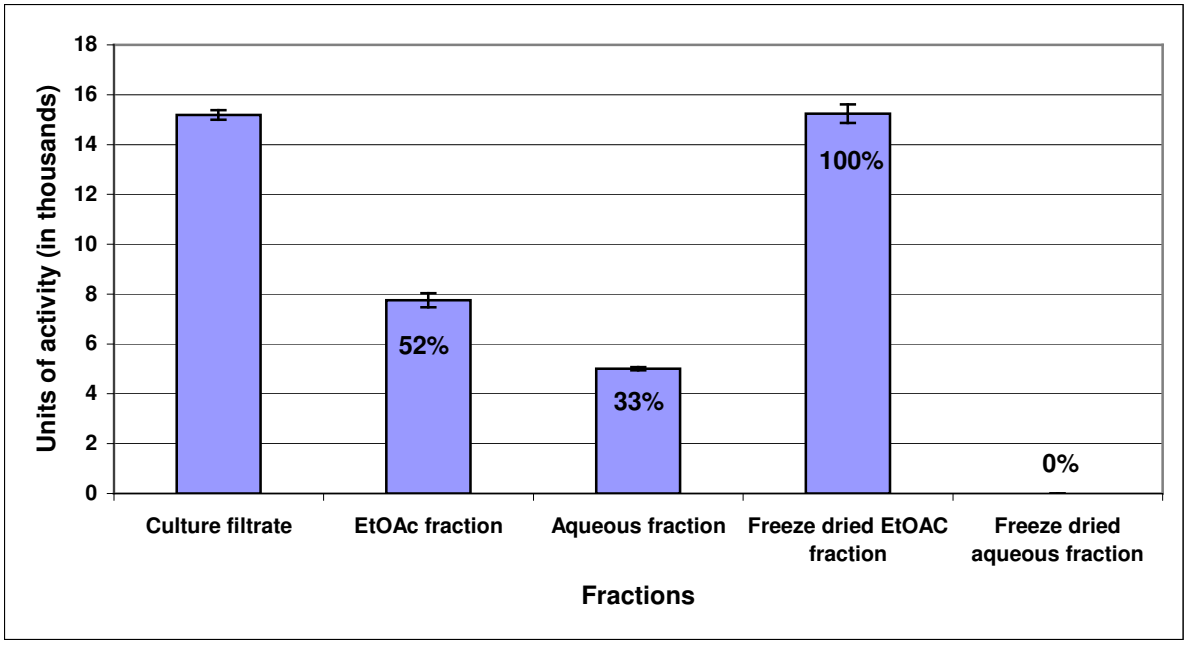

Fig. 3.3 Effect of freeze drying on EtOAc and aqueous fractions obtained by partitioning culture filtrates (3 times against EtOAc) of FOC 5. Percentages on columns refer to percentage of the original culture filtrate.

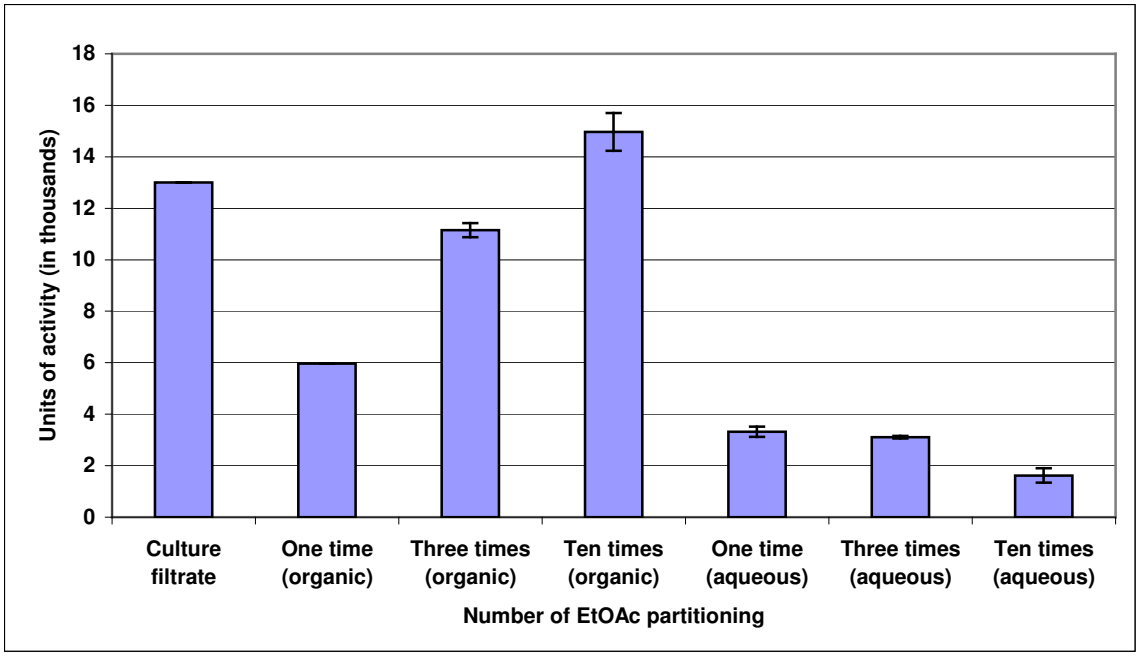

Fig. 3.4 The effect of multiple partitioning against EtOAc on the recovery of toxic activity

Fig. 3.5 Colourless and red cultures of FOC 5

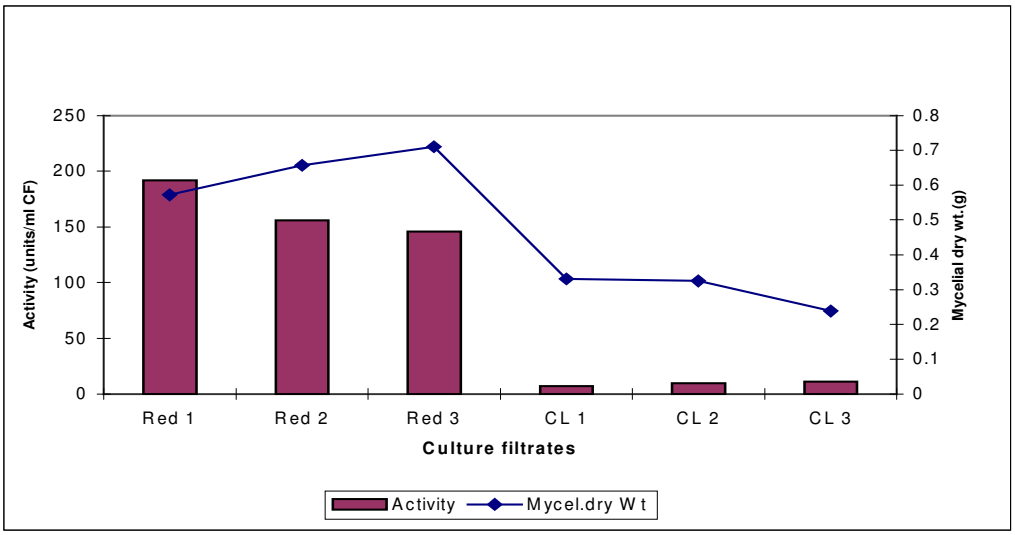

Fig. 3.6 Activity vs. mycelia dry weight of red and colourless culture filtrates of FOC 5

3.4 Discussion

Many phytotoxins found in culture filtrates of fungal pathogens are lipophilic, such as solanapyrones A, B and C, produced by *Ascochyta rabiei* and fumonisins, produced by *Fusarium moniliforme.* They can therefore be extracted into immiscible organic solvents from aqueous solutions such as culture filtrates. Various organic solvents have been used by researchers for the extraction of different toxins. Drummond and Pinnock (1990) used chloroform for extraction of aflatoxins, produced by *Aspergillus parasiticus* and *A. flavus* and Alam *et al.,* (1989) used ethyl acetate (EtOAc) to extract the solanapyrones from culture filtrates of *A. rabiei*. Hamid and Strange (1997) also used this method for extraction of the solanapyrones and were able to go on to purify the compounds by flash chromatography.

In chapter 2 a time course experiment was conducted on culture filtrates of five isolates of FOC, race 1, race 2, V2, Jabalpur and FOC 5 in order to determine the optimal temperature and incubation times for toxin production. In this chapter experiments to determine if the toxic activity partitioned from culture filtrates into EtOAc are reported. These showed that toxic activity was extracted from culture filtrates by EtOAc and there was a good match of activity between these results and those obtained by freeze drying of culture filtrates. For example, greater toxin titres were obtained at 20°C than 30°C for all isolates except Jabalpur and very much greater titres were obtained for FOC 5 grown at 20°C on day 12 than at any time for cultures grown at 30ºC. In several instances, toxic activity was recorded for EtOAc fractions whereas none was detected in the comparable freeze-dried culture filtrates (compare Fig. 2.6 with Fig. 3.1).

Since the toxic activity of culture filtrates of FOC 5 was so high, it was possible to assay them without concentration by freeze-drying. Accordingly, direct comparisons of the

activity of crude culture filtrates, their ethyl acetate fractions and the residual activity remaining in aqueous phases after ethyl acetate extraction were made. Initial results showed that after extracting culture filtrates three times in one-third volumes of EtOAc only about half of the activity was found in the EtOAc phase and 30% in the aqueous phase. When the two fractions were assayed together, there was no synergy between the two fractions, rather inhibition since the activity was just under half of the intact culture filtrate (Fig. 3.2).

When both the EtOAc and the residual aqueous fraction were freeze-dried there was a complete loss of activity in the latter but an increase in the activity of the EtOAc fraction (Fig. 3.3). One interpretation of these findings is that a volatile toxic component(s) is present in both EtOAc and aqueous fractions, which is competitive with a non-volatile toxic component of the EtOAc fraction.

In order to determine if recovery of toxin in the EtOAc fraction could be improved, culture filtrates were extracted multiple times with the organic solvent. These experiments showed that increasing the number of extractions did increase the toxin titres of the organic phase but even after 10 extractions nearly 10% of the activity remained in the aqueous phase (Fig. 3.4). In these experiments rather higher titres for the organic phase resulted from extraction three times (85% of the crude culture filtrate was extracted) compared with previous experiments (52%; Figs. 3.2 and 3.3). This may be the result of using smaller volumes of sample and consequently better mixing of the two phases.

A surprising result was that two types of cultures, red and colourless, were always obtained from FOC 5 even when these were derived from single spores. The colourless cultures grew less well than the red ones and had toxin titres which were only about one-tenth that of the red cultures.

In conclusion, FOC 5 produced the highest toxin titres when grown at 20°C for 12 days and the majority of the activity partitioned from aqueous solution to EtOAc. Despite single sporing, cultures of FOC 5 segregated into those that were red or colourless, the red cultures being far more toxic than the colourless. Therefore in order to purify the toxin, it was necessary to grow FOC 5 at 20ºC for 12 days and select those cultures with the red pigmentation.

CHAPTER IV

Toxin purification by solid-phase extraction and high performance liquid chromatography

4.1 Introduction

As concluded in chapters 2 and 3, toxins of FOC were produced in liquid culture on a defined medium. In order to determine the structure of the toxin(s) it was necessary to separate it from other components of the culture filtrate.

Solid-phase extraction (SPE) is an extraction method that uses a solid phase to adsorb analytes from solution. The analytes are then desorbed selectively in a small volume of an appropriate solvent, thus purifying and concentrating the sample. The technique is usually used to clean up a sample before using a chromatographic or other analytical method to quantitate the amount of analyte(s) of interest in the sample. The general procedure is to load a solution onto the SPE medium, wash away undesired components and then elute the desired analyte(s) with another solvent into a collection tube. Compared to the classic liquid-liquid extraction using a separation funnel SPE offers several advantages. These include:

1. Speed
2. Ease of manipulation
3. Reduction of amount of organic solvent, obviating the need to dispose of it
4. No problems with the miscibility of solvents
5. No problems with incomplete phase separation and emulsion formation.

The major purposes of the SPE are:

1. **Sample concentration:** In some instances, the component of interest is present in levels too low for detection. SPE may be used to concentrate the component to levels that are adequate for measurement.
2. **Removal of contaminants in the sample**: The presence of interfering compounds and materials used in sample preparation such as silica or sodium sulphate may mask the analysis of the component of interest. Many of these contaminants may be removed by the judicious choice of loading and eluting solutions for the SPE cartridge. SPE is particularly appropriate as a clean-up procedure before the use of HPLC since the same type of solid stationary phase may be used in both the SPE cartridge and the HPLC column, reducing the risk of permanent adsorption of constituents of the preparation on the HPLC column.
3. **Changing the phase of the sample solution**: For most analyses such as HPLC, GC, Spectrophotometry and Radio Immuno Assay, the sample must be prepared in an appropriate solvent that may differ from that of the original solution. SPE may be used to change the phase of the sample solution, e.g. from aqueous to organic.

Therefore solid-phase extraction was evaluated for the concentration and partial purification of toxic compound(s) from culture filtrates of FOC 5.

In order to further purify the toxic compounds in the SPE fraction, another method of chromatography was used viz. High Performance Liquid Chromatography (HPLC). HPLC is a separation technique in which a mixture of compounds is introduced into a column of adsorbent material (the stationary phase) and is washed

through the column with a solvent (the mobile phase). Compounds separate according to their relative affinities for the stationary phase and the mobile phase.

In the present investigation, the stationary phase of the HPLC consisted of either octodecyl silica or cyano silica particles (5µ in diam.). Because of the small size of these particles, considerable pressure has to be applied to the mobile phase to pump it through the column e.g. 100-200 bar (HPLC is some times called high pressure liquid chromatography). The separated compounds elute from the end of the column and are fed continuously into a photocell illuminated with light in the UV range. This allows absorbance of light by the eluting compounds to be measured between 190 and 390 nm.

The data are collected by a computer and represented on the screen as a three-dimensional graph, termed a chromascan, in which the x axis is elution time, the y axis is absorption of light and the z axis is wave length. From the graph it is possible to extract the UV spectrum of a compound eluting at a given retention time. It is also possible to extract a chromatogram at a given wavelength. The spectra may be compared with those of spectra of authentic compounds stored in the memory of the computer and the area under the peak in a chromatogram can be used to quantify the compound which is responsible for it.

Analytical HPLC is suitable for quantifying low-microgram quantities of low molecular weight compounds within a short separation time and uses small columns e.g. 150 mm X 4.6 mm diam. However, HPLC may be used semi-preparatively or preparatively to obtain milligram or even gram quantities, respectively, of pure

compounds. In these cases, larger columns and faster flow rates of the mobile phase are used.

There are several types of HPLC viz. reversed phase chromatography, normal phase chromatography, ion-pair chromatography and ion-exchange chromatography. Since normal or reversed phase chromatography was used in this experiment, these are discussed briefly. Reversed and normal phase chromatography refers to the polarity of the stationary and mobile phase components. Normal phase chromatography consists of a polar (hydrophilic) stationary phase and a non-polar (hydrophobic) mobile phase. In this system hydrophobic compounds elute more quickly than hydrophilic ones. In contrast, reversed phase chromatography consists of a non-polar (hydrophobic) stationary phase and a polar (hydrophilic) mobile phase. Here hydrophilic compounds elute more rapidly than hydrophobic compounds.

A flow diagram of all the methods used in toxin purification is provided in Appendix IX.

4.2 Materials and methods

4.2.1 Fractionation of ethyl acetate extracts of culture filtrates of FOC 5 by solid phase extraction (SPE) cartridges

Solid phase cartridges [5g, C_{18} end-capped (EC) or cyano Isolute (Jones Chromatography, UK)] were prepared by solvating with 30 ml of methanol (MeOH) or acetonitrile (ACN), respectively, and equilibrating with 30 ml of distilled water. Samples of ethyl acetate extracts of culture filtrates (2-3 L) in MeOH or ACN (10 ml; section 3.2.1) were removed from storage in the freezer and a 500 µl aliquot reserved for assay in order to determine its activity. The remaining 9.5 ml was mixed with 90 ml distilled water and introduced into the cartridge at a steady flow rate of 10-15 ml per minute. The discharge (hereafter called non-adsorbed fraction [NAF]) was retained and tested for toxic activity. Elution of the C_{18} cartridge was with 10% MeOH (20 ml) followed by 20 ml incremental MeOH concentrations from 20% -100%. Similarly, cyano cartridges were eluted with 10% ACN (20 ml) followed by 20 ml incremental ACN concentrations from 20% - 100%. Each incremental fraction was collected individually and assayed (section 2.2.6). Samples for assay were prepared by diluting 100 µl volumes of each fraction with 900 µl holding buffer (HB; Appendix I). Controls were a dilution series of 10% MeOH or 10% ACN in HB.

4.2.2 Scouting with Genesis columns

In order to find an HPLC system that separated the FOC 5 toxins with reasonable retention and good peak geometry, several reversed and normal phase columns (Jones Chromatography, UK) were used. They were of 2 cm in length and without a guard column. Data were acquired on a Philips HPLC system consisting of a PU4100 quaternary pump,

PU4021 diode array detector and a personal computer equipped with PU6003 diode array software for data handling. The columns and their respective mobile phases (suggested by the manufacturer of Genesis columns, Jones Chromatography, UK) used in the HPLC were:

Reversed phase columns	**Mobile phase**
Cyano	35% ACN
Phenyl	45% ACN
C_4EC	45% ACN
C_8	50% ACN
C_8EC	50% ACN
AQ	60% ACN
C_{18}	60% ACN

Normal phase columns	**Mobile phase**
Cyano	1% ACN in Heptane
Phenyl	1% ACN in Heptane
NH_2	1% ACN in Heptane

4.2.3 Separation of SPE fractions on an HPLC cyano column

The most active SPE fractions from material adsorbed onto the C_{18} cartridges (60% and 70% MeOH fractions) and those of cyano cartridges (40% and 50% ACN) and, in this case, activity that was not adsorbed, were further fractionated on an analytical cyano column (150 X 4.6 mm diam.; Jones Chromatography, UK) and semi-preparative cyano columns (150 X 10 mm diam.; Jones Chromatography, UK).

In general, the mobile phase used with the analytical cyano column was 40% ACN and with the semi-preparative cyano column 30% ACN. Flow rates with mobile phases degassed with helium for equilibrating the columns and running samples were 1000 µl/min for the analytical column and 4000 µl/min for the semi-preparative column. At the start of day and when changing mobile phases the system, without the column, was flushed at a flow rate of 4000 µl/ml. Solvents for mobile phases were HPLC grade, obtained from Merck, England and ultra pure water obtained from Maxima (Maxima, II AN, Elga Ltd., High Street Lane End, High Wycombe, Bucks., HP14 3JH, England).

4.2.3.1 Separation of C_{18} SPE fractions

Ten ml of the 60% and 70% MeOH fractions from the C_{18} SPE cartridge were evaporated on a rotovapour and dissolved in 2 ml of 50% ACN separately. These were injected (20 µl samples) into a analytical cyano column separately using 40% ACN as the mobile phase. Fractions were collected corresponding to the peaks and the troughs on the chromoscan. The collected fractions were evaporated on a rotovapour to remove the ACN and the remaining aqueous solutions were adjusted to pH 3.0 and partitioned three times against EtOAc. The organic phases (EtOAc phases) from nine runs were dried over anhydrous sodium sulphate and finally evaporated on a rotovapour and collected in a small volume (1 ml) of 100% ACN. All fractions were bio-assayed as described in section 2.2.6 and the entire experiment was replicated. Purity of compounds in the HPLC fractions was checked by HPLC using the same conditions as in the original separation.

4.2.3.2 Preparation of 1g C_{18} SPE cartridges

C_{18} SPE cartridges (1g) were used at various stages in the isolation of toxic compounds, mainly for the purpose of concentrating samples. Cartridges were solvated with 6 ml of 100% MeOH or 6 ml of 100% ACN followed by equilibration with 6 ml of ultra pure water. Once cartridges were prepared, samples were injected and eluted with 100% MeOH or 100% ACN, depending upon the requirement.

4.2.3.3 Preparation of samples for HPLC

All the SPE fractions that were to be further separated on HPLC needed concentration, as most of the fractions were so dilute that their constituents were not detectable by the diode array detector. In order to concentrate samples, they were diluted with water so that the organic solvent was not >10% and the diluted samples were introduced into 1g C_{18} SPE cartridges (section 4.2.3.2). After washing with 10% MeOH or 10% ACN compounds were eluted in a small volume of 100% MeOH/ACN (usually 3 X 1 ml). These samples were injected into the HPLC.

4.2.3.4 Separation of SPE fractions from cyano cartridges by HPLC on an analytical cyano column

Samples (10 ml) of the 40% and 50% ACN and NAF fractions from cyano SPE cartridges were concentrated (section 4.2.3.3) and the first 1 ml that was eluted and which showed the maximum toxic activity was injected into the HPLC. The stationary phase was the analytical cyano column and the mobile phase 30% ACN. Fractions were collected corresponding to peaks and troughs as before. They were evaporated on a rotovapour to

remove ACN, concentrated on a 1g C_{18} SPE cartridge (section 4.2.3.3.) and collected in 2 X 1 ml of 100% ACN. Samples were diluted 1:9 with HB before being assayed.

4.2.3.5 Separation of SPE fractions from cyano cartridges by HPLC on a semi-preparative cyano column

Active fractions from the 5g cyano cartridge viz. NAF, 40% and 50% ACN were further separated on a semi-preparative cyano column (150 X 10 mm diam.) in order to obtain sufficient material for bioassay.

SPE fractions (20 ml) were concentrated on 1g C_{18} SPE cartridges as per section 4.2.3.3. The cartridges were eluted successively with 3 ml and 2 ml of 100% ACN (named SM1 and SM2 respectively). A sample of SM1 (500 µl) was separated by HPLC on a semi-preparative cyano column with 30% ACN as mobile phase. Fractions were collected corresponding to the peaks and troughs of the chromascans. All the HPLC fractions were evaporated on a rotovapour to remove ACN and they were concentrated by the EtOAc partitioning method (section 3.2.1). The residues were collected in 1 ml of ACN.

In some instances the eluate from a whole HPLC run was collected as one fraction in order to determine recovery and for checking the possibility of any synergy among the HPLC fractions. Removal of ACN and concentration was as described in the previous paragraph.

The concentrated samples and fractions (all finally dissolved in 1 ml ACN) were diluted 1:9 with HB and assayed.

4.3 Results

4.3.1 Fractionation of EtOAc extracts of culture filtrates of FOC 5 by solid phase extraction on C_{18} and cyano cartridges

When EtOAc fractions of culture filtrates, which had been evaporated, dissolved in MeOH (10 ml) and diluted with 90 ml of water, were introduced to C_{18} SPE cartridges, just under 10% of the activity was not adsorbed. Of the remainder, only 35% could be recovered by elution in 10% incremental steps of MeOH in water, the majority eluting with 60-90% MeOH (Fig. 4.1 and appendix VII). Activity in the fractions appeared to be matched by their red colouration (Fig. 4.2) Similar results were obtained when samples were dissolved in ACN, diluted with water to 10% ACN and applied to a cyano SPE cartridge. However, here the eluent was 10% incremental steps of ACN in water. In this case only about 6% of the activity was not adsorbed by the cartridge and 45% was recovered in the ACN-H_2O fractions, predominantly in those eluted with 40% and 50% ACN (Fig. 4.3 and appendix VII). Again the intensity of the red colour in the fractions appeared to correspond with toxic activity (Fig. 4.4).

4.3.2 Scouting with Genesis columns

None of the normal phase Genesis columns (silica, amine [NH_2], cyano [CN] and phenyl [Phe]) aided the separation of toxins found in either the non-adsorbed or the adsorbed fractions obtained from C_{18} SPE cartridges, UV absorbing material appearing in the effluent as long tailing peaks.

In contrast separation was obtained with the same sample and reversed phase columns, the cyano column giving the best separation. Here there were three peaks that were

relatively sharp and with retention times of 29 sec, 53 sec and 1 min 39 sec and absorption maxima of 224 nm, 226 and 269 nm and 224 and 269 nm respectively. Hence the cyano column was selected for further purification of toxic compounds.

4.3.3 Separation of fractions from C_{18} SPE cartridges by analytical HPLC on a cyano column

When the most active fractions from the stepwise elution of C_{18} SPE cartridges in MeOH were chromatographed on a cyano HPLC column the 60% eluate from the cartridge gave three peaks (Fig. 4.5). The first of these eluted at 1 min 13 sec and had λ max at 200, 226 and 271 nm (Fig. 4.6), the second at 2 min 31 sec and had λ max of 224 nm and a shoulder at 271 nm (Fig. 4.7) and the third at 6 min 1 sec and had λ max of 224 and a shoulder at 271 nm (Fig. 4.8). Activity was found only in the effluent corresponding to the third peak.

When the 70% MeOH fraction from a C_{18} SPE cartridge was further chromatographed on the cyano HPLC column, two peaks of major absorption were found (Fig. 4.9). The first of these eluted at 2 min 34 sec and had λ maxima at 224 and a shoulder at 270 nm (Fig. 4.10) while the second eluted at 3 min 37 sec and had λ maxima at 237 nm and 260 nm (Fig. 4.11). Eluent corresponding to the first peak was not active in the assay but that corresponding to the second peak was.

Rechromatography by HPLC of active fractions that had UV absorption gave results that were inconsistent with the original separation. For this reason, this approach to purifying the toxin was abandoned.

4.3.4 Separation of fractions from cyano SPE cartridges by analytical HPLC on a cyano column

The most active fractions from cyano SPE cartridges, i.e. 40% and 50% ACN and NAF (Fig. 4.3), were further fractionated on an analytical cyano HPLC column. Four UV absorbing peaks were observed in the 40% ACN eluate and seven fractions were cut on the basis of these and the corresponding troughs (Fig. 4.12). Most activity was found in fraction 3 which eluted as a broad band at about 5 – 8 min but there was some activity in fractions 2, 4 and 5 (Fig. 4.13). The spectrum of fraction 3 had absorption maxima at 200, 224 and 269 nm (Fig. 4.14). When fraction 3 was rechromatographed on the same column, it again eluted as a broad peak with the same retention time.

Separation of the 50% ACN fraction from the cyano cartridge on an analytical cyano column yielded three major UV absorbing peaks and six fractions were cut, essentially on the basis of these and the corresponding troughs (Fig. 4.15). Fraction 3 was the most active but some activity was also present in fraction 2. In one of the two replicate separations, some activity was also found in fraction 5 (Fig. 4.16). The retention times of the second, third and fifth fractions were 2 min 43 sec, 6 min 55 sec and 17 min 25 sec, respectively. Absorption maxima were 204 nm for the second fraction and 200, 224 and 270 nm for the third fraction and for the fifth fraction 224 and 270 nm (Figs 4.17, 4.18 and 4.19). Upon rechromatography on the analytical cyano column in order to determine the purity of the active fractions, no peaks were found. One reason for loss might have been the use of a C_{18} cartridge to concentrate the sample before rechromatography since trial experiments showed that this allowed only 25-36% recovery of toxic activity. Despite this, it was calculated that there

would have been sufficient to visualise the components on the HPLC suggesting that they were labile.

Attempts were also made to purify a toxin from the fraction that was not adsorbed by a cyano SPE cartridge (NAF). Only one major peak was found but five fractions were cut (Fig. 4.20). Only the third of these, which corresponded to the major peak and had a retention time of 5 min 16 sec and absorption maxima of 200, 224 and 270 nm was active (Figs. 4.21 and 4.22). Upon rechromatographing on an analytical cyano HPLC column this peak also was not found.

4.3.5 Separation of fractions from cyano SPE cartridges by semi-preparative HPLC on a cyano column

In order to obtain sufficient material for bioassay and detection on HPLC, a semi-preparative cyano column was used. Several samples (500 µl each) of the 40% ACN fraction from a cyano SPE cartridge were injected into a semi preparative cyano column and collected individually. Seven fractions were cut, of which fraction three, as in the case of the smaller samples on the analytical column, was the most active (Fig. 4.13). The retention time of the peak was 5 min 25 sec and the absorption maxima were 200, 224 and 270 nm, as found previously with the analytical column. Minor amounts of activity were also found in fractions 2, 4, 5 and 6 (Fig. 4.23). Although recovery of toxic activity in three replicates was 180%, 107% and 77% no peaks were seen when fraction 3 was rechromatographed.

When the same experiments were performed with the 50% ACN fraction from a cyano SPE cartridge, of the five fractions that were cut only fractions 3 and 4 were active (Fig. 4.24), corresponding to the result obtained with the analytical cyano column (Fig. 4.16).

With this sample, however, recovery of activity was less than 20% and on rechromatographing more than one peak was observed.

Upon chromatography of the NAF fraction from a cyano SPE cartridge on a semi-preparative cyano column, a similar separation to that found with the analytical column with only one major peak was obtained (Fig. 4.20). Of the five fractions that were cut, only fraction three, corresponding to the large peak, was active (Fig. 4.25). Recovery of toxic activity was 119 and 45% in two replicates. On rechromatography this peak appeared clean but only 38% of the activity of the original activity was recovered.

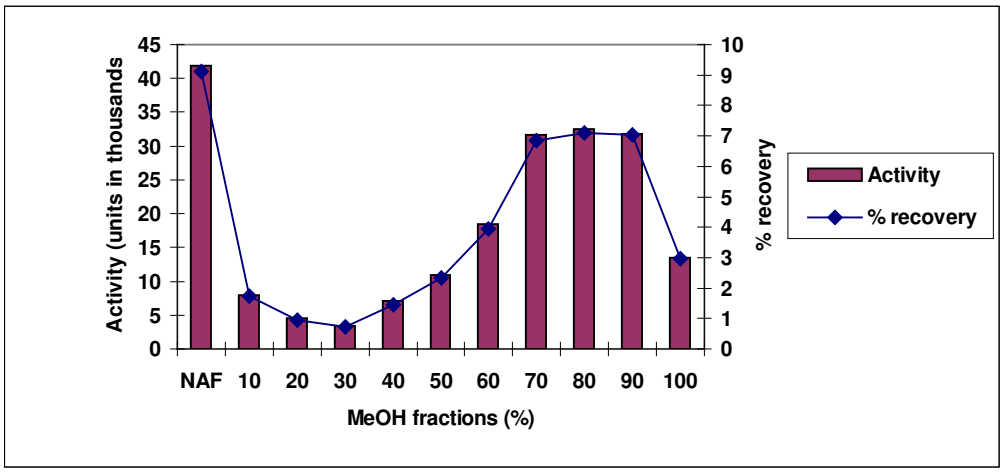

Fig. 4.1 Separation of the ethyl acetate phase of a culture filtrate of isolate FOC 5 on a solid phase C_{18} cartridge (5g). After adsorption the cartridge was eluted successively with 20 ml volumes of MeOH solutions in increasing 10% increments. The starting materials contained 203,594 units of activity (100%).

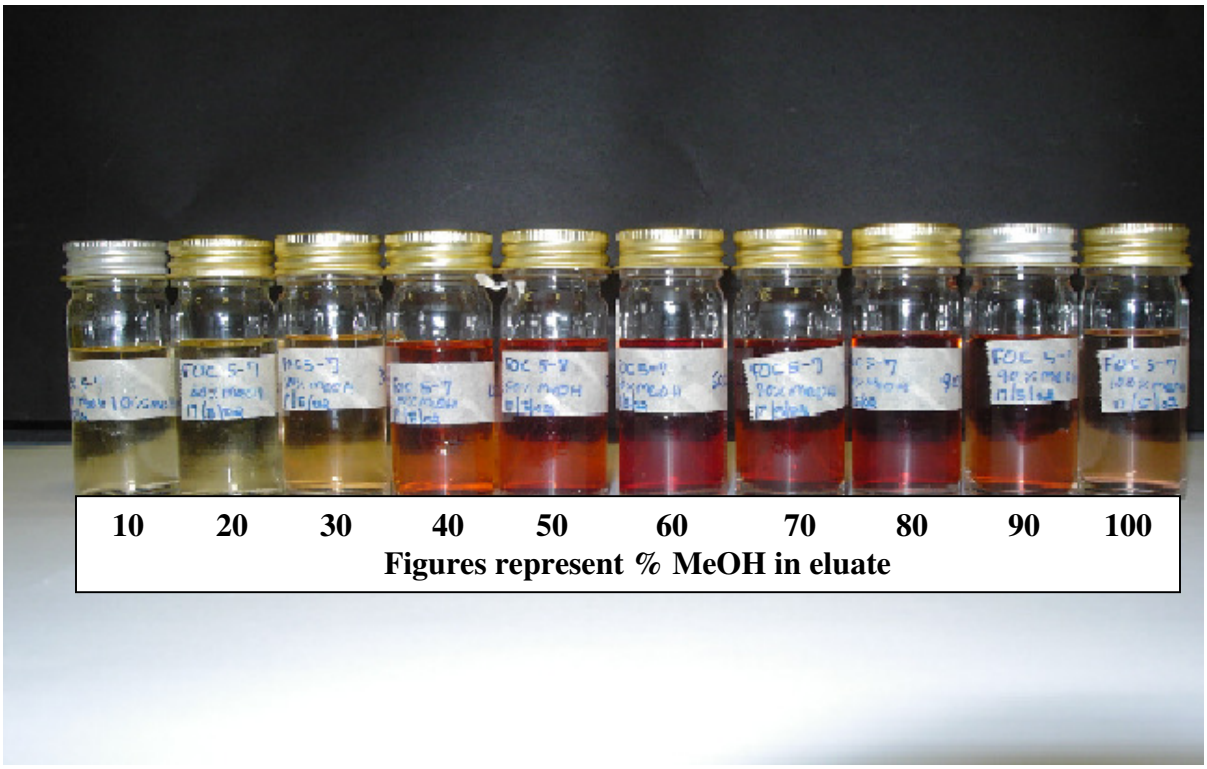

Fig. 4.2 Fractions from Solid Phase Extraction (SPE) of toxin (EtOAc fraction from culture filtrate) on a C_{18} cartridge. Note that the red colouration was present in fractions with activity (Fig. 4.1).

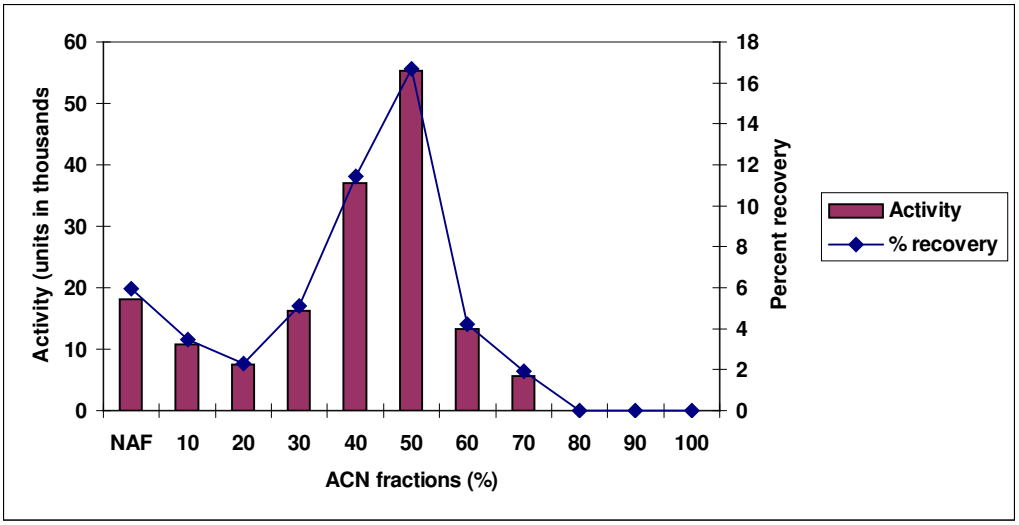

Fig. 4.3 Separation of the ethyl acetate phase of a culture filtrate of isolate FOC 5 on a solid phase cyano cartridge (5g). After adsorption the cartridge was eluted successively with 20 ml volume of ACN solutions in increasing 10% increments. Starting materials contained 319,102 units of activity (100%).

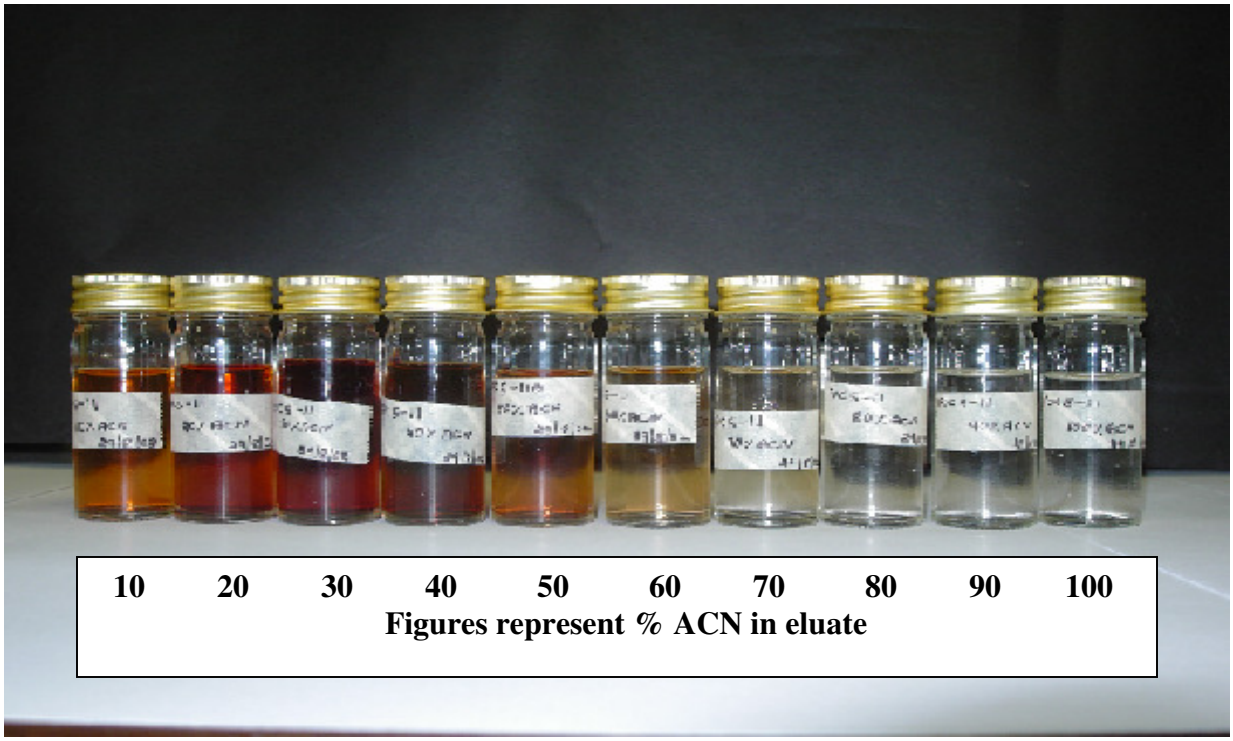

Fig. 4.4 Fractions from Solid Phase Extraction (SPE) of toxin (EtOAc fractions from culture filtrate) on a cyano cartridge. The red colouration was present in fractions with activity (Fig. 4.3).

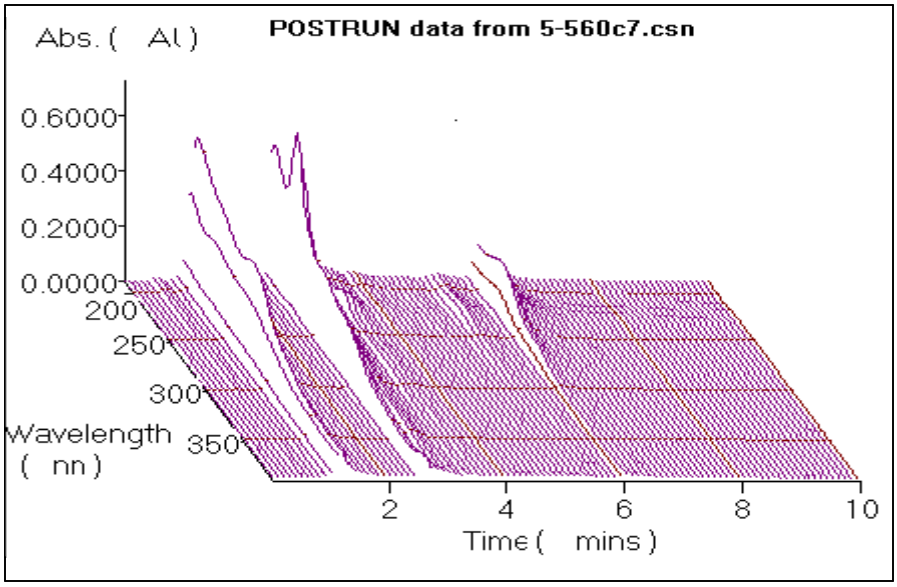

Fig. 4.5 Chromatography of the 60% MeOH eluate from a C_{18} SPE cartridge on an analytical cyano HPLC column

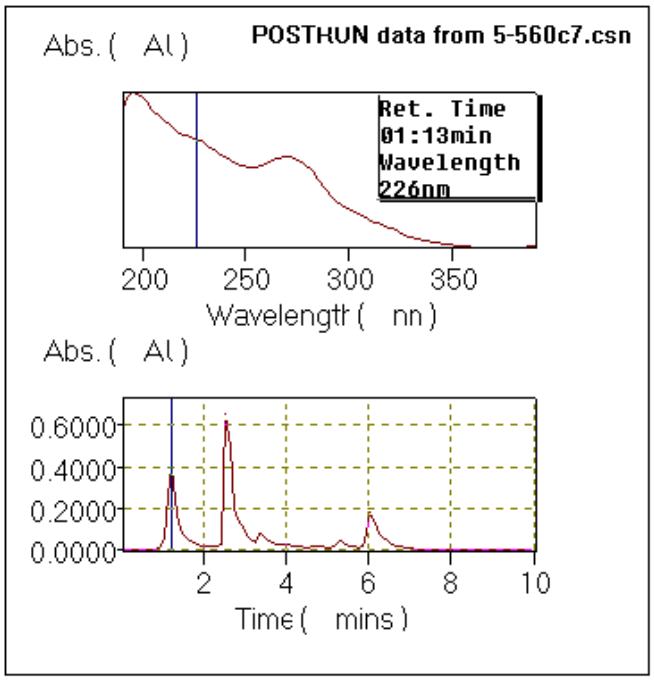

Fig. 4.6 Retention time and absorption maxima of the first peak found in the 60% MeOH eluate when chromatographed on an analytical cyano HPLC column

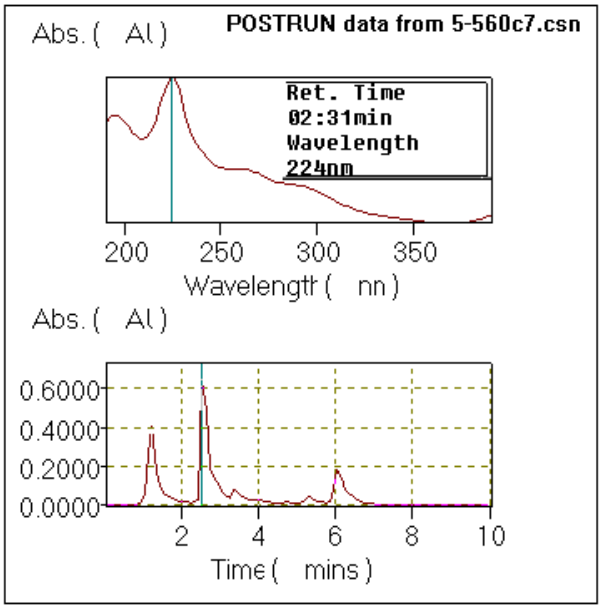

Fig. 4.7 Retention time and absorption maxima of the second peak found in the 60% MeOH eluate when chromatographed on an analytical cyano HPLC column

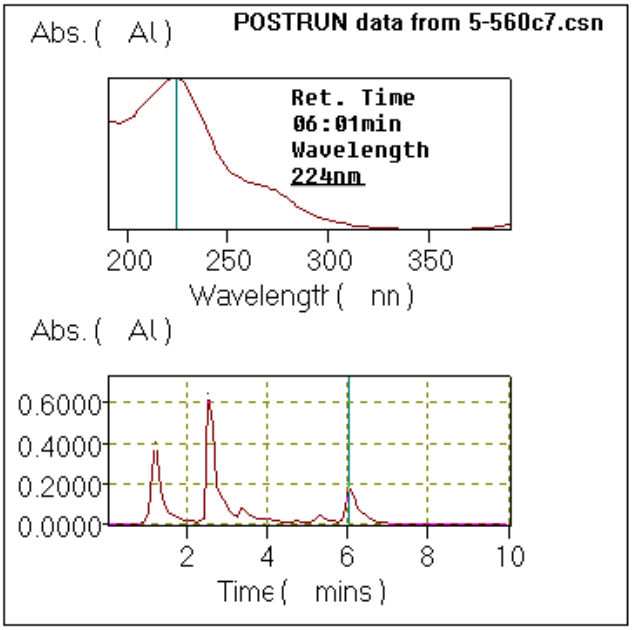

Fig. 4.8 Retention time and absorption maxima of the third peak found in the 60% MeOH eluate when chromatographed on an analytical cyano HPLC column

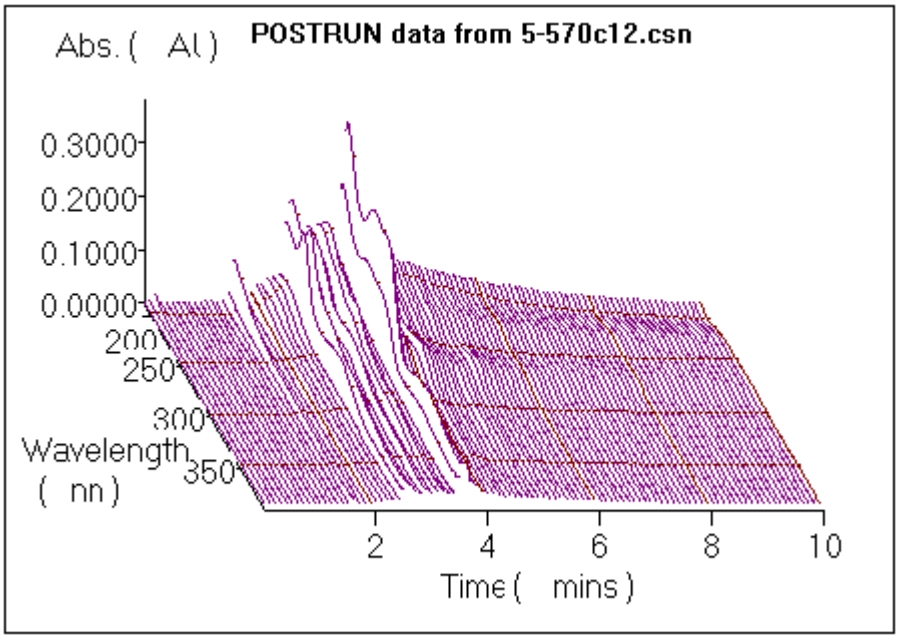

Fig. 4.9 Chromatography of the 70% MeOH eluate from a C_{18} SPE cartridge on an analytical cyano HPLC column

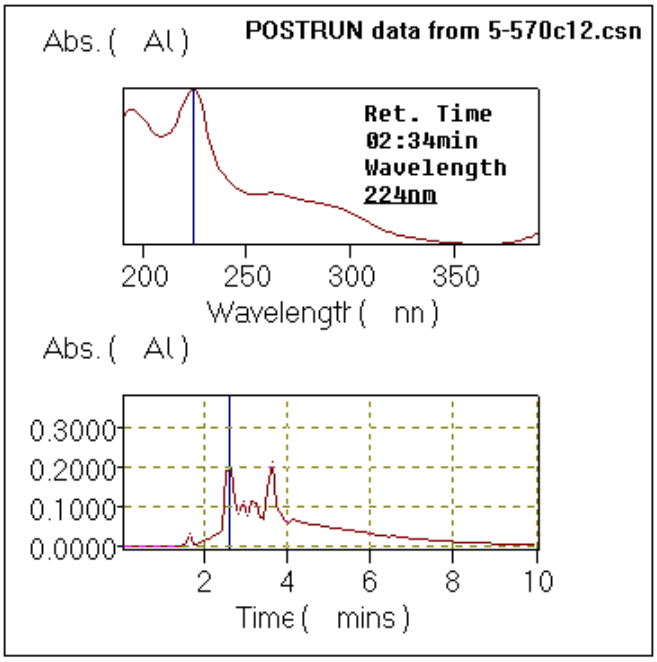

Fig. 4.10 Retention time and absorption maxima of the first peak found in the 70% MeOH eluate when chromatographed on an analytical cyano HPLC column

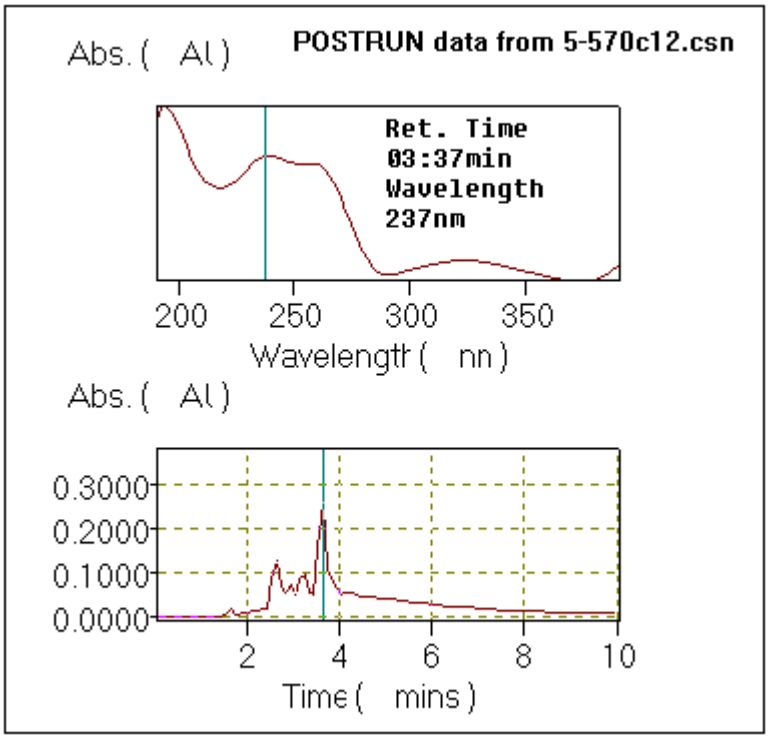

Fig. 4.11 Retention time and absorption maxima of the second peak found in the 70% MeOH eluate when chromatographed on an analytical cyano HPLC column

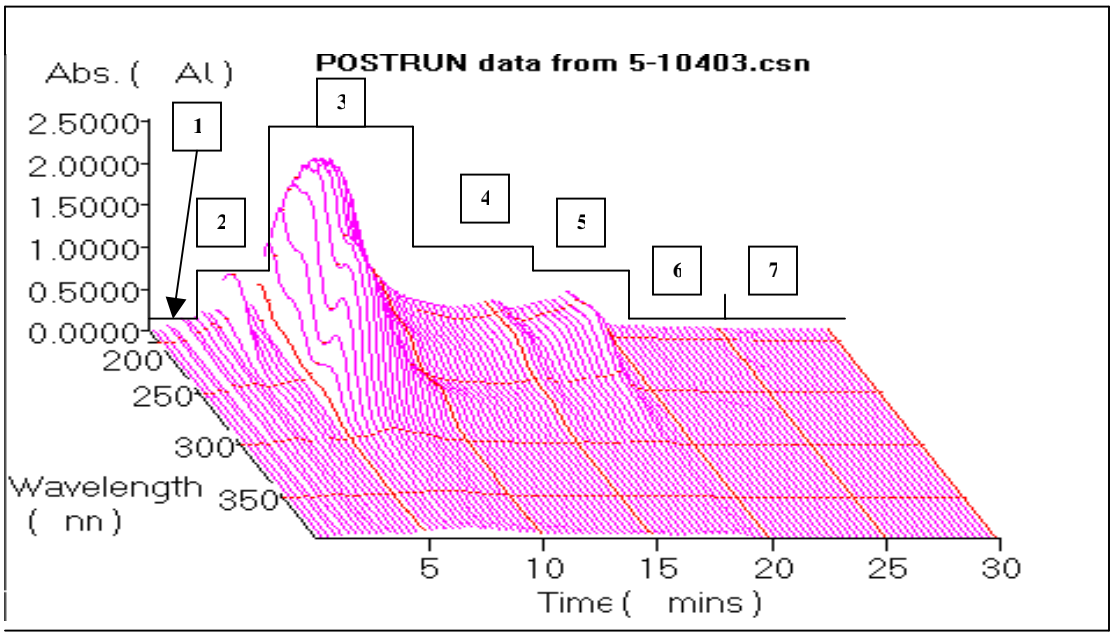

Fig. 4.12 Chromatography of the 40% ACN eluate from a cyano SPE cartridge on an analytical cyano HPLC column

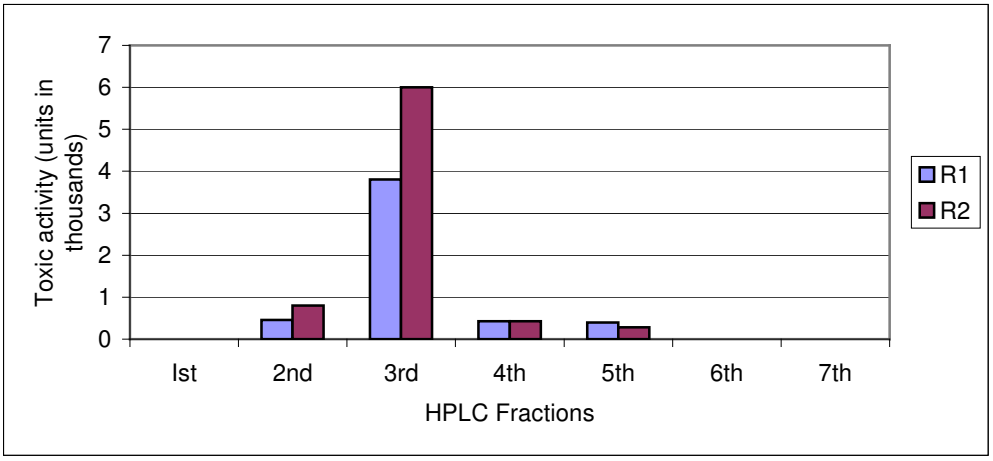

Fig. 4.13 Toxic activity present in the HPLC fractions of 40% ACN eluate of cyano SPE cartridge

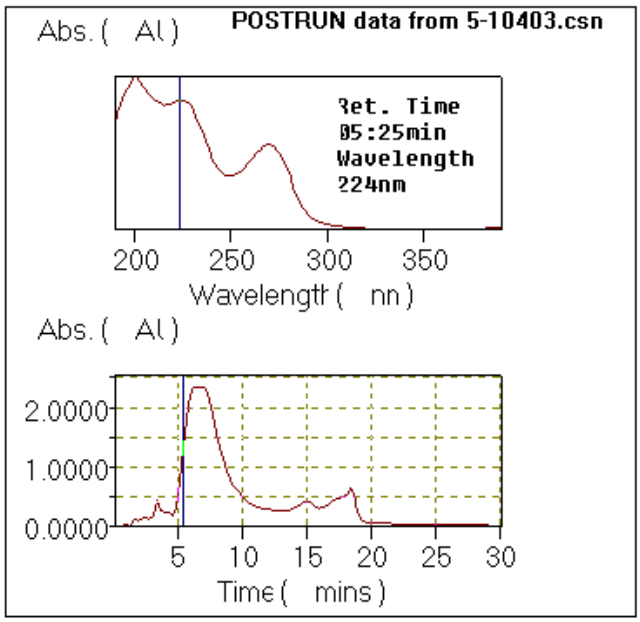

Fig. 4.14 Retention time and absorption maxima of the HPLC fraction 3 found in the 40% ACN eluate when chromatographed on an analytical cyano HPLC column

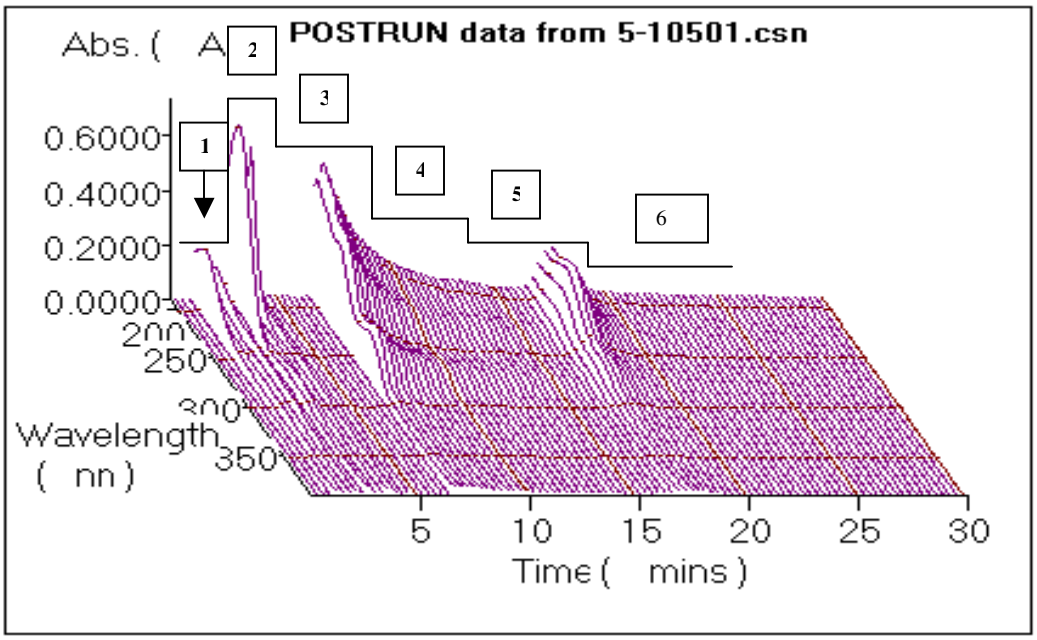

Fig. 4.15 Chromatography of the 50% ACN eluate from a cyano SPE cartridge on an analytical cyano HPLC column

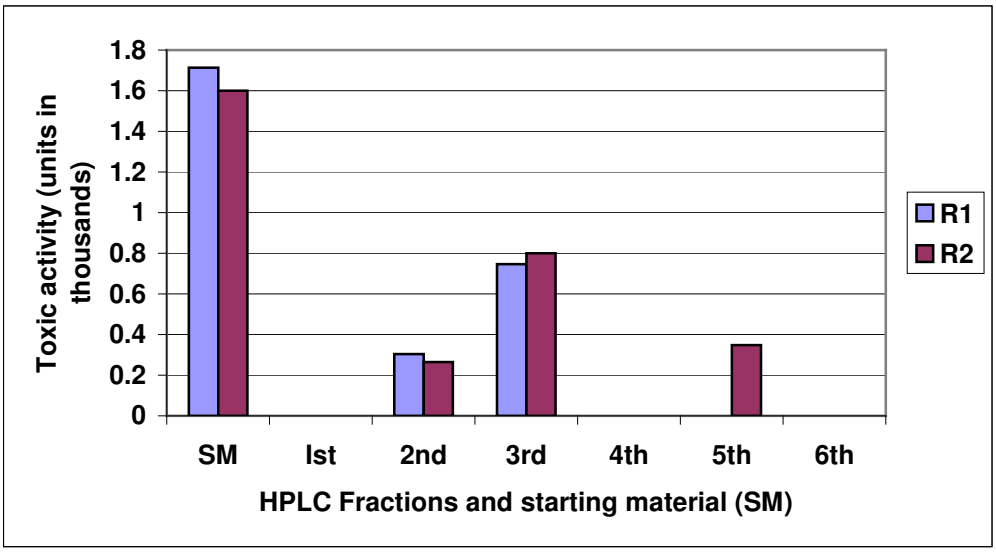

Fig. 4.16 Toxic activity present in the HPLC fractions of 50% ACN eluate of cyano SPE cartridge

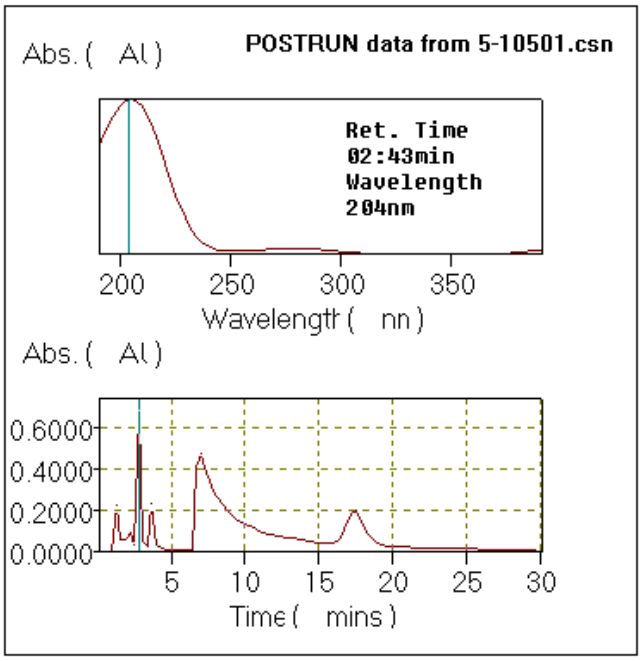

Fig. 4.17 Retention time and absorption maxima of the HPLC fraction 2 found in the 50% ACN eluate when chromatographed on an analytical cyano HPLC column

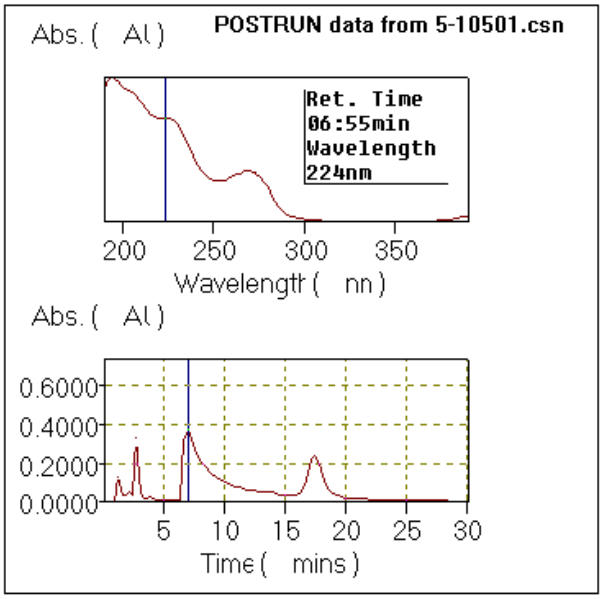

Fig. 4.18 Retention time and absorption maxima of the HPLC fraction 3 found in the 50% ACN eluate when chromatographed on an analytical cyano HPLC column

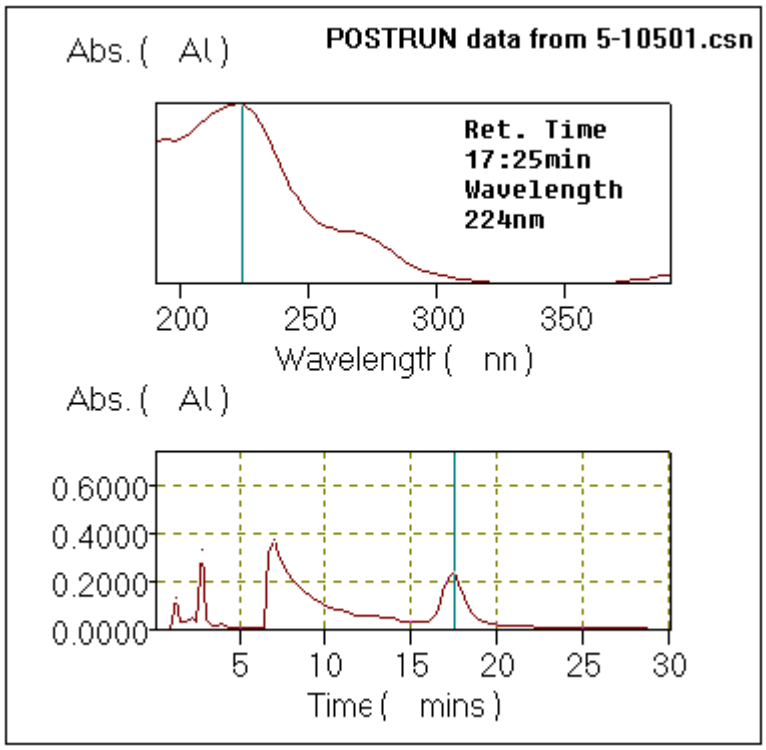

Fig. 4.19 Retention time and absorption maxima of the HPLC fraction 5 found in the 50% ACN eluate when chromatographed on an analytical cyano HPLC column

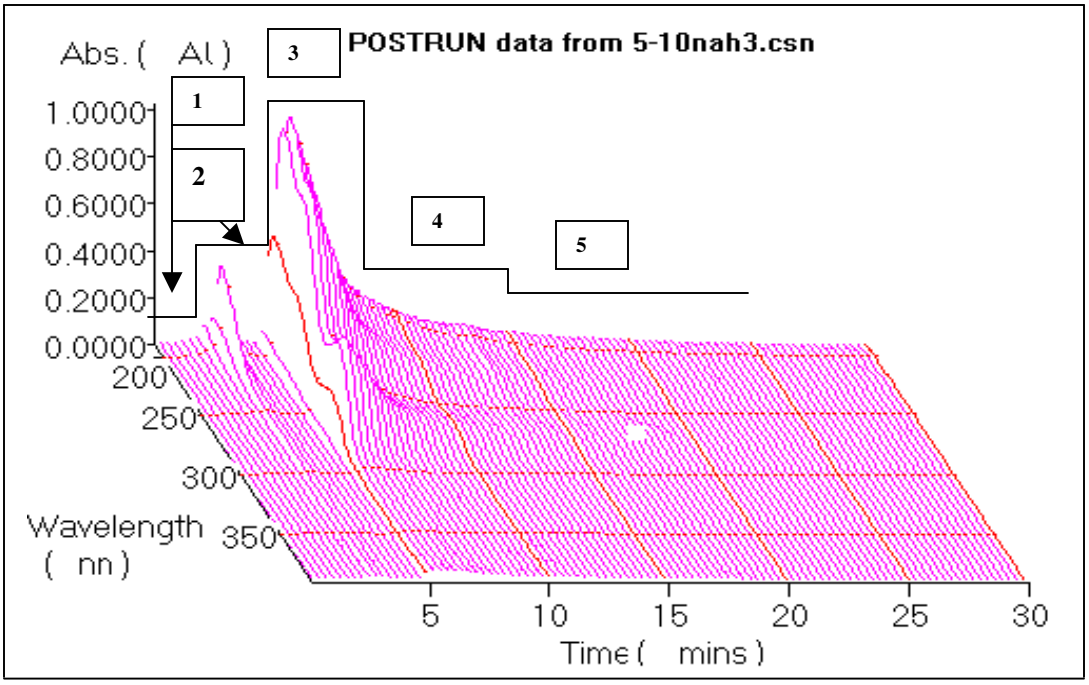

Fig. 4.20 Chromatography of the non-adsorbed eluate from a cyano SPE cartridge on an analytical cyano HPLC column

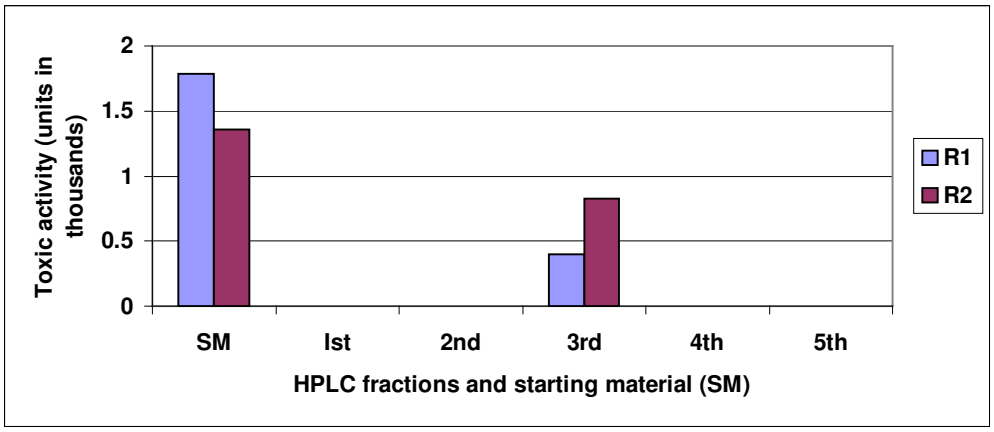

Fig. 4.21 Toxic activity present in the HPLC fractions of non-adsorbed eluate from the cyano SPE cartridge

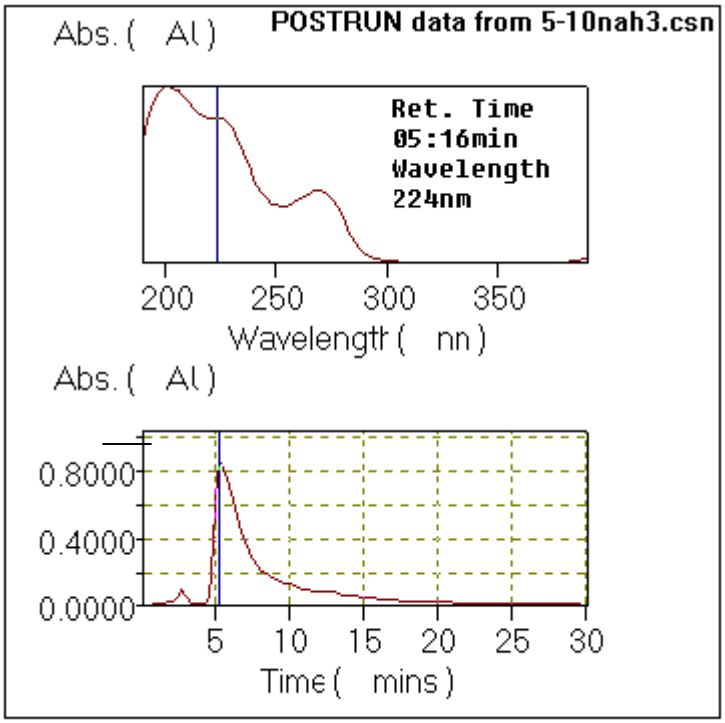

Fig. 4.22 Retention time and absorption maxima of the HPLC fraction 3 found in the non-adsorbed eluate when chromatographed on an analytical cyano HPLC column

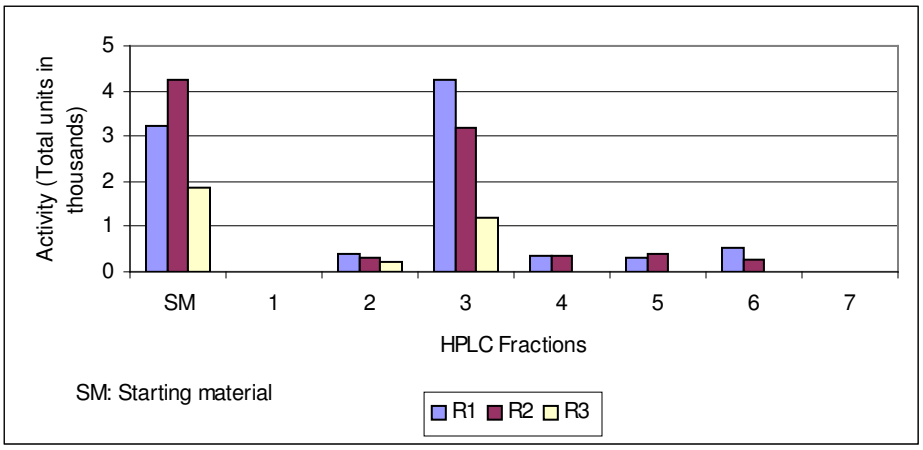

Fig. 4.23 Toxic activity present in the HPLC fractions of 40% ACN eluate of cyano SPE cartridge on a semi-preparative cyano column

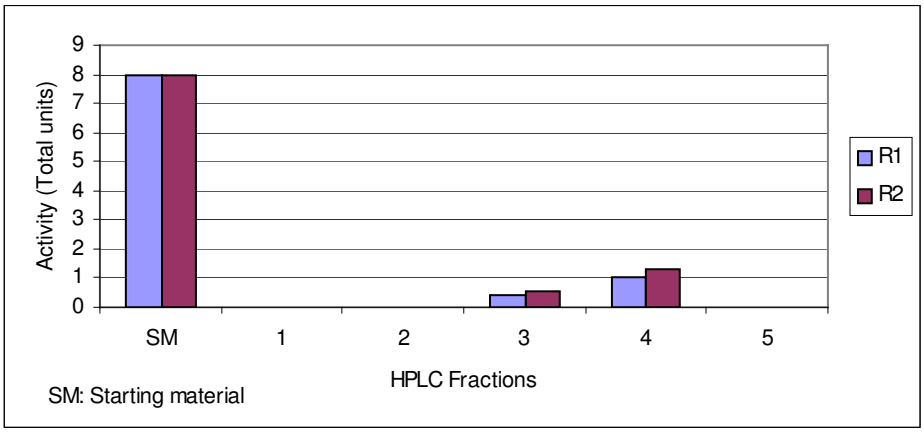

Fig. 4.24 Toxic activity present in the HPLC fractions of 50% ACN eluate of cyano SPE cartridge on a semi-preparative cyano column

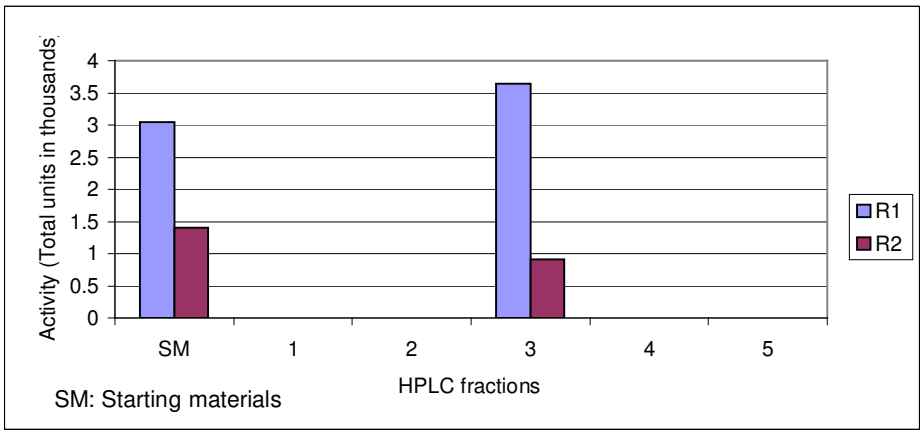

Fig. 4.25 Toxic activity in the HPLC fractions of NAF eluate of cyano SPE cartridge on a semi-preparative cyano column

4.4 Discussion

Plant pathogens produce a variety of secondary metabolites in culture that show phytotoxic activity but only a small proportion of these have a demonstrated role in plant disease. Knoche and Duvick (1987) described phytotoxin as a low molecular weight compound produced by a microorganism that may cause necrosis, chlorosis, wilting, or a combination of these symptoms in susceptible hosts. Toxins may be classified as host-selective (host-specific) or non-selective (non-specific). Host-selective toxins are toxic to those plant species or cultivars that serve as hosts for the toxin producing pathogen and lack toxicity towards non-hosts but a non-selective toxin may exhibit differential toxicity towards various plant species but toxicity is not highly correlated with the toxin-producer's host range.

Most host-selective toxins belong to fungi of the genera *Alternaria* and *Cochliobolus* (= *Helminthosporium*). For e.g. *Alternaria alternata* produce epoxy-decatrienoic esters group toxin on Japanese pear called AK-toxin, on strawberry called AF-toxin, on tangerine called ACT-toxin, cyclic tetrapeptide group toxin on apple called AM-toxin, aminopentol esters group toxin on tomato called AAL toxin and terpenoid group toxin on rough lemon called ACR(L)-toxin. While *Cochliobolus carbonum* produce cyclic tetrapeptide group toxin on corn called HC-toxin, *C. heterostrosphus* produce linear polyketols group toxin on corn called T-toxin and C. *victoriae* produce cyclized chlorinated peptide group toxin on oats called victorin (Wolpert *et al*., 2002).

Non-host selective toxins such as fungi belong to the genera *Fusarium* and *Ascochyta* and bacteria of the genera *Streptomyces* and *Pseudomonas.* For examples, *Fusarium monliforme* produce aminopentol esters group toxins on maize called fumonisins, *F.*

graminearum produce terpenoids group toxins, potent inhibitor of protein synthesis, on wheat called trichothecene and *Ascochyta rabiei* produce polyketide group toxins on chickpea called solanapyrones a, b and c. Some of the bacteria that include *Pseudomonas syringae* produce amino-acid derived toxin (dipeptide of either threonine or serine linked to tabtoxinine-β-lactam) on tobacco called tab-toxin and *Streptomyces scabies* also produce amino-acid derived toxin (4-nitotryptophan and phenylalanine groups linked in an L, L-configured cyclodipeptide) on potato called thaxtomins toxins (Strange, 1993 and 2003).

A variety of chromatographic methods, such as thin layer chromatography (TLC), flash chromatography, high performance liquid chromatography (HPLC) and solid phase extraction (SPE), have been used to purify various toxic compounds. For instances, Schaafsma *et al*. (1988) showed that TLC methods for identifying and quantifying the mycotoxins, deoxynivalenol, zearalenone and the fumonisins, often found in cereals infected by species of *Fusarium*, were cheaper than ELISA and HPLC methods and equally reliable. Milat and Blein (1995) used flash chromatography to purify the toxin cercosporin, a red coloured compound, extracted from the mycelium of *Cercospora beticola*, the causal agent of a leaf spot disease of sugar beet.

Solid phase extraction has been used to separate and concentrate toxins from several fungi and in some cases pure compounds may be obtained by the judicious use of SPE media and eluting solvents. For example Poling and Plattner (1996; 1999) obtained pure preparations of fumonisins B_3 and B_4, produced by *Fusarium moniliforme,* using successively NH_2 and C_{18} SPE cartridges, which were eluted with 5% acetic acid in MeOH and a gradient of ACN in water, respectively.

More generally, SPE is used as a clean-up process prior to other methods of separation. For example, Chen and Strange (1994) used C_{18} SPE cartridges to purify partially and concentrate the solanapyrone toxins from the culture filtrates of *Ascochyta rabiei* before quantitative analysis by reversed phase HPLC. Similar techniques have been used with toxins from species of *Fusarium.* For example, Mateo *et al*. (2001) used Silica and Florisil SPE cartridges as a means of preparing samples for chromatographic analysis of type B trichothecenes produced by *Fusarium graminearum* and *F. culmorum* in cereals. Radova *et al*. (1998) found that SPE provided a better method for the clean up of seven trichothecene toxins than gel permeation chromatography prior to analysis of the mycotoxins by gas chromatography of their trifluoroacyl derivatives.

In the present investigation, C_{18} and cyano SPE cartridges were used to purify the toxin of FOC 5 from EtOAc extracts of culture filtrates of the fungus. In order to exploit the selectivity of the media in the cartridges as far as possible, they were eluted in water containing stepwise increments of increasing organic solvent. Attempts were then made to isolate the active compound by semi-preparative HPLC on stationary phases consisting of the same type of materials as in the cartridges but with smaller particle size.

These experiments showed that toxic activity was better adsorbed by the cyano cartridges than the C_{18} cartridges, the non-adsorbed fraction averaging just under 6% of the toxin applied to the former compared with more than 9% of that applied to the latter. Moreover, when the cyano cartridge was eluted with ACN, most of the adsorbed activity that was recovered (about 45%) came off as a fairly sharp peak in the 40% and 50% ACN eluates. In contrast when C_{18} cartridges were eluted low activity appeared in many fractions.

In agreement with the results from the SPE cartridges, separation on cyano HPLC columns was better than C_{18} columns. However, attempts to obtain a pure compound by semi-preparative HPLC with a cyano stationary phase were frustrated by poor recoveries and loss of activity. Therefore, alternative techniques were sought such as flash chromatography, which is described in the next chapter.

CHAPTER V

Purification of the FOC toxin by flash chromatography and HPLC

5.1 Introduction

In chapters 2 and 3 it was concluded that out of the five isolates tested, FOC 5 produced the highest toxin titres. In chapter 4 the toxin was partially purified by solid phase extraction (SPE), but elution from the C_{18} and cyano cartridges in aqueous methanol and aqueous acetonitrile, respectively did not result in the production of a pure compound. Therefore a different chromatography procedure was required.

Flash chromatography is widely used in the pharmaceutical industry for separation of natural products and organic synthetic compounds from other constituents of the reaction mixture. The essence of flash chromatography is the use of columns of large particle size silica (40-60 μm) through which appropriate solvents, under low pressure (0.35-1.05 bar) usually from a compressed air line, are percolated. This allows quick separation of mixtures of compounds with reasonable resolution. Fractions are collected manually and may be monitored by a variety of techniques according to the product sought. In the present investigation, a commercial apparatus was used consisting of a solvent reservoir, a cylinder containing a disposable cartridge of pre-packed silica or C_{18} resin and an injection port (Fig. 5.1).

Both normal phase chromatography on silica gel and reversed phase chromatography on a C_{18} resin were used. Normal phase was used for purification of the toxin from the ethyl acetate (EtOAc) fraction of the culture filtrate of FOC 5 (section 3.2.1) while reversed phase

was used for purification of toxin from the fraction that was not adsorbed by a C_{18} solid phase extraction cartridge (section 4.2.1 and fig. 4.1). Fractions that were active in the bioassay (see section 2.2.6 for method) were examined by HPLC (see sections 4.2.2. and 4.2.3 for methods).

A flow diagram of all the methods used in toxin purification is provided in Appendix IX.

5.2 Materials and methods

5.2.1 Finding suitable mobile phases for flash chromatography

In order to find an appropriate mobile phase for flash chromatography on silica gel it was necessary to perform scouting runs using Si-gel thin layer chromatography. Normally, Rf values of between 0.15 and 0.35 are optimal for using flash chromatography. Ethyl acetate fractions from liquid-liquid extractions and active fractions from the stepwise elution of C_{18} SPE cartridges in aqueous methanol (50-100%; section 4.3.1 and fig. 4.1) were spotted on silica gel thin layer plates (silica gel 60 F_{254}, Merck, Germany) and the plates developed in various solvent systems (Table 5.1). After allowing the plates to dry, they were observed under short (254 nm) and long (350 nm) wavelength UV light.

Solvent systems	Ratio of constituents						
	DCM	Cyclohexane	Isopropan-2-ol	EtOH	MeOH	Butanol	Acetone
1	4	-	-	-	1	-	-
2	8	-	-	-	1	-	-
3	4	4	-	-	1	-	-
4	4	4	-	1	-	-	-
5	4	4	-	-	-	1	-
6	1	1	-	-	-	-	-
7	1	1	-	-	-	-	1
8	4	4	1	-	-	-	-
9	8	8	1	-	-	-	-
10	1	1	1	-	-	-	-
11	49.5	49.5	1	-	-	-	-
12	49	49	2	-	-	-	-

Table 5.1 Solvent systems tested for their suitability as mobile phases for normal phase flash chromatography

5.2.2 Purification of the EtOAc fraction of the culture filtrate of FOC 5 on Si-gel flash chromatography

The EtOAc fraction from 2-3 L of culture filtrate of FOC 5 was dissolved in ACN (4 ml) and 200 µl retained for bioassay. The remainder was injected onto a dry cartridge of silica (40 g, Biotage UK Ltd., Hertford, UK) on a flash chromatography apparatus (Fig. 5.1). Solvents used to elute the column were 100% cyclohexane (100 ml) followed by dichloromethane/cyclohexane/isopropanol 8:8:1 (1L) and finally MeOH (200 ml). The eluates were collected as 25 ml fractions and samples (5 ml) were film-evaporated to dryness. Residues were dissolved in 1 ml ACN and assayed (section 2.2.6).

5.2.3 Purification of the active Si-gel flash fraction on HPLC

Active fractions from flash chromatography were examined by analytical HPLC on a cyano column with 30% ACN as mobile phase (section 4.2.3). Flash fractions that gave similar chromoscan profiles were pooled and evaporated on a rotovapour. The residue was dissolved in ACN (5 ml) and samples (500 µl) chromatographed on a semi-preparative cyano HPLC column (150 X 10 mm diam.) with 30% ACN as mobile phase. Recovery of activity was checked by running the HPLC for 30 min and collecting the effluent as one fraction. Thereafter, peaks recorded by the diode array detector with similar retention times and spectra were amalgamated and the ACN removed by film evaporation. Compounds were recovered from the remaining aqueous solutions by partitioning into EtOAc (section 3.2.1). After film evaporation of the EtOAc, the residues were dissolved in 1 ml of ACN and checked for purity by running on an analytical HPLC cyano column and for activity by the cell assay (section 2.2.6).

5.2.4 Separation of compounds present in the non-adsorbed fraction (NAF) from C_{18} SPE cartridges by flash chromatography on a C_{18} stationary phase

The C_{18} cartridge was prepared as per the guidelines of the manufacturer (Biotage, 1500 Avon Street Extended, Charlottesville, VA 22902, USA) by flushing successively with 30 ml volumes of 100%, 80% and 50% MeOH. A small volume (500 µl) of the sample (NAF of the C_{18} SPE cartridge; section 4.3.1 and fig. 4.1) was retained for bioassay in order to determine its activity and the remaining 99.5 ml was adjusted to pH 3.0 with dilute sulphuric acid. After partitioning against EtOAc (section 3.2.1) the EtOAc fraction was evaporated to dryness on a rotovapour and the residue dissolved in 0.5 ml MeOH. The MeOH solution was diluted to 4.5 ml with water and applied to the cartridge.

The cartridge was eluted in 10% MeOH with 0.1% triethylamine (TEA; 200 ml) followed by MeOH (100 ml) and the eluent was collected as 25 ml fractions. Fractions containing aqueous MeOH and TEA were evaporated on a rotovapour in order to remove the organic constituents of the solvent and concentrated by partitioning against EtOAc as described before (section 3.2.1) and dissolved in MeOH (1 ml). The MeOH solutions were examined for UV absorbing compounds on a spectrophotometer over a range of 220-350 nm and assayed as usual (section 2.2.6). They were also examined by reversed phase analytical HPLC with an Apex II C_{18} HPLC column (5µ, 150 X 4.6 mm diam.) as stationary phase and 5% MeOH + 0.1% TEA as mobile phase.

5.3 Results

5.3.1 Finding a suitable mobile phase for flash chromatography

When the EtOAc fraction, obtained from partitioning culture filtrates of FOC 5 against the solvent, was chromatographed on Si-gel TLC plates in 12 different solvent mixtures (Table 5.1) DCM: cyclohexane: isopropanol 8:8:1 (v/v/v) gave the best separation with prominent bands at Rf values 0.36 (mauve), 0.27 (mauve), yellow (0.23) and 0.13 (red). Rather different results were obtained when active fractions desorbed from SPE C_{18} cartridges were chromatographed. Here the prominent red band had moved further to 0.20, the yellow to 0.26 and one mauve at Rf 0.37 (faint) and the other at 0.49 (medium strength; fig. 5.2). Although the compound from the SPE fractions, which ran furthest in this solvent, had too high an Rf value for separation by flash chromatography, it was, nevertheless, selected as the other compounds were close to or within the range recommended.

5.3.2 Purification of the EtOAc fraction of culture filtrates on Si-gel flash chromatography

No toxic activity was found in fractions 1-3, which were eluted in cyclohexane but toxic activity was found in fractions 5-35 (with the exception of fraction 7) which were eluted in DCM/cyclohexane/isopropanol @ 8:8:1 and in fractions 45-47, which were eluted in MeOH. A prominent peak was noted in fractions 9-11 and comprised 30.5% of the total recovered. The remainder of the activity (43.6%) was found distributed mainly in a broad peak comprising fractions 12-35. A small amount of residual activity (7.5 %) was present in fractions 45-47 (Fig. 5.3). When the activity of the fractions was summed and compared with the starting material, recovery was 85%, 131% and 99% for three replicate experiments.

5.3.3 Examination of the active flash fractions on HPLC

When fractions 9-25 (see above and fig. 5.3), were examined by HPLC on an analytical cyano column with 30% ACN as mobile phase, fractions 9-11 had four peaks in common. These eluted at 1 min 55 sec (λmax at 195, 226 and 262 nm), 2 min 49 sec (λmax at 232 and 260 nm), 8 min 13 sec (λmax at 222 and 268 nm) and 10 min 7 sec which also had λmax at 222 and 260 nm (Figs 5.4-5.7.). In contrast, fractions 12-25 had three peaks in common. These eluted at 2 min 1 sec, 2 min 49 sec and 5 min 43 sec. The first two peaks had the same absorption maxima i.e. 224 and 281 nm but the third peak had absorption maxima of 224 and 269 nm (Figs. 5.8-5.10).

When flash fractions 12-25 were pooled and their constituents separated by HPLC on a semi-preparative cyano column (Fig. 5.11), most of the activity was found in fractions 2, 3 and 4. Fraction 2 was a double peak with similar spectra eluting between 2 min 1 sec and 2 min 49 sec. Fraction 3 was collected between 2 min 50 sec and 5 min 43 sec and corresponded to the majority of a large peak and fraction 4 was the tail of this peak which eluted between 5 min 44 sec and 9 min. Fractions 5 and 6 also had some activity (Fig. 5.12) but this may also have been caused by tailing of the compound which peaked in the third fraction. Recovery of toxic activity was 110, 61 and 69% in three replicates and the contribution of each fraction was additive rather than synergistic. However, on rechromatography of fractions 2, 3 and 4 single peaks were not obtained suggesting degradation of the compounds. For this reason this approach was not pursued.

5.3.4 Separation by reversed phase flash chromatography of components of the EtOAc fraction not adsorbed by C_{18} SPE cartridges

When the non-adsorbed fraction from C_{18} SPE cartridges (section 4.3.1 and fig. 4.1) was introduced into a reversed phase C_{18} flash cartridge and eluted initially with 10% MeOH + 0.1% TEA and finally with 100% MeOH, most of the toxic activity was found in fractions 3-9 (Fig. 5.13).

Upon checking these fractions on a spectrophotometer two peaks at 270 nm were observed, the first at fractions 6 or 7 (depending on sample) and the second at fraction 9. The first of these peaks corresponded with a peak of toxic activity, although lower levels of toxic activity were present in other fractions as well (Fig. 5.13).

HPLC of fraction 7, which had high activity in both runs, on an analytical Apex II C_{18} column with a mobile phase of 5% MeOH + 0.1% TEA showed there were two peaks of UV absorption. One was sharp and had a retention time of 355 sec and the other was broader with a retention time of 505 sec. Both had absorption maxima of 230 and 270 nm (Fig. 5.14).

Fig. 5.1 The flash chromatography apparatus (Biotage UK Ltd., Hertford, UK) consisting of a solvent reservoir reservoir, a cylinder containing a disposable cartridge of pre-packed silica or C_{18} resin and an injection port

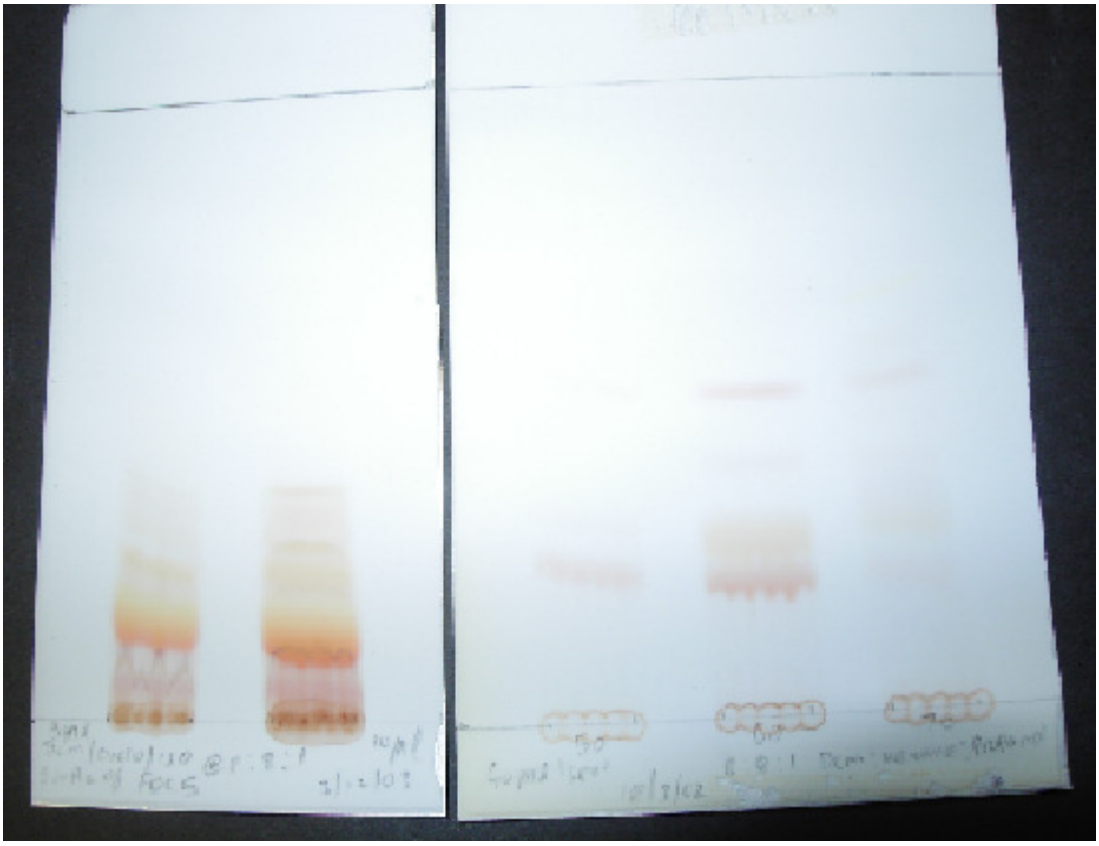

Fig. 5.2 Separation of toxic fractions of culture filtrates by Si-gel thin layer chromatography. The plate on the left is the EtOAc fraction from a culture filtrate of FOC 5 (814 units activity/spot) developed in DCM/cyclohexane/isopropanol 8:8:1 (v/v/v). The plate on the right shows the result of chromatography of SPE fractions from a C_{18} cartridge with, from left to right fractions eluting in 60%, 70% and 80% MeOH, corresponding to 37, 62 and 65 units of activity/spot, respectively (cf. fig. 4.1-chapter 4).

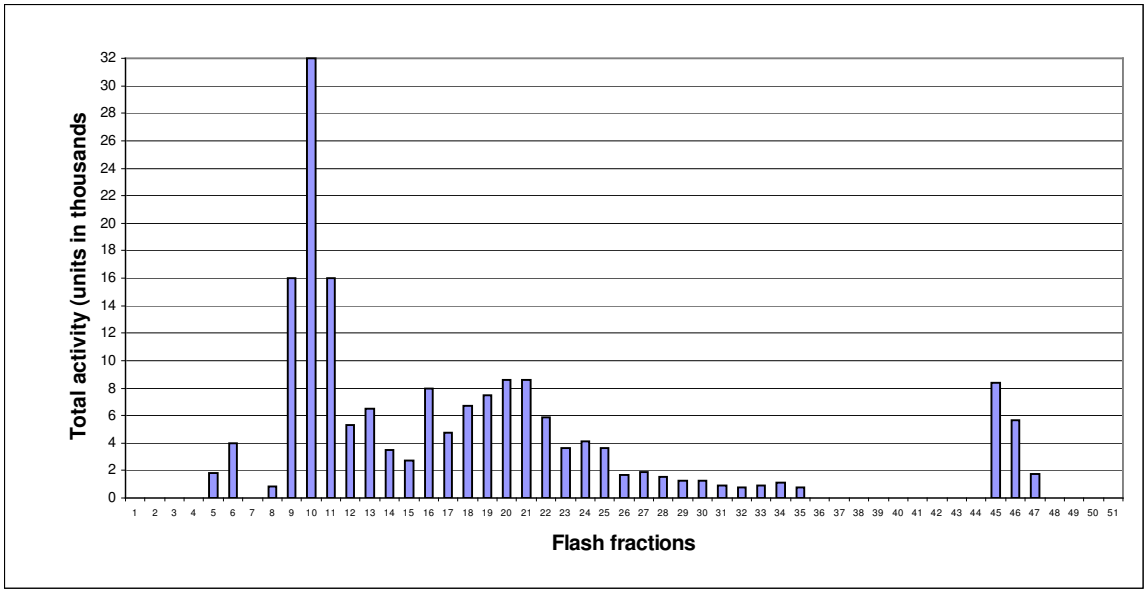

Fig. 5.3 A representative example (one of three) of purification of the EtOAc fraction of a culture filtrate of FOC 5 by flash chromatography on Si-gel. Fractions 1-3 were eluted with cyclohexane, fractions 4-43 with DCM/cyclohexane/isopropanol 8:8:1 (v/v/v) and 44-51 with MeOH. All fractions were 20 ml volumes. Total recovery of activity was 84.8% of the starting material (209,820 units).

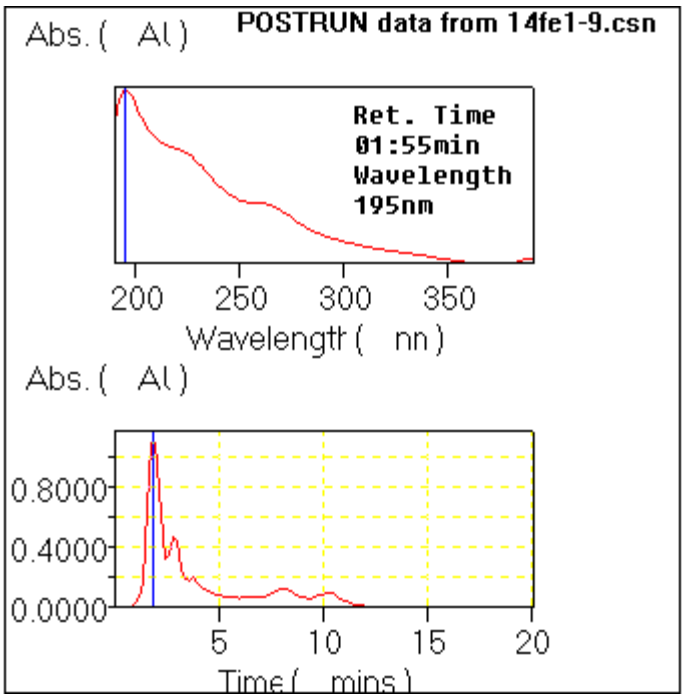

Fig. 5.4 HPLC of fraction 10 from flash chromatography of the EtOAc fraction of a culture filtrate of FOC 5 (see fig. 5.3). The lower figure shows the chromatogram cut at 195 nm and the upper figure the spectrum of the first peak of the chromatogram, which eluted at 1 min 55 sec with absorption maxima at 195, 226 and 262 nm. Fractions 9 and 11 gave similar results.

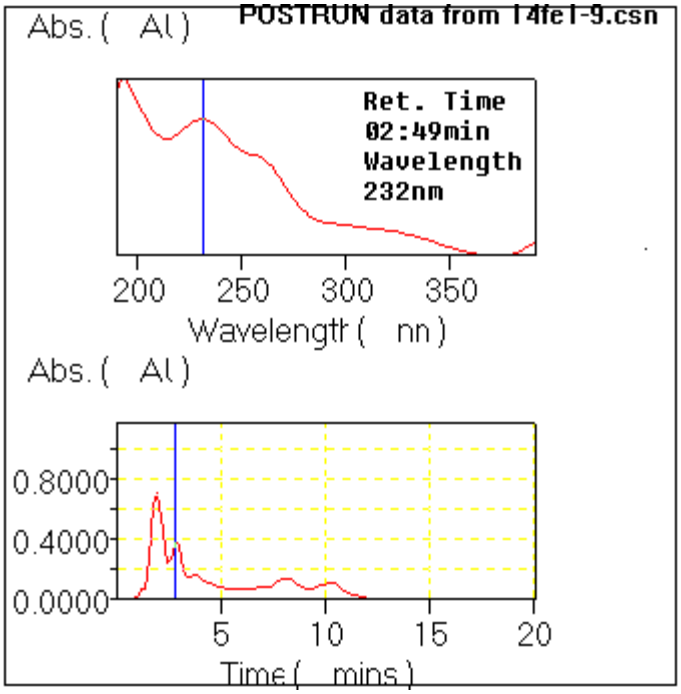

Fig. 5.5 Data as for fig. 5.4 except that the upper figure shows the spectrum of the second peak of the chromatogram which eluted at 2 min 49 sec but had absorption maxima at 232 and 260 nm

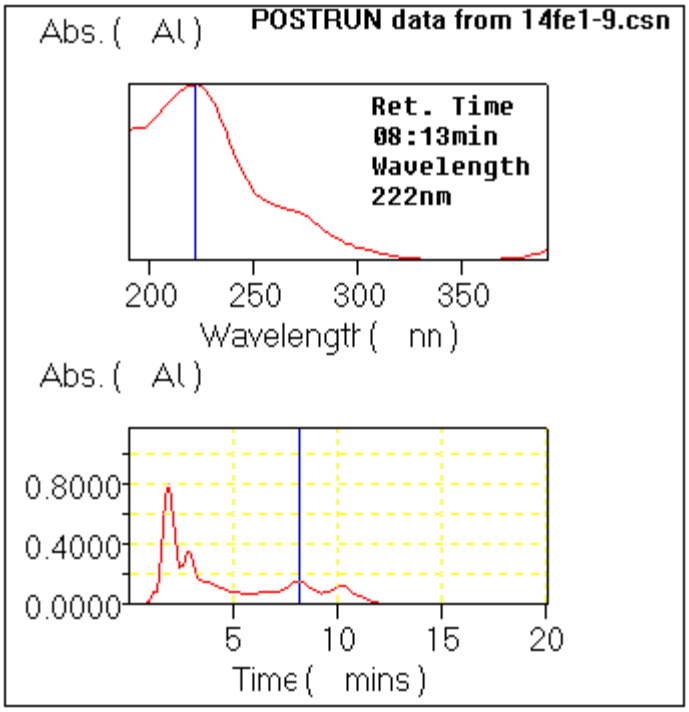

Fig. 5.6 Data as for fig. 5.4 except that the upper figure shows the spectrum of the third peak of the chromatogram which eluted at 8 min 13 sec but had absorption maxima at 222 and 268 nm

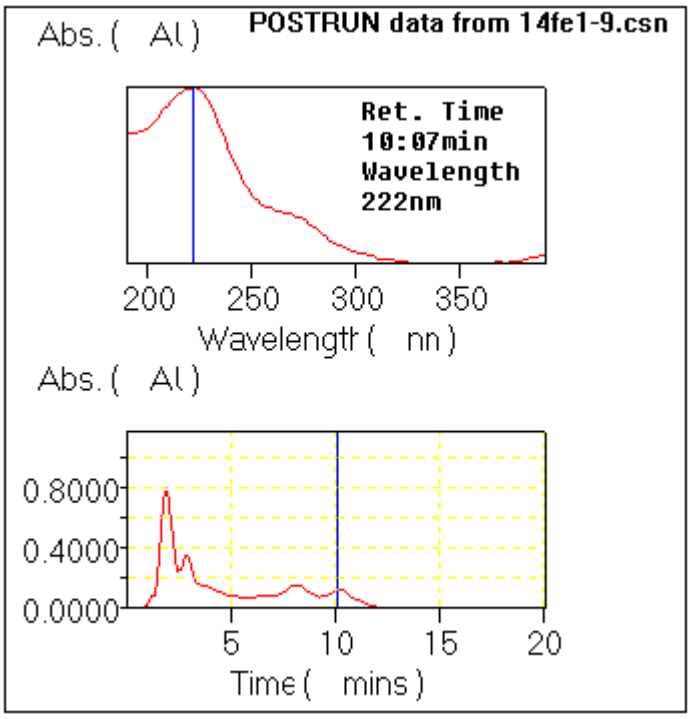

Fig. 5.7 Data as for fig. 5.4 except that the upper figure shows the spectrum of the fourth peak of the chromatogram which eluted at 10 min 7 sec but which had a similar spectrum to the third peak with absorption maxima at 222 and 268 nm

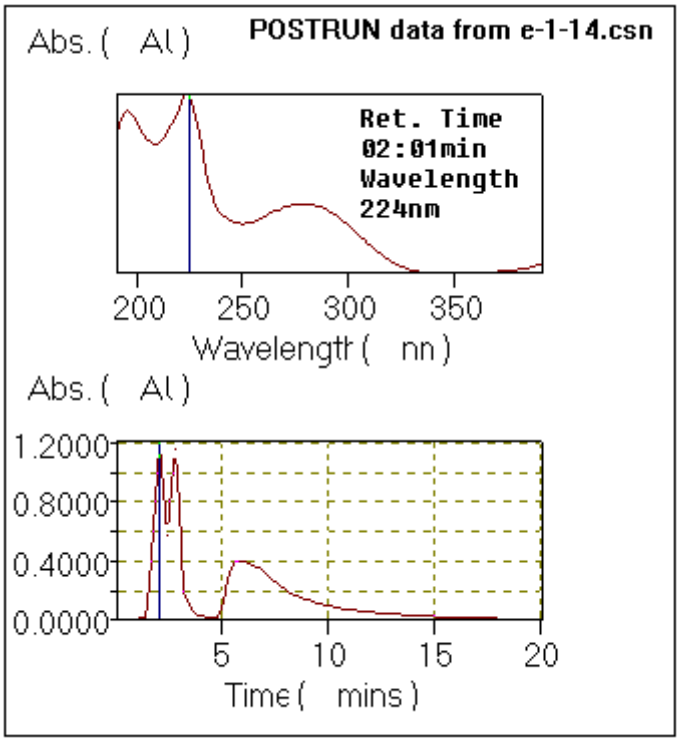

Fig. 5.8 HPLC of fraction 14 from flash chromatography of the EtOAc fraction of a culture filtrate of FOC 5 (see fig. 5.3). The lower figure shows the chromatogram cut at 224 nm and the upper figure the spectrum of the first peak of the chromatogram, which eluted at 2 min 1 sec with absorption maxima at 224 and 281 nm. Fractions 12,13 and 15-25 inclusive gave similar results.

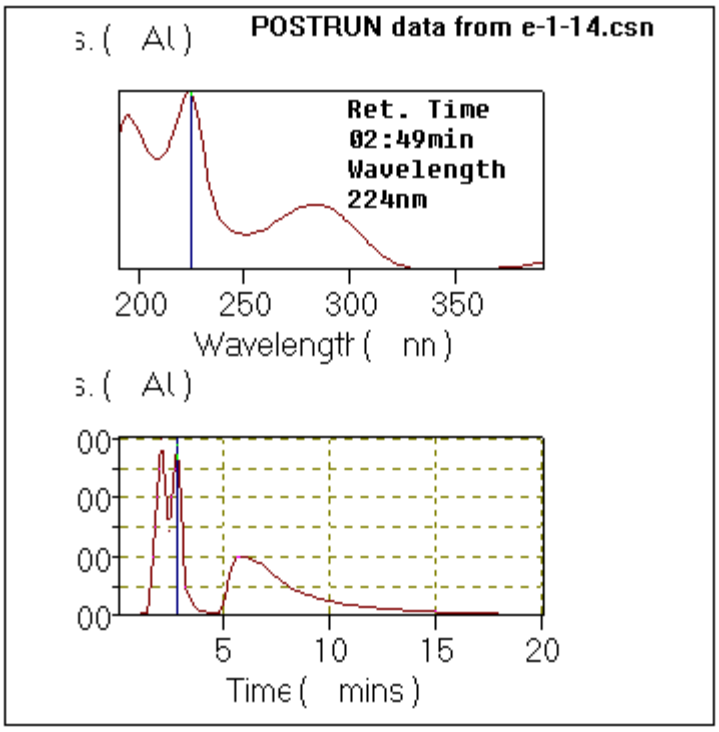

Fig. 5.9 Data as for fig. 5.8 except that the upper figure shows the spectrum of the second peak of the chromatogram which eluted at 2 min 49 sec but which had a similar spectrum to the first peak with absorption maxima at 224 and 281 nm

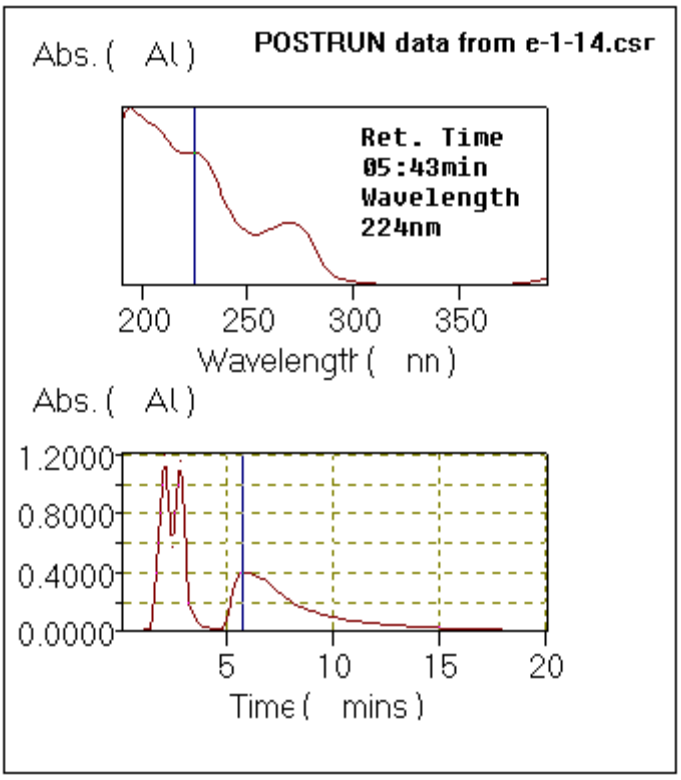

Fig. 5.10 Data as for fig. 5.8 except that the upper figure shows the spectrum of the third peak of the chromatogram which eluted at 5 min 43 sec but which had absorption maxima at 224 and 269 nm

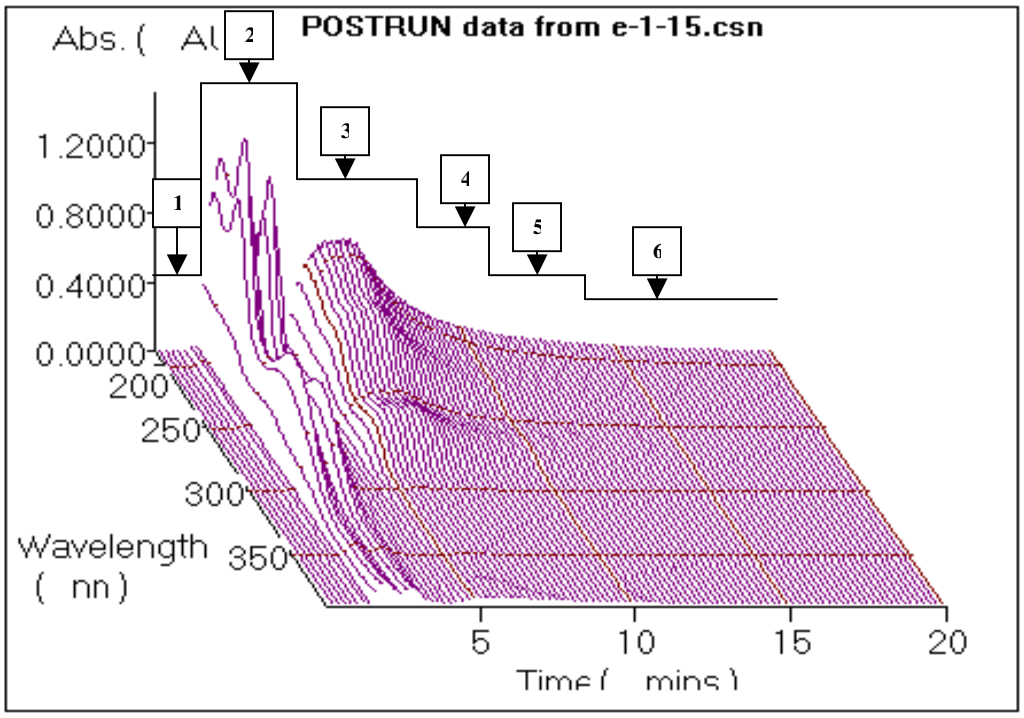

Fig. 5.11 Chromascan showing the result of chromatographing the amalgamated fractions 12-25 inclusive from flash chromatography (see fig. 5.3) on a semi-preparative cyano HPLC column. Fractions 1–6 were cut as indicated and tested for cell killing activity.

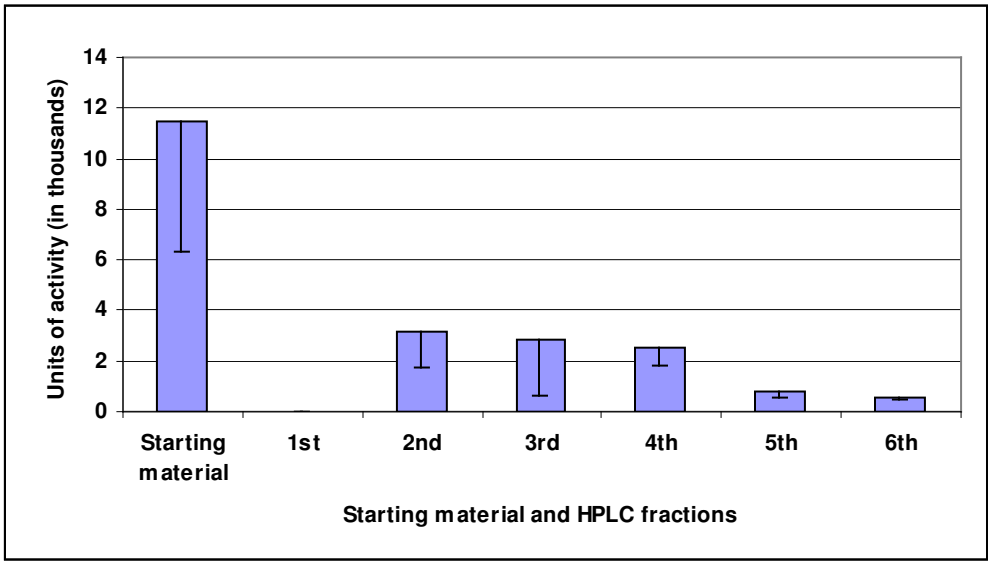

Fig. 5.12 Toxic activity of the six fractions as described in fig. 5.11 (mean of 3 replicates; error bars are standard deviations)

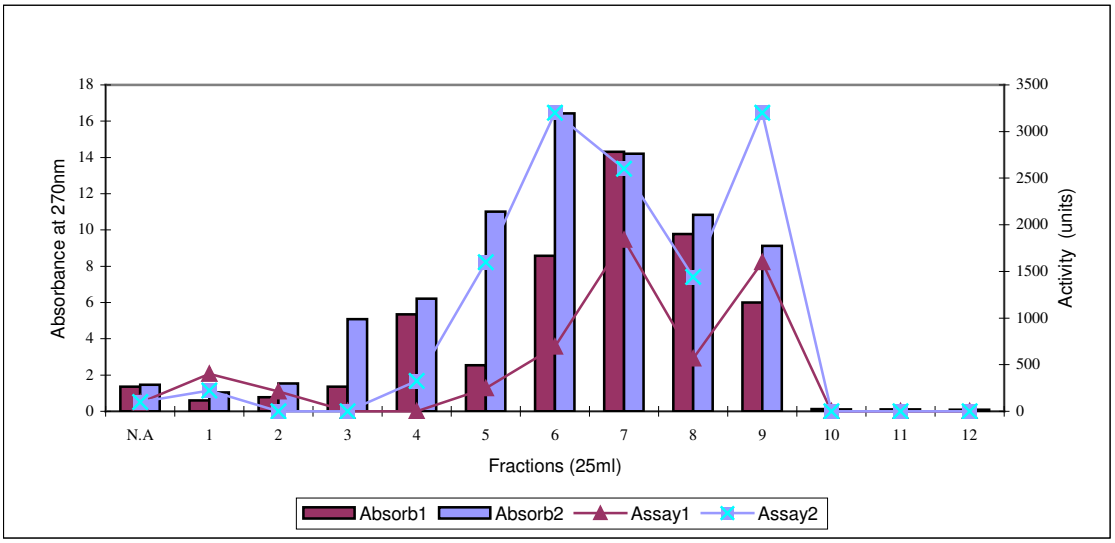

Fig. 5.13 The EtOAc fraction of a culture filtrate of FOC was adsorbed onto a C_{18} cartridge and eluted in increments of increasing MeOH (see fig. 4.1-chapter 4). The present figure shows the result of chromatographing the fraction that was not adsorbed by the cartridge (NAF). Fractions (25 ml) were assayed for activity and their absorbance at 270 nm was recorded.

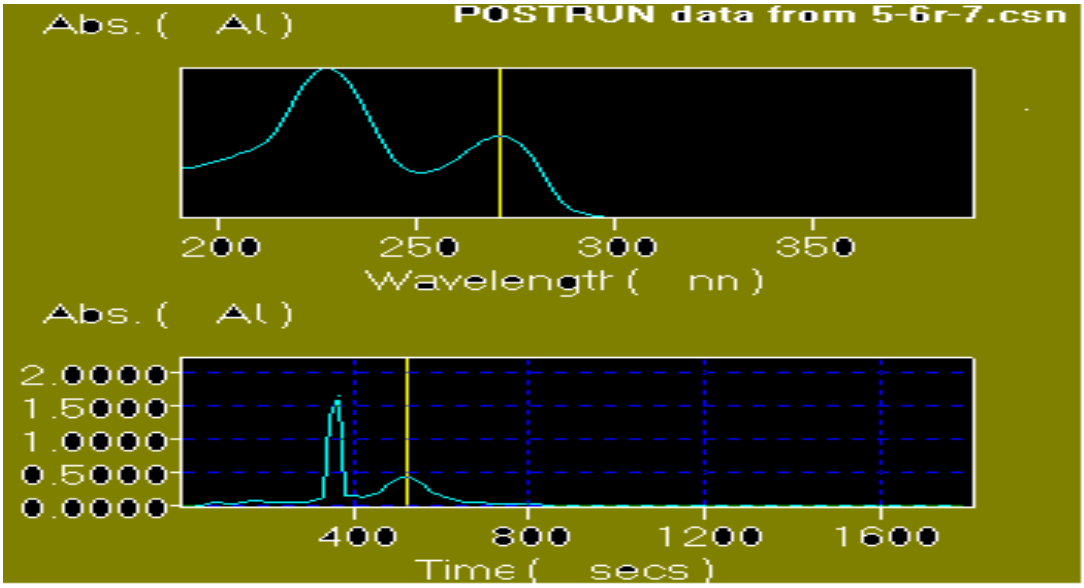

Fig. 5.14 HPLC of fraction 7 (see fig. 5.13) on a C_{18} reversed phase column (Apex II, 15 X 4.6 mm diam) with 5% MeOH containing 0.1% triethylamine as mobile phase. The chromatogram cut at 270 nm showed two peaks one of which eluted at 355 sec and the other, which was broader, with a retention time of 505 sec. Both had absorption maxima at 230 and 270 nm.

5.4 Discussion

Flash chromatography has been used to separate and concentrate several toxins produced by various fungi and, in some cases, pure compounds have been obtained. For example, Alam *et al*. (1989) described a technique by which solanapyrones A and C, produced by *Ascochyta rabiei*, were isolated by solvent partitioning of culture filtrates with EtOAc and flash chromatography of the organic fraction on silica. Hamid and Strange (1997) showed, using the same EtOAc preparation and appropriate solvents, that pure samples of solanapyrone B as well as solanapyrones A and C could be obtained by flash chromatography. Recovery for solanapyrones A, B and C was 75%, 43% and 33%, respectively. Milat and Blein (1995) also used flash chromatography to purify the toxin cercosporin, a red coloured compound, extracted from the mycelium of *Cercospora beticola,* the causal agent of a leaf spot disease of sugar beet.

Flash chromatography is also used as a clean-up process prior to other methods of separation. For example, Edwards *et al*. (1996a, b) purified the toxins, known as microcystins, from MeOH extracts of cyanobacterial scum by reversed phase flash chromatography on a C_{18} cartridge and elution with either 70% aqueous MeOH or with a stepwise gradient of MeOH in water. Purification to homogeneity was achieved by subsequent HPLC on a preparative C_{18} column.

In the present investigation, a Si-gel flash cartridge was used for the clean-up of the EtOAc fraction of culture filtrates of FOC 5. The cartridge was initially eluted with DCM/cyclohexane/isopropanol 8:8:1 (v/v/v) and finally with MeOH. Toxic activity was tested in the bioassay (section 2.2.6) and fractions that were active were examined by reversed phase HPLC on a cyano analytical column. Fractions from the Si-gel flash

chromatography that had similar profiles were combined and further purified by semi-preparative HPLC on a cyano column.

The recovery of toxic activity from Si gel flash chromatography was 85%, 113% and 99% in three replicates compared with the starting material, i.e. EtOAc fraction of culture filtrates. Bioassay of the Si-gel flash fractions showed that activity was divided into two groups. The first group, comprising 30.5% of the activity, eluted at fractions 9 - 11 and each had at least four compounds with different retention times and spectra on reversed phase HPLC. Comparison of these parameters showed that the same four compounds were represented in each fraction (Figs. 5.4-5.7). The second group, comprising 43.6% of the activity consisted of fractions 12-25 and each had at least three compounds as demonstrated by different retention times on reversed phase HPLC, which were represented in all 14 of the Si-gel flash fractions. However, here the first two compounds to elute (from the reversed phase HPLC column) had identical spectra but the last differed (Figs. 5.8-5.10). A small amount of activity (7.5%) was also found when the Si-gel cartridge was finally eluted with 100% MeOH.

Fractions 12-25 from Si-gel flash chromatography which, in aggregate, had the most activity, were pooled and further purification was attempted by semi-preparative HPLC on a cyano column. These experiments suggested that all three peaks seen in the analytical HPLC (Figs. 5.8 – 5.10) were active and that recovery of activity was good (61%, 69% and 110% in three replicates). However, when the fractions were concentrated and rechromatographed on an analytical cyano column no trace of the compounds was seen suggesting that they were unstable.

In the other flash chromatography technique employed, a reversed phase (C_{18}) flash cartridge was used to purify active components of the fraction not adsorbed by a C_{18} SPE cartridge. This yielded a broad band of activity which coincided with a peak of absorption of light at 270 nm but there was also a second peak of absorption at this wavelength. In these latter fractions activity was less (Fig. 5.13). HPLC of the fraction with the most activity on a C_{18} analytical column gave two peaks. However, since this component of the EtOAc fraction of cultural filtrates represents only <10% of the total activity, further purification was not pursued.

Both the HPLC and the flash chromatography proved to be disappointing in terms of finding a combination of stationary and mobile phases that gave a pure preparation of a toxin from FOC. Moreover, these experiments suggested that the toxin may be unstable.

CHAPTER VI

Purification of the toxin by thin layer chromatography and characteristics of the purified toxin

6.1 Introduction

In chapters 2 and 3 it was concluded that out of the five FOC isolates tested, FOC 5 produced the highest toxin titres and that most of the activity could be partitioned into ethyl acetate. Chapter 4 reported the fractionation of this ethyl acetate fraction by solid phase extraction (SPE) using C_{18} and cyano cartridges and stepwise elution with incremental increases of solvent in aqueous solvent mixes. However, isolation of the toxin by semi preparative HPLC of active fractions derived from SPE was unsuccessful. Similarly, as reported in chapter 5, semi-preparative HPLC of active fractions derived from active flash fractions was also unsuccessful. Therefore attempts were made to isolate the toxin by thin layer chromatography. A flow diagram of all the methods used in toxin purification is provided in Appendix IX.

As previously explained (section 2.1), although FOC causes a wilting syndrome in chickpea, a cell bioassay technique was used to monitor the purification of the toxin from culture filtrates of FOC 5 as this offers several technical advantages as well as being quantitative. However, in this chapter, the effect of the purified toxin and culture filtrates of FOC 5 on cuttings of chickpea shoots was tested as well.

6.2 Materials and methods

6.2.1 Thin layer chromatography (TLC) of the EtOAc fraction of the culture filtrate of FOC 5

Samples of the EtOAc fraction (40 µl containing 1200 - 1600 units of activity) from the culture filtrate of FOC 5 (section 3.2.1) were spotted as 1 cm bands alternating with 1 cm non-spotted areas on silica gel TLC plates (Silica gel 60 F_{254}, Merck, Germany). They were developed in a mobile phase consisting of DCM/MeOH at 8:1 (v/v). When the solvent had run to within 1 cm of the top of the plate, the solvent front was marked and the plates were immediately removed from the chromatography tank. After drying, they were examined under normal light, and short (254 nm) and long (350 nm) wave ultraviolet light. Conspicuous bands were marked with a pencil and Rf values were calculated.

The bands were scraped separately from the TLC plates, transferred into 4 X 1.5 ml Eppendorf tubes and agitated with acetonitrile (ACN; 500 µl). Other areas of the plate adjacent to the bands and areas devoid of the sample, as controls, were also scraped from the TLC plate and agitated with ACN. Tests and controls were centrifuged at 6500*g* for 5 min. The supernatants were transferred into a vial and the remaining silica agitated again in ACN (500 µl) and centrifuged. The process was repeated once or twice more and the supernatants were amalgamated.

6.2.2 Checking the TLC fractions for toxic activity and examining their HPLC elution profiles

All the above TLC fractions were tested for toxic activity by the assay (section 2.2.6) and examined on HPLC using an analytical cyano column with 30% ACN as mobile phase (section 4.2.3).

6.2.3 Alternative method of purification of the FOC 5 toxin avoiding the use of EtOAc

Since preliminary Nuclear Magnetic Resonance (NMR) spectra suggested that solvent contaminants from EtOAc might have accounted for some of the peaks, an alternative method for toxin purification avoiding this solvent was sought.

FOC 5 culture filtrates were obtained as per section 3.2.3. Culture filtrates (50 ml) were introduced into a cyano SPE cartridge (5 g) after solvation and equilibration of the cartridge with 30 ml each of ACN and ultra purified water, respectively. The residual aqueous culture filtrate was removed by passing water and then air through the cartridge. The toxin was eluted in ACN (10 ml) and the volume reduced to 2 ml on a rotovapour. In order to minimise contamination, the following precautions were taken before chromatographing the above sample on silica gel TLC.

1. TLC plates were developed in 100% MeOH, air-dried and activated by placing in an oven for 1 h at 70°C.
2. No plastics were used in any steps of purification.

The ACN eluates were spotted as before on the silica gel TLC plates using a glass Pasteur pipette and developed as per section 6.2.1. The bands and spots were collected as per section 6.2.2 but instead of Eppendorf tubes, glass vials of 7 ml capacity were used. All TLC

fractions were checked for their toxic activity using the assay (section 2.2.6) and examined on HPLC using an analytical cyano column with 10% ACN as mobile phase.

6.2.4 Dry weight and heat stability of the pure toxin

Vials of 7 ml capacity were heated in an oven at 80°C for 1 hr. These were transferred into a desiccator for 30 min and weighed. The process was repeated until the weight of the vials was constant. Once the constant weights of the vials were obtained, purified toxin preparation of known activity (sections 6.2.1-3) was transferred to them and the vials were dried at 80°C to constant weight.

Once the dry weight of the toxin had been obtained the contents of the vials were re-dissolved in ACN (1 ml) and assayed (section 2.2.6) to check for loss of toxic activity.

In order to test further the heat stability of the toxin, samples (1 ml) of the crude EtOAc fraction (section 3.2.1) and purified toxin (1 ml) from TLC (sections 6.2.1-3) were dissolved separately in 9 ml of HB (appendix 1) and boiled in a boiling water bath for one hour before assaying (section 2.2.6).

6.2.5 Effect of purified toxin and culture filtrate of FOC 5 on plant shoot cuttings

Purified toxin of known activity in ACN was diluted to 10% ACN in water and 5 ml of this solution placed in three graduated centrifuge tubes (15 ml). Undiluted culture filtrate of FOC 5 (5 ml) was place in three further graduated centrifuge tubes (15 ml). Shoots of three-week-old chickpea plants, cultivar ILC 482, were weighed and placed in each tube. Tubes containing diluted ACN (10 %) or water were used as controls. The tubes containing the shoots were incubated at 20 ± 3°C in a green house for 4 hours and the amounts of the

solutions taken up measured. Also at this time the shoots were weighed again and transferred to new tubes containing water (5 ml) and monitored for symptoms for 24 hours.

6.2.6 TLC of EtOAc fractions from the culture filtrates of race 1, race 2, V2 and Jabalpur

The four FOC isolates other than FOC 5 studied in chapters 2 and 3, namely race 1, race 2, V2 and Jabalpur, were grown separately on CDCLM in Roux bottles (Appendix I; 100 ml/Roux bottle; 10 bottles/isolate) for 12 days at 20^{o}C. These were filtered through four layers of muslin cloth, centrifuged at 10,000*g* for 20 min and the supernatants collected. The supernatants were adjusted to pH 3.0, partitioned three times against EtOAc and the organic phases amalgamated. They were dried over anhydrous sodium sulphate, evaporated to dryness on a rotovapour and the residues dissolved in ACN (5 ml) before assaying (section 2.2.6).

The EtOAc fractions dissolved in ACN of the four isolates as well as FOC 5 were spotted on Silica gel TLC plates (Silica gel 60 F_{254}, Merck, Germany) and the plate was developed in DCM/MeOH at 8:1 (v/v). The TLC plate was air dried for a few minutes before observing under short and long wavelength UV light as per section 6.2.1.

6.3 Results

6.3.1 TLC of the EtOAc fraction of the culture filtrate of FOC 5

DCM/cyclohexane/isopropanol at 8:8:1 (v/v/v) separated the components of the EtOAc fraction of FOC 5 well but DCM/MeOH at 8:1 (v/v) also gave good separation and so this simpler combination of solvents was used as the mobile phase for further purification of the toxin by TLC (Fig. 6.1).

When the EtOAc fraction of culture filtrates of FOC 5 were chromatographed in this solvent nine bands of different colours were visible, the most prominent of which was red and had an Rf value of 0.57 (Fig. 6.1). Other bands were at Rf 0.0, 0.07, 0.2, 0.33, 0.47, 0.64, 0.68, and 0.74. When examined under short wave UV light, a dark band at the same Rf as the red band seen in visible light (Rf 0.57) was again the most prominent feature of the plate but a band at Rf 0.33 was also visible. Under long wavelength UV there was a fluorescent band at Rf 0.64.

6.3.2 Checking the TLC fractions for toxic activity

When the areas of silica corresponding to the visible bands on the TLC plates and also areas above and below these were scraped from the plate, the silica eluted and the eluates checked for toxicity, only the red band with Rf 0.57 was active.

6.3.3 Examination of the active TLC fraction on HPLC

Upon examining the active TLC fraction on an analytical cyano HPLC column using 30% ACN as mobile phase, there was only one peak which eluted at 2 min 43 sec and which

had absorption maxima of 224 and 281 nm. Since the peak was eluting very early, it was decided to find a solvent that would increase the retention time but maintain good peak geometry. When a 20% ACN mobile phase was used, the compound eluted at 5 min 46 sec and upon using a 10% ACN mobile phase, the compound eluted at 15 min 37 sec (Figs. 6.2 and 6.3). When spectra were cut at four different time intervals through the peak all had essentially the same spectrum, suggesting the compound was pure (Fig. 6.4).

6.3.4 Alternative method of purification of the FOC 5 toxin

Very similar results were obtained when culture filtrates were fractionated by solid phase extraction on a cyano cartridge rather than by solvent extraction in EtOAc. The ACN eluate from the cartridge gave a comparable chromatogram to that obtained when the toxin was solvent extracted. Also when the red band was eluted and examined on HPLC a compound with similar retention time and UV spectrum was evident (Fig. 6.5). When spectra were cut at different time intervals through the peak they were identical and were also the same as those derived from samples that had been prepared by solvent extraction (section 6.3.3; fig. 6.6).

6.3.5 Heat stability of purified toxin

When samples containing 3900 units of purified toxin (i.e. the red band from TLC; section 6.3.4) were dried to constant weight at 80°C the values obtained ranged between 0.8 and 1.0 mg (0.23 and 0.33 mg/ml) in three replicates. However, the samples maintained 62–71% of their activity suggesting that the toxin was heat stable (Table 6.1).

Reps.	Weight of vials (g)	Weight of vials + purified toxin (g)	Weight of purified toxin (g)	Total units of activity	Recovery compared with starting material (3,900 units)
R1	6.4244	6.4252	0.0008	2426	62.2%
R2	6.3740	6.3747	0.0007	2426	62.2%
R3	6.3790	6.3800	0.0010	2786	71.4%

Table 6.1 Recovery of toxic activity after drying purified toxin to constant weight

The heat stability of the purified toxin and the EtOAc fraction of the culture filtrates of FOC 5 was further tested by immersing these preparations for one hour in a boiling water bath. In these experiments 90% of the activity of the purified toxin was recovered and > 100% of the EtOAc fraction (Table 6.2).

Samples	Units of activity in **EtOAc fraction** (% recovery)	Units of activity in **Purified toxin** (% recovery)
Before boiling	2718	240
After boiling R1	3223.8 (118.6%)	216 (90%)
After boiling R2	3704.4 (136.3%)	216 (90%)
After boiling R3	2710.8 (99.7%)	216 (90%)

Table 6.2 Heat stability of the toxin in the EtOAc fraction obtained from solvent partitioning of culture filtrates and of purified toxin

6.3.6 Effect of purified toxin and culture filtrate of FOC 5 on cuttings of chickpea shoots

Shoots of chickpea, cultivar ILC 482, placed in purified toxin solution containing 2426-2786 units/ml wilted within 4 h whereas controls remained healthy (Figs. 6.7a and 6.7b). Shoots in culture filtrate containing 216 units/ml also wilted within 4 h. Water uptake by shoots in purified toxin solution and culture filtrate was considerably reduced (Fig. 6.8).

The shoot cuttings did not recover from wilting even after transferred into tubes containing water for 24 hours.

6.3.7 TLC of EtOAc fractions from the culture filtrates of isolates race 1, race 2, V2 and Jabalpur

The FOC isolate race 1 produced the highest toxin titre (more than 8000 units of activity/L culture filtrate) compared to race 2, V2 and Jabalpur (Fig. 6.9). However, this was considerably less than that normally obtained from FOC 5 ~ 80,000 units of activity/L culture filtrate). After solvent extraction in EtOAc and TLC, the typical red band of FOC 5 associated with toxicity was not detectable in preparations from any of the other four isolates although a number of pale yellow and green bands were visible (Fig. 6.10).

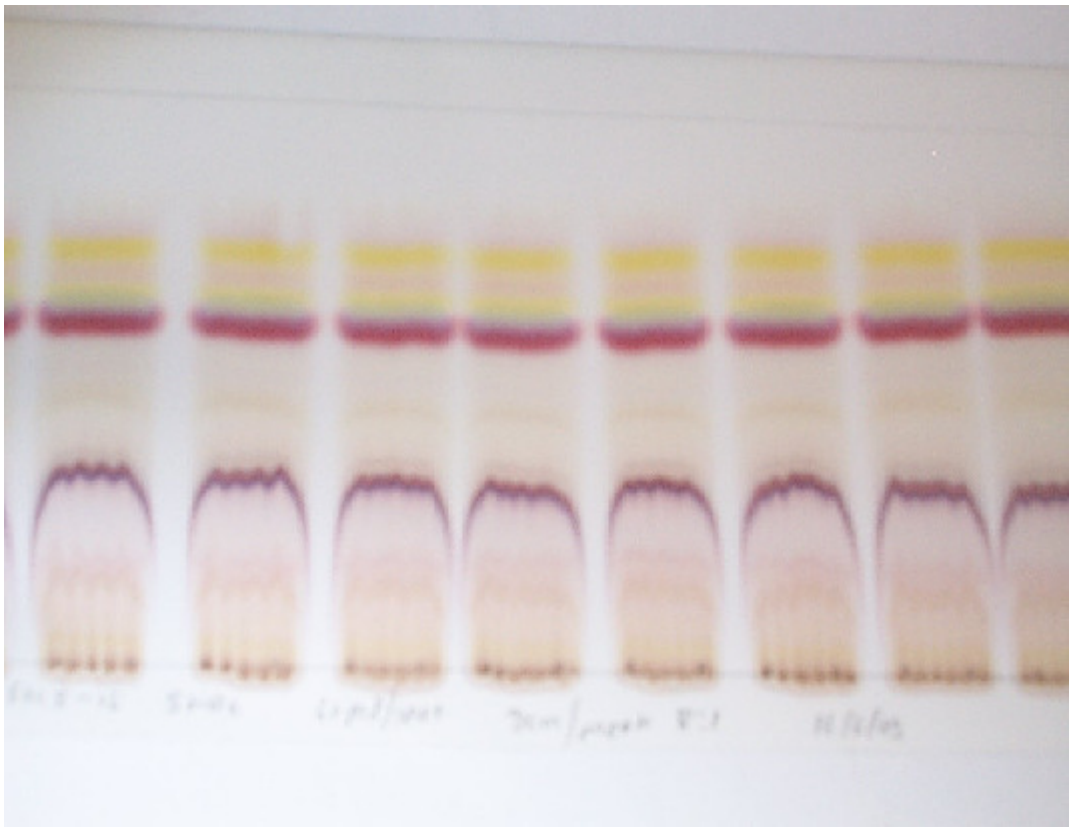

Fig. 6.1 Thin layer chromatography of the EtOAc fraction of the culture filtrates of FOC 5 with DCM/MeOH 8:1 (v/v) as mobile phase

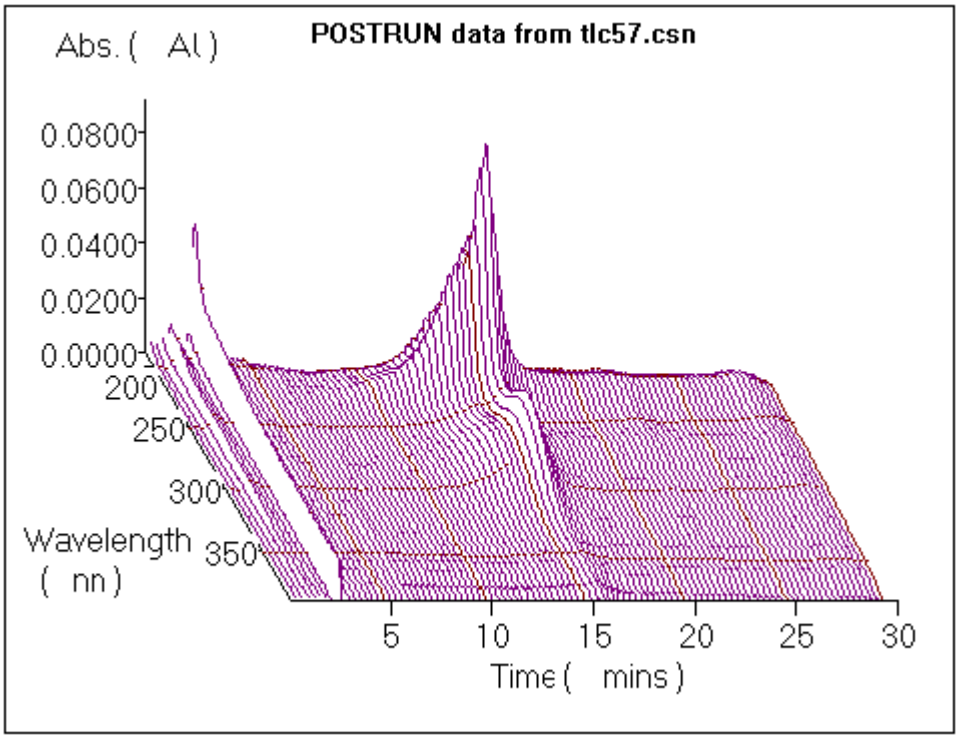

Fig. 6.2 A chromascan of the active TLC fraction showing a single peak with UV absorption maxima at 224 and 281 nm. The active TLC fraction was chromatographed on an analytical cyano HPLC column with 10% ACN mobile phase.

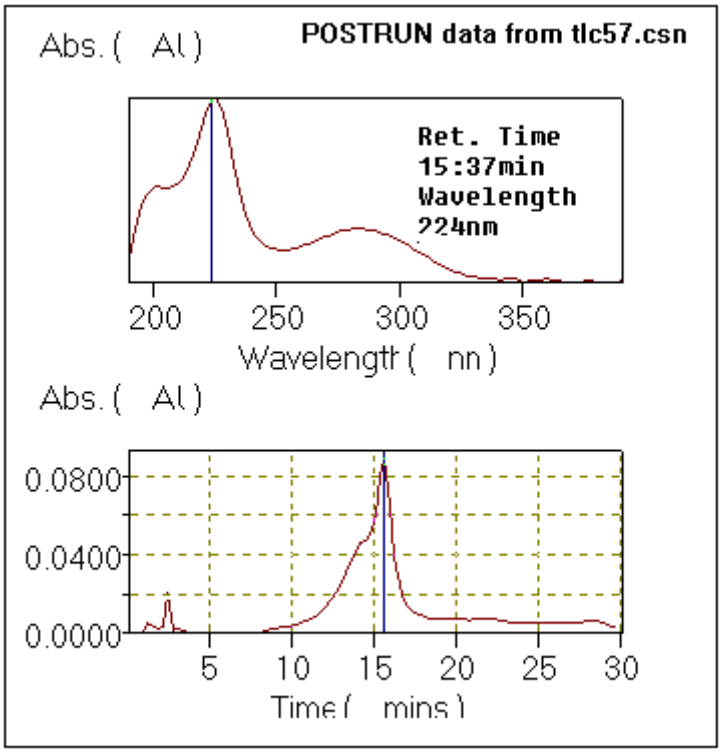

Fig. 6.3 Upper figure; spectrum of the purified toxin and lower figure; chromatogram of the purified toxin cut at 224 nm (purification by solvent partitioning method)

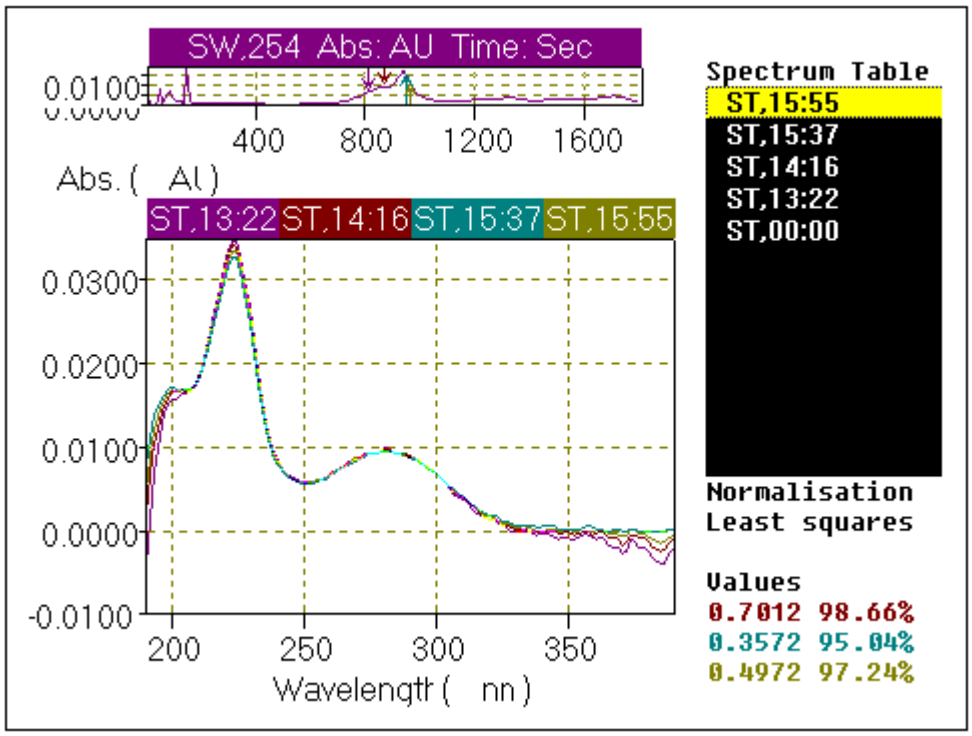

Fig. 6.4 Spectra of the purified toxin (lower figure) cut at different retention times (upper figure), purified by solvent partitioning method. The four spectra at different time intervals showed 95-98% similarity suggesting the compound was essentially pure.

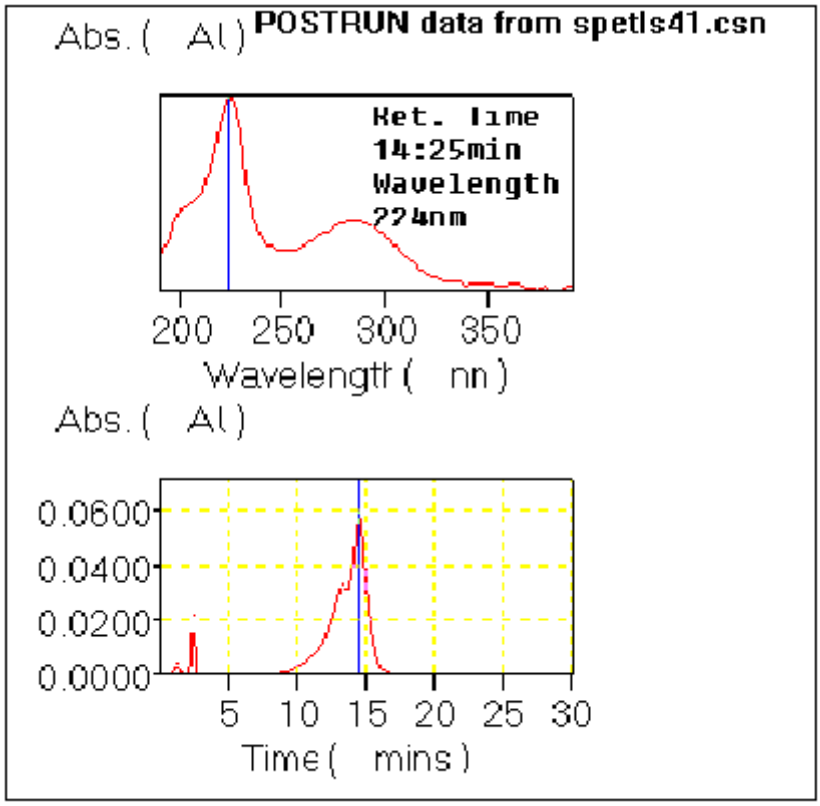

Fig. 6.5 Upper figure; spectrum of the purified toxin and lower figure; chromatogram of the purified toxin at 224 nm (purification by SPE method)

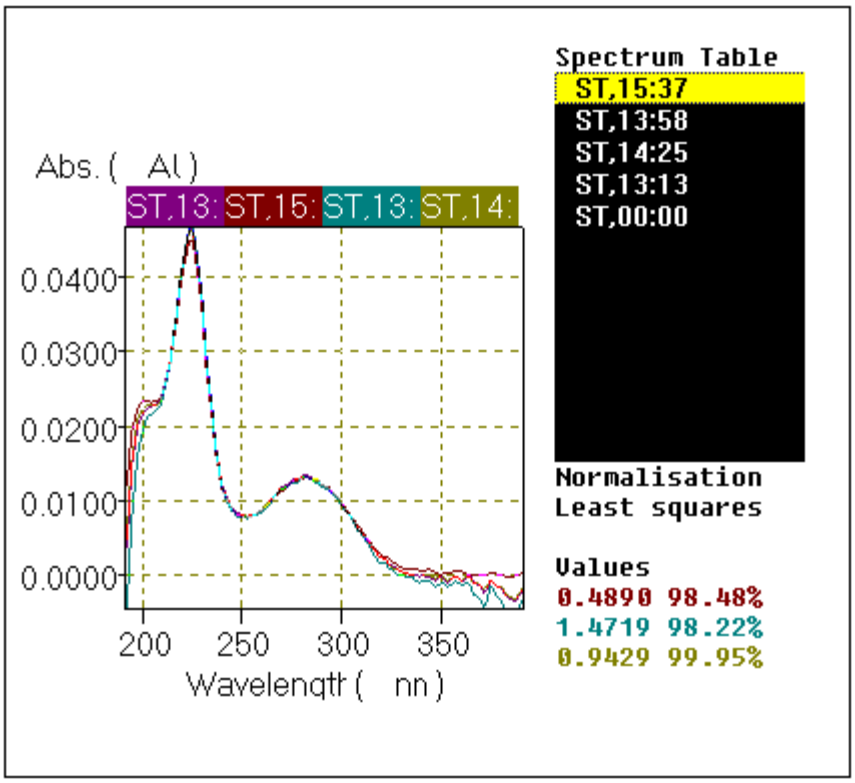

Fig. 6.6 Comparison of the spectra of the toxin purified the EtOAc partitioning method (ST13 purple and ST15 red) with toxin prepared by the SPE method (ST13 blue and ST14 green). The four spectra at different time intervals showed 98-99% similarity suggesting the compound prepared by the two methods was the same and essentially pure**.**

Fig. 6.7a Shoots, which had wilted within 4 h of treatment of a solution containing 2426 units of activity of purified toxin. The amount of toxin absorbed by the shoots of chickpea plants was 68.8 units of activity equivalent to 25.5 µg**.**

Fig. 6.7b Control shoots which remained healthy

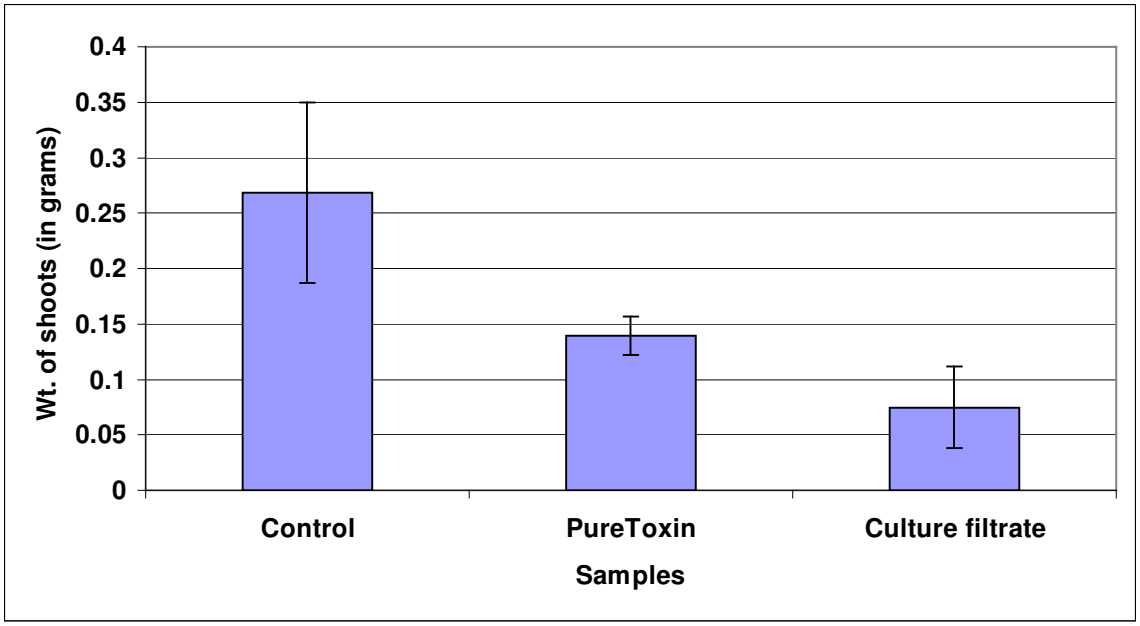

Fig. 6.8 Weight of chickpea shoots after treatment for 4h with purified toxin, culture filtrate of FOC 5 or 10% acetonitrile (control)

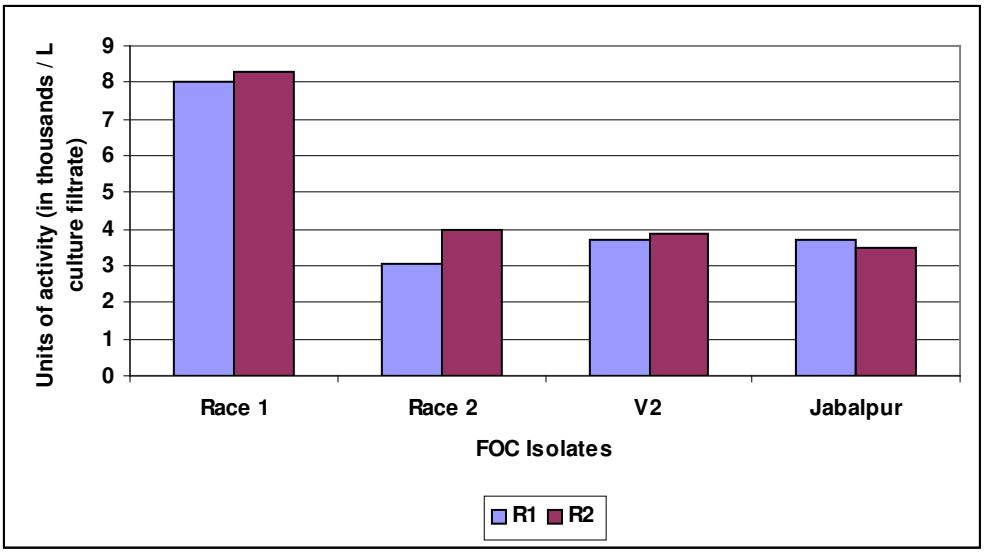

Fig. 6.9 Toxicity (units) of the EtOAc fractions from 1 L samples of culture filtrates of two replicate assays of four isolates of FOC

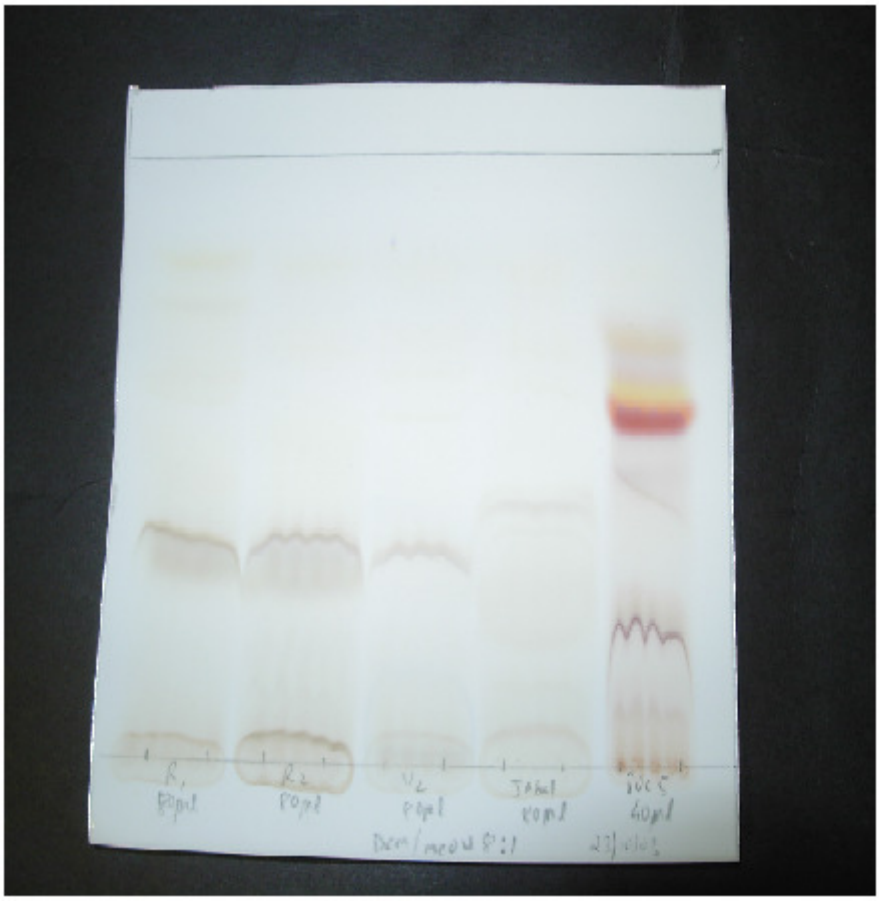

Fig. 6.10 Thin layer chromatography of the EtOAc fraction of the culture filtrates of the four isolates of FOC and FOC 5. From left to right race 1, race 2, V2, Jabalpur and FOC 5. The samples were chromatographed on DCM/MeOH 8:1 (v/v) on a silica gel TLC plate.

6.4 Discussion

Thin layer chromatography (TLC) has been used as a facile, fast and cost effective method for the analysis of a wide range of toxins produced by various fungi. For example, Milat and Blein (1995) used TLC to monitor the purity of cercosporin in flash fractions of extracts of the mycelium of *Cercospora beticola*. Also, Schaafsma *et al*. (1998) showed that TLC methods for identifying and quantifying the mycotoxins, deoxynivalenol, zearalenone and the fumonisins, often found in cereals infected by species of *Fusarium,* were cheaper than enzyme linked immuno-sorbent assay (ELISA) and HPLC methods and equally reliable.

In the present investigation, materials from culture filtrates partitioning into EtOAc or adsorbed on cyano solid phase extraction (SPE) cartridges and desorbed with ACN were chromatographed on thin layer silica gel plates with the solvent DCM/MeOH 8:1 (v/v). Activity of both preparations was associated with a red band that ran at an Rf of 0.57. When this band was eluted and examined by HPLC on an analytical cyano column using 10% ACN as mobile phase a single peak was obtained which eluted at about 15 min. The absorption spectrum of the peak had λ max at 224 and 281 nm (see Figs. 6.3 and 6.5 for spectra of the compound prepared by partitioning and solid phase extraction, respectively). Moreover, the spectra were homogeneous throughout the peak and identical in both cases (Figs 6.4 and 6.6). When the other isolates of FOC were examined i.e. race 1, race 2, V2 and Jabalpur the red compound was absent (Fig. 6.10).

The toxin was heat stable and one unit of activity was given by 327 ng in the assay.

Purified toxin and culture filtrates of FOC 5 wilted the chickpea shoot cuttings within 4h of their dipping in toxin preparation and water uptake by shoots in toxin preparation was also considerably reduced (Figs. 6.7a, 6.7b and 6.8). The amount of toxin absorbed by the

shoots of chickpea plants was 68.8 and 4.71 units of activity for purified toxin and culture filtrates, equivalent to 25.5 and 1.5 μg, respectively. Since the cuttings wilted earlier in the culture filtrates of FOC 5 than the pure toxin, it is likely that culture filtrates contained other virulence factors.

Other workers have provided evidence for the toxicity of culture filtrates of FOC. Rao and Padmaja (2000) showed that crude culture filtrates of FOC, when diluted to 30% with water, caused wilting of 1-week-old chickpea seedlings within 4-5 days. Kaur *et al.* (1987) partitioned the culture filtrate of FOC against EtOAc and discarded the organic phase, leaving an aqueous phase which inhibited callus growth of chickpea. Hamid *et al.* (2001) showed that culture filtrates of FOC killed cells isolated from leaflets of chickpea plants and in agreement with the results of this thesis but in contrast to the work of Kaur *et al.* (1987), toxic activity partitioned into EtOAc.

In conclusion, the results of this chapter show that a toxin has been isolated from culture filtrates of FOC 5 which kills isolated cells of chickpea and wilts chickpea cuttings in low concentration. The purified compound at the time of writing is in the hands of Dr Mike Beale of Rothamsted and is undergoing spectral analysis for identification purposes. However, the absence of this compound in other isolates of *F. oxysporum* f. sp. *ciceri* raises the question as to the identity of FOC 5 and this is examined in the next chapter.

CHAPTER VII

Genotypic comparison of the five isolates of *Fusarium oxysporum* f. sp. *ciceri*

7.1 Introduction

Comparison of culture filtrates of five fungal isolates sent to University College London as *Fusarium oxysporum* f. sp. c*iceri,* demonstrated that those of the isolate designated FOC 5 were at least 10 times more toxic to cells isolated from chickpea leaflets (Chapter 2). Toxic activity from the culture filtrates partitioned into ethyl acetate (Chapter 3) and could be adsorbed from them on reversed phase solid phase extraction (SPE) cartridges. Activity was associated with a red compound, which was finally purified by thin layer chromatography on silica gel (Chapter 6). Examination by reversed phase HPLC with diode array detection demonstrated that the toxin had a UV spectrum with λ max at 224 and 281 nm.

Comparison of the ethyl acetate fraction of FOC 5 with ethyl acetate fractions of the four other isolates showed that the red band associated with toxic activity was absent. This finding called into question the identity of the isolates and, consequently, it was decided to investigate this by sequencing the ITS regions of ribosomal DNA of all five isolates.

The ribosomal DNA gene cluster encodes RNA and proteins required for the assembly of ribosomes. This gene cluster is repetitive with each repeat consisting of three main conserved regions, a large subunit, the 28 S gene (LSU), a small subunit, the 18 S gene (SSU) and the 5.8 S gene. The three genes within the repeat are separated by variable internal

transcribed spacer regions, ITS 1 and ITS 2 and the repeats themselves are separated by the intergenic transcribed spacer regions (IGS; fig.7.1).

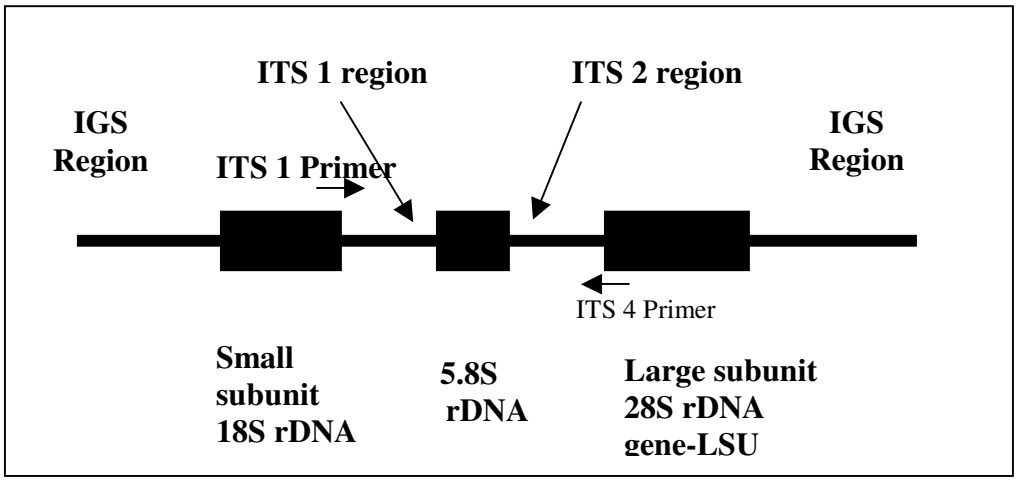

Fig. 7.1 Diagram of the eukaryotic ribosomal repeat region, showing the annealing positions of the ITS 1 and ITS 4 primers

The presence of conserved regions in the SSU and LSU has allowed the generation of universal primers for use in PCR that give rise to amplicons spanning regions of variability such as ITS 1 and ITS 2. Gardes and Bruns (1991) showed that the primers designated as ITS 1 and ITS 4, when used in PCR of DNA from Ascomycetes, gave good yields of amplicons and therefore these primers were used in the present investigation. Sequencing of such amplicons may be used not only to identify fungi to the species level but, in some cases, may also allow differentiation within populations of a given species from different areas or the differentiation of races that may attack different cultivars of host plants (White *et al*., 1990).

When grown in culture, FOC initially produces colourless to pale yellow mycelium that turns pink or purple with age. Di Pietro *et al*. (2003) stated that no sexual stage has been observed in any formae speciales of *Fusarium oxysporum.* Nelson (1981) described macroconidia of FOC as thin walled, 2-5 septate, produced branched or singly on aerial mycelium while microconidia occur as kidney shaped structures on short microconidiophores. Chlamydospores may be present. These are thick-walled resting spores produced in hyphae either in intercalary or terminal positions or in conidia. FOC has not been reported to infect any crop plant other than chickpea and no other forma specialis of *Fusarium oxysporum* has been reported as a pathogen of chickpea.

7.2 Materials and methods

7.2.1 Extraction of DNA

Race 1, race 2, V2, Jabalpur and FOC 5 were inoculated by spotting spore suspensions (10 µl at 10^7 spores/ml) from the liquid nitrogen bank (section 2.2.2.) in the centre of plates of potato dextrose agar (PDA; Oxoid Ltd., Basingstoke, Hampshire, England). Plates were incubated at 20^{o}C for 1 week. Plugs of mycelium were cut from the edge of the colonies with a No. 3 cork borer and as much agar removed from the bottoms of the plugs as possible. For each isolate, five plugs were transferred to a 1.5 ml Eppendorf tube containing one measure of glass beads (Sigma, Sigma-Aldrich company Ltd., Fancy Road, Poole, Dorset, BH12 4QH, England; approximately up to the 0.1 ml mark on the Eppendorf tube).

One ml of Chelex-Tris suspension (Biorad Laboratories Ltd, Biorad House, Maylands Avenue, Hemel Hempstead, Herts, HP2 7TD; 3% Chelex w/v, 1 mM Tris, pH 8.0) was added to the Eppendorf tube and mixed well by vortexing for 1 min. The tubes were frozen in liquid nitrogen for 1 min and boiled for another 1 min. Freezing and boiling was repeated twice more but the last boiling was extended to 5 min. Care was taken not to transfer Eppendorf tubes immediately from liquid nitrogen to the boiling water bath since this may cause the tubes to crack. Tubes were mixed well by vortexing for 1 min and incubated at 55°C for 30-40 min. After incubation, the tubes were centrifuged at 13,000*g* on a Microfuge for 5 min and the supernatant (100 µl), free of Chelex, was transferred to a fresh Eppendorf tube and placed on ice.

7.2.2 Concentration of DNA

The concentration of DNA was estimated spectrophotometrically using the following formula:

E = A x B x C

Where E = DNA concentration in μg/ml; A = absorbance at 260 nm in a 1 cm light path; B = 50, which is a constant for double stranded DNA and C = dilution factor

7.2.3 Amplification of the extracted DNA

Two primers, ITS 1 and ITS 4 were obtained from MWG (Mill Court, Featherstone Road, Wolverton Mill South, Milton Keynes, MK12 5RD) and were used in the polymerase chain reaction. The reaction mixture consisted of template DNA (2.0 – 2.5 pg, 4 μl of each primer at 2.5 picomoles/μl and one "Ready to Go" PCR bead (Amersham Pharmacia Biotech Inc., Sweden). In some cases $MgCl_2$ was added to a final concentrations of 1.5, 2.5 and 3.5 mM. Water was added to give a final volume of 25 μl. PCR was performed in a Progene Thermal Cycler, (Techne) using a programme consisting of 2 min at 94°C (denaturation) followed by 35 cycles of 30 sec at 94°C (denaturation), 30 sec at 55°C (annealing) and 2 min at 72°C (extension). At the end of the programme the tubes were held at 4°C until required. λ DNA (2 μl from a stock solution of 25 pg/μl; New England Biolabs (UK) Ltd., 73 Knowl Piece, Wilbury Way, Hitchin, Hertfordshire SG4 0TY, England, UK) was used as a positive control and tubes lacking template DNA as a negative control (Table 7.1).

Sample No.	FOC Isolate	ITS 1 (µl)	ITS 4 (µl)	$MgCl_2$ from a 10mM stock (µl)	DNA of the FOC isolates (µl)	Sterile distilled water (µl)
1	Race 1	4	4	0	5	12
2	Race 1	4	4	2.5	5	9.5
3	Race 1	4	4	5	5	7
4	Race 2	4	4	0	5	12
5	Race 2	4	4	2.5	5	9.5
6	Race 2	4	4	5	5	7
7	V 2	4	4	0	5	12
8	V 2	4	4	2.5	5	9.5
9	V 2	4	4	5	5	7
10	Jabalpur	4	4	0	5	12
11	Jabalpur	4	4	2.5	5	9.5
12	Jabalpur	4	4	5	5	7
13	FOC 5	4	4	0	5	12
14	FOC 5	4	4	2.5	5	9.5
15	FOC 5	4	4	5	5	7
-ve control	-	2 (primer 1)	2 (primer 2)	-	-	21
+ve control	-	2 (primer 1)	2 (primer 2)	-	2 (λ DNA)	19

Table 7.1 Constituents of Eppendorf tubes used in PCR.

7.2.4 Checking the quality of DNA in extracts of fungal isolates and PCR products

DNA, extracted from fungi, was eletrophoresed in an agarose gel (1%) containing 2 µg/ml ethidium bromide with Tris-acetate-EDTA as buffer (TAE buffer; appendix I). Flanking wells contained a λ DNA-Hind III digest as a size marker. Amplicons from PCR reactions were also electrophoresed under similar conditions. Gels were run at 80 V for 1.5 h and were examined on a transilluminator (UVP, Inc., Cambridge, England) and photographed.

7.2.5 Purification of amplicons from PCR

Amplicons (10 µl; section 7.2.3) were purified with a QIAquick PCR purification kit (Qiagen Ltd., Boundary Court, Gatwick Road, Crawley, West Sussex, RH10 9AX). They were mixed with buffer PB (50 µl) and applied to QIAquick columns connected to a 2 ml collection tube. The combined column and collection tube were centrifuged at 13,000*g* for 60 sec and the flow through was discarded. PE buffer (750 µl) was applied to the column and the column was centrifuged and the flow through discarded. The column was centrifuged at 13,000*g* for an additional 60 sec in order to remove the PE buffer completely before placing the column on a clean 1.5 ml Eppendorf tube. Elution buffer (30 µl) was applied to the centre of the column and the column was allowed to stand for 1 min before centrifuging at 13,000*g* for 60 sec. The purified DNA in the Eppendorf tubes was checked by agarose gel electrophoresis (section 7.2.4).

7.2.6 Sequencing and sequence analysis of the purified PCR products

Lola Martinez, Wolfson Institute of Biomedical Research, University College London, conducted the sequencing using both primers ITS 1 and ITS 4. The purified PCR products were sequenced with the WellRed dye-labelled dideoxy-terminator cycle sequencing kit. This contained the dye-labelled terminators ddUTP, ddGTP, ddCTP, ddATP, dNTP mix solution, thermostable DNA polymerase and pyrophosphatase-sequencing buffer (Beckman Coulter Inc. California, USA). Sequencing was performed on a Beckman CEQ2000XL instrument with an eight capillary automated DNA sequencing and fragment analysis system. The read length of the capillaries (DNA separation array, 33-75B) is 700 bp with an accuracy of 98.5%. Sequences were aligned using Sequencher 4.1. (Gene Codes Corporation, Michigan, USA). Homologies with sequences in the GenBank database were identified using BLAST (http://www.ncbi.nlm.nih.gov/blast). Alignment was carried out using the Sequencher program and Clustal W (http://www.ebi.ac.uk/clustalw).

7.2.7 Morphology of the isolate FOC 5

FOC 5 (50 µl of a spore suspension containing 10^7 spores/ml) was inoculated on the centre of the Petri plates containing potato dextrose agar (PDA) and incubated at 20°C for one week. After incubation macroscopic and microscopic observations were made.

7.3 Results

7.3.1 Extraction of DNA from the five isolates of FOC

The DNA of all five FOC isolates, race 1, race 2, V2, Jabalpur and FOC 5, was successfully extracted by the Chelex-Tris suspension method, giving about 40 – 50 ng DNA/100 µl; Table 7.2).

Isolate of FOC	Concentration of DNA (ng/100 µl)
Race 1	40.5
Race 2	41.0
V2	42.5
Jabalpur	43.5
FOC 5	49.5

Table 7.2 The amount of DNA extracted from five plugs of mycelium of the five isolates of FOC growing on potato dextrose agar

7.3.2 Amplification of the extracted DNA

Amplicons were obtained from all five isolates at all three levels of $MgCl_2$ but isolates differed in the amount that was optimal. As the $MgCl_2$ was increased the amount of DNA amplified also increased in the cases of race 2, V2 and FOC 5 while it was the reverse in race 1. There was little effect of $MgCl_2$ on Jabalpur (Fig. 7.2).

7.3.3 Purification of the DNA obtained from the PCR product

When the PCR products of all the FOC isolates were purified with a QIAquick PCR purification kit an amplicon running at the same position as the original unpurified product was obtained (Fig. 7.3)**.**

7.3.4 Alignment of sequences of the amplicons obtained by primers ITS 1 and ITS 4 in PCR reactions

The amplicons obtained for each isolate from sequence reactions with primers ITS 1 and ITS 4 were aligned using CLUSTAL W (1.82). Where disagreements occurred between the sequences these were resolved by inspection of the electropherograms in Chromas (version 1.45). The corrected sequences for each isolate and various combinations were then aligned (Figs 7.4-7.7). From these alignments it was clear that FOC 5 differed markedly from the other isolates. When the sequences were aligned without FOC 5 there was a minimum of 99% homology and race 1 and V2 were identical (Table 7.3). Jabalpur was also very similar with only two base pair differences from race 1 and V2 and all isolates had 392 bp in this region. In contrast, race 2 gave an amplicon that was 8 bp longer and that of FOC 5 was 16 bp longer (Table 7.4)

7.3.5 Identification of the five fungal isolates

When the sequences of the five isolates of FOC were submitted to a BLAST database search, all the isolates except FOC 5 were matched nearest as formae speciales of *Fusarium oxysporum.* Race 1 and V2 which were identical were also identical with *F. oxysporum* f. sp. *vasinfectum* and race 2 was also matched nearest as this forma specialis. The isolate Jabalpur differed from *F. oxysporum* f. sp. *vanillae* by only 1 base out of 392. In contrast, FOC 5 was matched nearest as *Fusarium acutatum* (Table 7.5).

7.3.6 Morphology of the isolate FOC 5

After incubation for one week at 20°C on PDA, the morphology of isolate FOC 5 was observed as follows: Colony margin entire, aerial mycelium pinkish-white, pigmentation

in reverse light pinkish to pinkish-white. Both micro- and macroconidia were present, the latter often septate. Chlamydospores were also found and these were single or in pairs.

Fig. 7.2 Result of electrophoresis of the amplicons obtained for the five isolates of FOC with ITS 1 and ITS 4 as the primers.

Amplicons were electrophoresed in a 1% agarose gel. Lanes 1 and 14, λ DNA-Hind III; lanes 2-4, race 1; lanes 5-7, race 2; lanes 8-10, V2; lanes 11-13, Jabalpur and lanes 15-17, FOC 5. Lanes 2,5,8,11 and 15 had 1.5 mM $MgCl_2$, lanes 3, 6, 9, 12 and 16 had 2.5 mM $MgCl_2$, and lanes 4, 7,10, 13 and 17 had 3.5 mM $MgCl_2$.

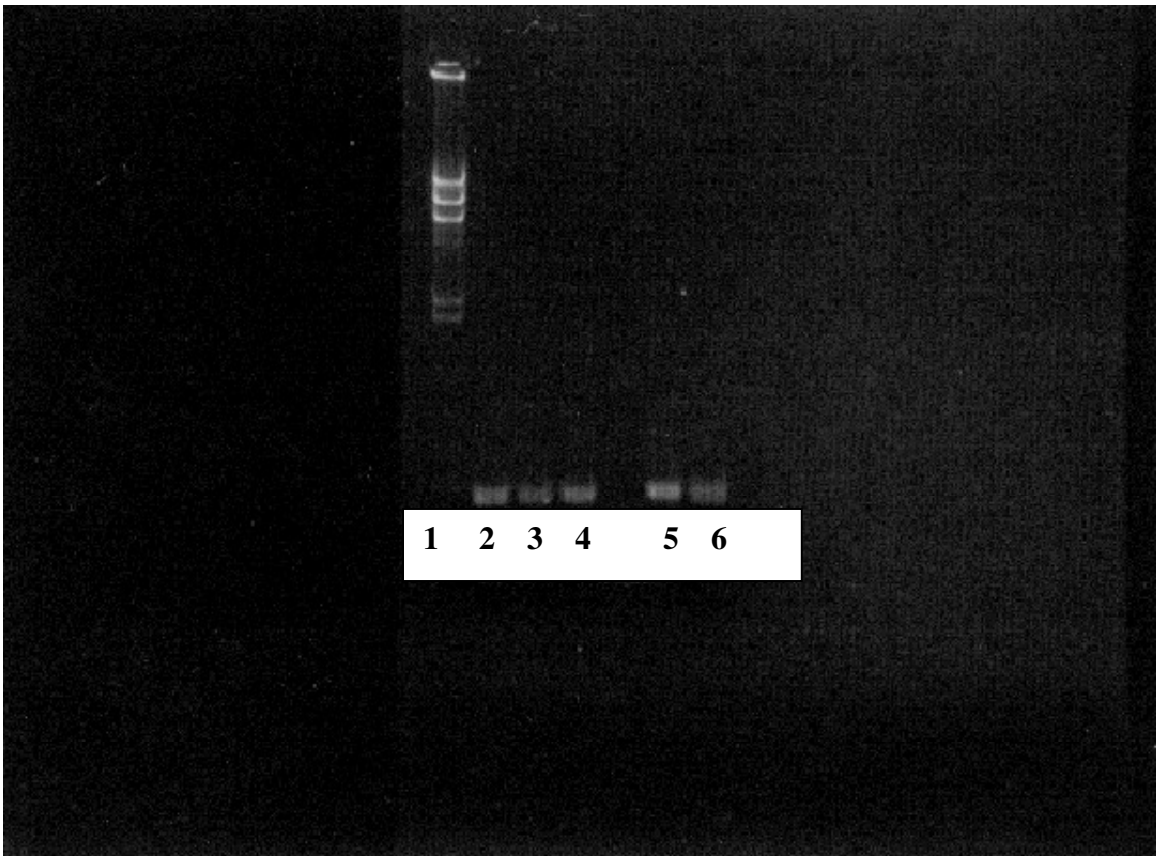

Fig.7.3 Result of electophoresis of amplicons obtained for the five isolates of FOC with ITS 1 and ITS 4 primers after treatment with the QIAquick purification kit.
Lane 1, λ DNA-Hind III; lane 2 race 1; lane 3, race 2; lane 4, V2; lane 5, Jabalpur and lane 6, FOC 5.

```
Race1           AGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATAT 60
V2              AGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATAT 60
                ************************************************************

Race1           GTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCT 120
V2              GTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCT 120
                ************************************************************

Race1           GGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA 180
V2              GGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA 180
                ************************************************************

Race1           TCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGA 240
V2              TCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGA 240
                ************************************************************

Race1           GCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCTC 300
V2              GCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCTC 300
                ************************************************************

Race1           AAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGT 360
V2              AAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGT 360
                ************************************************************

Race1           AATCGTCGCGGCCACGCCGTTAAACCCCAACT 392
V2              AATCGTCGCGGCCACGCCGTTAAACCCCAACT 392
                ********************************
```

Fig.7.4 Alignment of sequences obtained from race 1 and V2 isolates of FOC using the Clustal W program showing a 100% match

```
Race1           AGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATAT 60
V2              AGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATAT 60
Jabalpur        AGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATAT 60
                ************************************************************

Race1           GTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCT 120
V2              GTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCT 120
Jabalpur        GTAACTTCTGAGTAAAACTATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCT 120
                ****************** ***************************************

Race1           GGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA 180
V2              GGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA 180
Jabalpur        GGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA 180
                ************************************************************

Race1           TCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGA 240
V2              TCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGA 240
Jabalpur        TCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGA 240
                ************************************************************

Race1           GCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCTC 300
V2              GCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCTC 300
Jabalpur        GCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCCC 300
                ************************************************************ *

Race1           AAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGT 360
V2              AAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGT 360
Jabalpur        AAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGT 360
                ************************************************************

Race1           AATCGTCGCGGCCACGCCGTTAAACCCCAACT 392
V2              AATCGTCGCGGCCACGCCGTTAAACCCCAACT 392
Jabalpur        AATCGTCGCGGCCACGCCGTTAAACCCCAACT 392
                ********************************
```

Fig.7.5 Alignment of sequences obtained from the race 1, V2 and Jabalpur isolates of FOC using the Clustal W program showing that Jabalpur differs by two base pairs from the other two isolates

```
Race1           AGCCCGCTCCCGG-TAAAACG-GGACGGCCCGCC---AGAGGACCCCTAAACTCTGTTTC 55
V2              AGCCCGCTCCCGG-TAAAACG-GGACGGCCCGCC---AGAGGACCCCTAAACTCTGTTTC 55
Race2           AGCCCGCTCCCGGATAAAACGAGGGCGGCCCGCCCAAAGAGGACCCCTAAACTCTGTTTC 60
Jabalpur        AGCCCGCTCCCGG-TAAAACG-GGACGGCCCGCC---AGAGGACCCCTAAACTCTGTTTC 55
                ************* ******* ** *********   ***********************

Race1           -TATATGTAACTTCTGAGTAAAACCATAAA--TAAATCAAAACTTTCAACAACGGATCTC 112
V2              -TATATGTAACTTCTGAGTAAAACCATAAA--TAAATCAAAACTTTCAACAACGGATCTC 112
Race2           ATATATGTAACTTCTGAGTAAAACCATAAAATTAAATCAAAACTTTCAACAACGGATCTC 120
Jabalpur        -TATATGTAACTTCTGAGTAAAACTATAAA--TAAATCAAAACTTTCAACAACGGATCTC 112
                 ********************** *****  ****************************

Race1           TTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAAT 172
V2              TTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAAT 172
Race2           TTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAAT 180
Jabalpur        TTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAAT 172
                ************************************************************

Race1           TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGC 232
V2              TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGC 232
Race2           TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGC 240
Jabalpur        TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGC 232
                ************************************************************

Race1           CTGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCG 292
V2              CTGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCG 292
Race2           CTGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCG 300
Jabalpur        CTGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCG 292
                ************************************************************

Race1           CGTTCCTCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCG 352
V2              CGTTCCTCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCG 352
Race2           CGTTCCTCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCG 360
Jabalpur        CGTTCCCCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCG 352
                ****** ******************************************************

Race1           TTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACT 392
V2              TTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACT 392
Race2           TTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACT 400
Jabalpur        TTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACT 392
                ****************************************
```

Fig.7.6 Alignment of sequences obtained from the race 1, race 2, V2, and Jabalpur isolates of FOC using the Clustal W program showing that race 2 has interpolations of 1,1, 3, 1 and 2 base pairs compared with the other three isolates

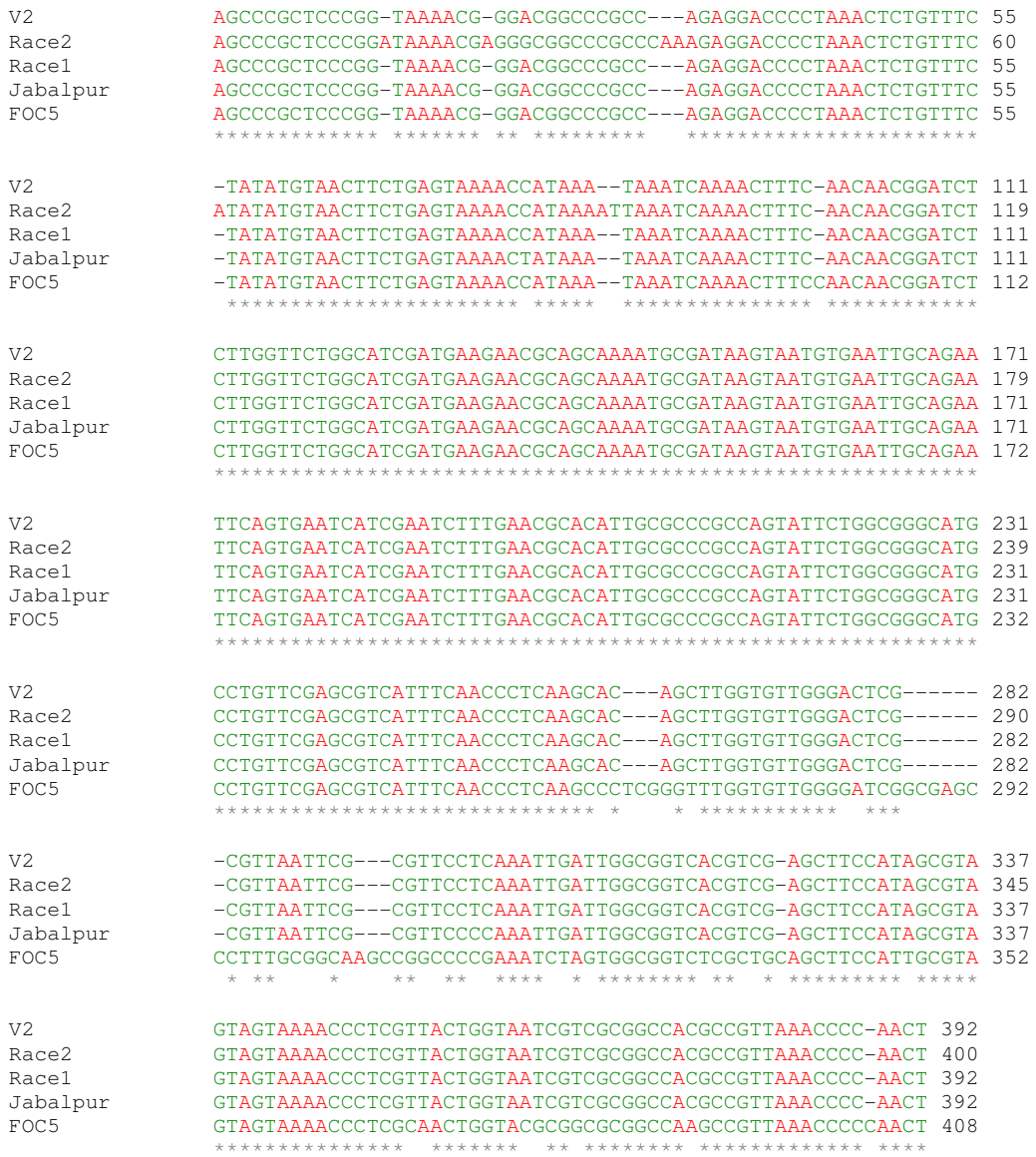

Fig.7.7 Alignment of sequences obtained from all five isolates of FOC using the Clustal W program showing that FOC 5 has multiple interpolations of bases and substitutions compared with the other isolates.

Combinations	Sequences aligned	Score (percent match)
1	Race 1 X Race 2	99
2	Race 1 X V2	100
3	Race 1 X Jabalpur	99
4	Race 1 X FOC 5	92
5	Race 2 X V2	99
6	Race 2 X Jabalpur	99
7	Race 2 X FOC 5	90
8	V2 X Jabalpur	99
9	V2 X FOC 5	92
10	Jabalpur X FOC 5	92

Table 7.3 Pair wise alignments of sequences of FOC isolates and score of matching between pairs of isolates as percentages

Isolate of FOC	Number of base pairs (bp)
Race 1	392
Race 2	400
V2	392
Jabalpur	392
FOC 5	408

Table 7.4 Numbers of base pairs present in the amplicons of the five isolates of FOC

Isolate of FOC	Best match on the BLAST	% Match	Number of bases	Reference
Race 1	*Fusarium oxysporum* f. sp. *vasinfectum*	100%	392	Catal and Schilder (2003)
Race 2	*Fusarium oxysporum* f. sp. *vasinfectum*	97%	400	Catal and Schilder (2003)
V2	*Fusarium oxysporum* f. sp. *vasinfectum*	100%	392	Catal and Schilder (2003)
Jabalpur	*Fusarium oxysporum* f. sp. *vanillae*	99%	392	Wang *et al.* (2003)
FOC 5	*Fusarium acutatum*	99%	408	O'Donnell and Cigelnik (1997)

Table 7.5 Closest matching of the FOC isolates by BLAST analysis

7.4 Discussion

The isolation of a pure toxin from ethyl acetate fractions of culture filtrates of isolate FOC 5 was described in chapter 6. The compound was red in colour and had UV absorption maxima of 224 and 281 nm. However, the compound was not detectable in comparable preparations from culture filtrates of the other four isolates. This suggested considerable heterogeneity among the isolates of *F. oxysporum* f. sp. *ciceri.* So it was decided to compare the ITS regions of ribosomal DNA of all five isolates by sequencing. Attempts were also made to determine if the isolates belonged to the same anastomosis groups.

DNA (40-50 ng/100 µl) of all the five isolates was extracted by the Chelex-Tris suspension method which gave amplicons on PCR with ITS 1 and ITS 4 as primers. After treatment with the QIAquick PCR purification kit, good sequence data were obtained using the same primers.

Upon analysing the sequences, it was clear that FOC 5 differed markedly from the other isolates as it matched only 90-92% with other isolates. In contrast, the other four isolates matched 99% among themselves while race 1 and V2 were 100% matched (Table. 7.3). The amplicon from FOC 5 was 16 bp longer than race 1, V2 and Jabalpur and 8 bp longer than race 2 owing to multiple interpolations and base substitutions (Table 7.4). A BLAST search closely matched race 1, race 2, V2 and Jabalpur as *Fusarium oxysporum.* Sequences of race 1 and V2 were 100% and race 2 was 97% identical to the sequences of *F. oxysporum* f. sp. *vasinfectum* isolate PA3 (Catal and Schilder, 2003). The sequence of Jabalpur was a 99% match to that of *F. oxysporum* f. sp. *vanillae* isolate ML-1-1 (Wange *et al.,* 2003). In contrast, FOC 5 was a 99% match with *Fusarium acutatum,* a pathogen of pigeonpea (O'Donnell and Cigelnik, 1997). This was described as a new species by

Nirenberg and O'Donnell (1998). When the morphology of FOC 5 was studied in this investigation, aerial mycelium with simple, prostrate conidiophores producing conidia in false heads were found as observed by Nirenberg and O'Donnell (1988). The conidia were small to large and aseptate or with one septa. Chlamydospores were also found and these were single or in pairs.

The finding of complete homology of race 1 and V2 with *F. oxysporum* f. sp. *vasinfectum,* a pathogen of cotton, raises the interesting question as to whether the chickpea pathogen is also able to infect this crop but no literature was found in support of this hypothesis. Similarly, it would be interesting to know if the isolate Jabalpur is able to infect both vanilla and chickpea. A positive answer to both these questions might have important implications for rotations involving these species.

CHAPTER VIII

Overall discussion

Fusarium oxysporum causes wilting in many plant species and in order to differentiate them Snyder and Hansen (1945), Snyder and Tousson (1965) and Tousson and Nelson (1968, 1975 and 1976) classified them as formae speciales (f. sp.). As described in Chapter 1, the f. sp. name was often taken from the name of the species of plant infected (see Table 1.2). The forma specialis that infects chickpea is *F. oxysporum* f. sp. *ciceri* (Nelson, 1981).

Several formae speciales of *F. oxysporum* produce secondary metabolites that are toxic to plants and are involved in wilt diseases. These include fusaric acid produced by *F. oxysporum* f. sp. *cubense* on banana, beauvericin produced by *F. oxysporum* f. sp. *melonis* on muskmelon and a polyketide toxin produced by *F. oxysporum* f. sp. *vasinfectum* on cotton (Belisario *et al*., 2002; Bell *et al*., 2003; Thangavelu *et al*., 2001).

The symptoms of the Fusarium wilt of chickpea (section 1.6), caused by FOC, suggest that phytotoxins may be involved in the disease development. However, there are only a few experiments reported in the literature that support this hypothesis. Kaur *et al.* (1987) found that partially purified toxin of FOC inhibited callus growth of chickpea and Rao and Padmaja (2000) showed that crude culture filtrates of FOC, when diluted to 30% with water, caused wilting of 1 week old chickpea seedlings within 4-5 days. These results suggest that FOC is capable of producing a phytotoxic compound(s) when grown in culture. However, no further studies appear to have been made. The present investigation was

initiated in order to determine if the wilting symptoms associated with Fusarium wilt of chickpea could be explained, at least in part, by the production of a toxin by the pathogen.

This thesis provides strong evidence that five fungal isolates (race 1, race 2, V2, Jabalpur and FOC 5) originally identified by others as FOC were toxigenic. Time course experiments of the above isolates for production of toxin demonstrated that all five isolates secreted toxin(s) into liquid cultures that were lethal to cells isolated from leaflets of chickpea by enzymic digestion. Maximal toxicity (70 units/ml culture filtrate) was generated by the isolate designated as FOC 5 when cultured on Czapek Dox medium supplemented with five cations (CDCLM) for 12 days at 20ºC. Toxicity of this isolate was far less when cultured at 30ºC (<10 units/ml) as was the toxicity of all other isolates at either temperature.

Activity was recovered when culture filtrates of the isolates were freeze-dried and activity mainly partitioned into EtOAc when culture filtrates were shaken with the solvent. In isolates race 1 and race 2 activity was detected in EtOAc fractions after culture for some time periods at either temperature but not in the corresponding freeze-dried cultures. Moreover, the activity of the EtOAc fraction of 12 day-old cultures of FOC 5 grown at 20ºC was higher (equivalent to 110 units/ml culture filtrate) compared to the same culture when freeze-dried (70 units/ml culture filtrate).

Since the activity of 12 day-old cultures of FOC 5 grown at 20ºC was far higher than that of any other isolate grown at either temperature, attempts were made to isolate the toxin from culture filtrates of this isolate. Moreover, since activity was so high, it was possible to

assay the culture filtrates of this isolate without the complication of concentration. This allowed the effect of partitioning culture filtrates against EtOAc to be determined directly. When culture filtrates were partitioned three times, just over half of the activity was found in the organic phase and 30% in the aqueous phase. The effect of freeze-drying on both these fractions was surprising. All activity was lost in the aqueous phase and activity was increased in the EtOAc phase. One interpretation of these results is that there are two competing toxins. Toxin A partitions into EtOAc with ease and toxin B, which is volatile, with greater difficulty. Toxin B which is lost on freeze-drying thus removing it from competition with toxin A in the EtOAc fraction. In contrast when toxin B is removed from the aqueous phase by freeze-drying, no compound with toxic activity is left. Attempts were made to determine if multiple partitionings would allow complete extraction of activity from culture filtrates by EtOAc but even after 10 partitionings just under 10% of activity remained in the aqueous phase (Fig. 3.4).

Despite single sporing, cultures of FOC 5 segregated into those that were red and those that were colourless, suggesting that nuclei for both types of cultures were present in the single but multicellular conidia of the fungus. The red cultures were ten times more toxic than those that were colourless, necessitating the selection of cultures with red pigmentation for toxin extraction.

The toxin(s) of FOC 5 proved very difficult to purify. C_{18} and cyano SPE cartridges were used as a clean-up process and for concentrating the toxin. These experiments showed that the cyano cartridges adsorbed toxic activity slightly better than C_{18} cartridges, the

amount of activity not adsorbed being approximately 6% and 9% respectively of that introduced into the cartridges. When C_{18} cartridges were developed in a stepwise gradient of methanol in water, toxic activity eluted as a broad and shallow peak. In contrast, a rather sharper peak of toxic activity was obtained when cyano cartridges were eluted with a stepwise gradient of ACN in water (compare figs. 4.1 and 4.3). The potential of cyano columns as a stationary phase was confirmed by scouting with a variety of HPLC columns (Genesis columns) with different packing materials (Chapter 4). Consequently, attempts were made to purify the toxin by HPLC on cyano columns. However, attempts to obtain a pure compound by this means were frustrated by poor recoveries.

As an alternative, purification of the toxin was attempted using normal phase flash chromatography on Si gel. When the starting material was the EtOAc fraction of culture filtrates, recovery of toxic activity was 85%, 113% and 99% in three replicates. Most of the activity was recovered in two peaks one of which was sharp and eluted early and the other was broad and eluted later (Fig. 5.3). Examination of these by HPLC on a cyano column showed that the first peak had at least four constituents and the second three although this fraction had more activity. Further purification was attempted by semi-preparative HPLC on a cyano column. Although activity was recovered (61%, 69% and 110% in three replicates), no peak was seen when the three separated constituents were rechromatographed. Accordingly, attempts were made to purify the toxin by thin layer chromatography (TLC).

When the EtOAc fraction from culture filtrates or the fraction of such filtrates adsorbed to a cyano cartridge and desorbed with ACN was chromatographed on silica gel in a solvent system consisting of DCM/MeOH 8:1 (v/v) a red band that ran at an Rf of 0.57 was observed. Elution of the red compound from the silica and other areas of the plate showed

that only this band was active. HPLC of the red band on a cyano column with 10% ACN as mobile phase gave a single peak with λ max at 224 and 281 nm. When spectra were cut through the peak at different retention times, essentially the same spectrum was obtained suggesting that the compound was pure.

The purified toxin was heat stable and when taken up by chickpea cuttings (25.5 µg equivalent to 68.8 units in the bioassay) caused wilting within 4 h. Similar results were obtained with culture filtrates but here only 4.7 units of activity were taken up although cuttings wilted earlier than in pure toxin suggesting that other factors in the culture filtrate may also cause wilting.

The purified compound of FOC 5 at the time of writing is in the hands of Dr Mike Beale and Dr Jane Ward of Rothamsted and is undergoing spectral analysis for identification purposes. Preliminary data are consistent with the preparation being a mixture of bikaverin and norbikaverin but further confirmatory experiments are in progress (Fig. 8.1)

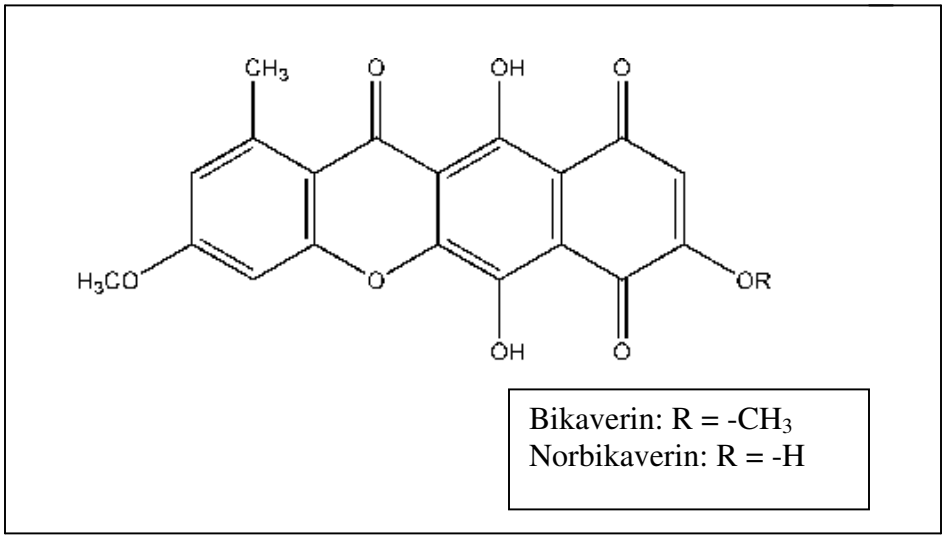

Fig. 8.1 Structures of bikaverin and norbikaverin (Bell *et al.,* 2003)

TLC of EtOAc fractions from the four other isolates of FOC did not show a red band comparable with that obtained from isolate FOC 5. This prompted an investigation of the identity of the five isolates. Ribosomal DNA sequences from FOC 5 differed markedly from those of the other isolates giving a match of only 90-92%. Of the other isolates, two were identical to each other (race 1 and V2) and were similar to Jabalpur (99.5% match). The sequence of race 2 had 8 additional bases interpolated in the ITS 1 region compared with race 1, V2 and Jabalpur. An even longer sequence was obtained from FOC 5 which had interpolations in both the ITS 1 and the ITS 2 regions (Fig. 7.7). A BLAST search matched all the isolates except FOC 5 as *Fusarium oxysporum.* Sequences of race 1 and V2 were identical and race 2 was 97% matched to the sequence of *F. oxysporum* f. sp. *vasinfectum* isolate PA3 (Catal and Schilder, 2003) but the sequence from Jabalpur was 99% matched to that of *F. oxysporum* f. sp. *vanillae* isolate ML-1-1 (Wange *et al.,* 2003). In contrast, FOC 5 was a 99% match with *Fusarium acutatum,* a pathogen of pigeonpea (O'Donnell and Cigelnik, 1997). This was described as a new species by Nirenberg and O'Donnell (1998) and morphology of this isolate in this investigation was quite matching with those found by them.

There are therefore two major findings of this thesis. The first that there is a toxin which may play an important role in wilting of chickpea caused by a species of *Fusarium.* The second is that none of the *Fusarium* isolates sent to London as *Fusarium oxysporum* f. sp. *ciceri* (FOC) were identified by molecular methods as this forma specialis. This throws open the question as to how many times wilt of chickpea has been erroneously ascribed to FOC. Three of the isolates were identified as the cotton pathogen, *Fusarium oxysporum* f. sp. *vasinfectum* which has also been reported from another legume, pigeonpea. However, it does

not seem to have been reported previously from chickpea. One isolate, Jabalpur was identified as *F. oxysporum* f. sp. *vanillae* which again has not been previously reported from chickpea. However, the isolate that was the principal subject of this thesis was identified as *F. acutatum.*

The identity and specificity of the species of *Fusarium* causing wilt of chickpea therefore appear to be in considerable doubt.

Future Work

The erroneous identification of all five isolates of *Fusarium* studied in this thesis as *F. oxysporum* f. sp. *ciceri* suggests that areas in which wilt is ascribed to this forma specialis should be thoroughly surveyed for fungal species capable of inducing these symptoms in chickpea. In the first instance their pathogenicity should be proved by Koch's postulates and then their identity established by molecular means. Furthermore, careful studies should be made of their host range. Where plants other than chickpea are found to be susceptible care should be taken not to include these in rotations with the legume.

It will be of interest to determine if toxins play a role in pathogenicity or virulence. This will necessitate isolation and identification of the toxins and demonstration of their role by the induction of wilting symptoms with the pure toxins and the attenuation of virulent strains in which toxin production has been negated by mutagenesis. Should toxins play a role in the disease syndromes elicited by these fungi it may prove possibly to select plants which are toxin insensitive. These would be expected to be more resistant than toxin sensitive cultivars. One reason for toxin insensitivity could be the possession of enzymes capable of degrading the toxin. Alternatively, such enzymes may be found in other species. In this

instance, they may be exploited by cloning the gene encoding them and incorporating the gene into chickpea.

ADDENDUM

After this thesis was written, a further and larger sample of the toxin was sent to Professor Mike Beale and Dr Jane Ward. This allowed them to obtain definitive structural information from ^{1}HNMR and ^{13}CNMR studies that identified the compound as 8-O-methylfusarubin (Fig. 8.2; Table 8.1 and 8.2).

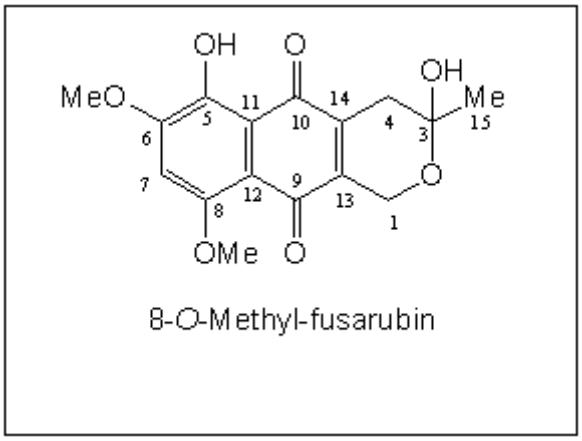

Fig 8.2 Structure of 8-O-methylfusarubin

Assignment	Literature Chemical Shift	Observed Chemical Shift
1-H_2	4.66 (J=18Hz and 2.7Hz)	4.75 (q, J=18 Hz and 2.8Hz)
4-H_2	2.50 d, J=18 Hz	2.53 (d, J=18Hz)
	2.78 d, J=18 Hz	2.84 (d, J=18Hz)
7-H	6.85 (s)	6.73 (s)
15-H_3	1.56 (s)	1.55 (s)
OCH_3	4.01 (s)	4.00 (s)
OCH_3	4.05 (s)	4.03 (s)
5-OH	13.16 (s)	13.16 (s)

Table 8.1 ^{1}HNMR data, spectrum run in $CDCl_3$

Carbon Atom	Literature Chemical Shift	Observed Chemical Shift
1	57.7	58.66
3	93.1	94.36
4	31.7	31.20
5	147.6	148.47
6	155.0	155.70
7	103.0	102.49
8	155.3	155.97
9	179.0	180.01
10	188.7	189.23
11	113.7	114.28
12	108.9	109.89
13	144.6	144.94
14	136.8	136.91
15	28.3	29.33
6-OMe	56.1	56.82
8-OMe	56.4	56.40

Table 8.2 ^{13}CNMR, spectrum run in $CDCl_3$

APPENDIX I

Media and Solutions

Czapek Dox Cation Liquid Medium (CDCLM)

Czapek Dox liquid medium (Oxoid)	33.4 g
Copper chloride ($CuCl_2.2H_2O$)	20 mg
Manganese chloride ($MnCl_2.4H_2O$)	20 mg
Calcium chloride ($CaCl_2.2H_2O$)	100 mg
Cobalt chloride ($C_OCl_2.6H_2O$)	30 mg
Zinc sulphate (Zn SO_4.7H2O)	50 mg
Distilled water	1000 ml

Holding Buffer (HB)

Citric acid	50 mM = 12.350 g
Magnesium sulphate ($MgSO_4$)	1 mM = 0.210 g
Potassium di-hydrogen phosphate (KH_2PO_4)	1 mM = 0.136 g
Sodium hydroxide (NaOH)	5.8 g
Glucose	100 g
Distilled water	1000 ml
$_P$H	5.8

Digestion solution

Holding buffer	100 ml

Pectolyase Y 23	50 μg/ml = 5 mg
Bovine Serum Albumin	500 μg/ml = 50 mg
Macerozyme R-10	15 mg/ml = 1.5 g

TAE buffer (Tris/acetate/EDTA) electrophoresis buffer; Current protocols in Molecular Biology, Vol. 1)

50X stock solution:

Tris base	242 g
Glacial acetic acid	57.1 ml
$Na_2EDTA\ .2H_2O$	37.2 g
Water	1 L

Loading buffer (Current protocols in Molecular Biology, Vol. 1)

10X stock solution

Ficoll 400	20%
Disodium EDTA, pH 8	0.1M
Sodium dodecyl sulphate	1%
Bromophenol blue	0.25%
Xylene cyanol	0.25% (optional; runs ~50% as fast as bromophenol blue and can interfere with visualization of bands of moderate molecular weight, but can be helpful for monitoring very long runs)

APPENDIX II

Transformation of percentages to probits

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.5	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.8	4.82	4.85	4.87	4.9	4.92	4.95	4.97
50	5	5.03	5.05	5.08	5.1	5.13	5.15	5.18	5.2	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.5
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
99	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

APPENDIX III

Toxic activity of freeze dried culture filtrates from cultures of FOC 5, race1, race2, V2 and Jabalpur grown at 20°C and 30°C

1. FOC 5

Sample	20°C				30°C			
	R1	**R2**	**R3**	**Mean**	**R1**	**R2**	**R3**	**Mean**
3rd day	2.3	0	0	0.77	4.6	0	0	1.53
6th day	29.86	29.86	4.92	21.55	0	3.25	2.73	1.99
9th day	-	-	-	-	-	-	-	-
12th day	119.04	87.43	6.73	71.19	4.6	7.72	9.18	7.16
15th day	13.45	119.42	3.25	45.37	4	12.13	4.28	6.8

Results expressed as units/ml culture filtrate

2. Race 1

Sample	20°C				30°C			
	R1	**R2**	**R3**	**Mean**	**R1**	**R2**	**R3**	**Mean**
3rd day	2.38	2.3	2.22	2.3	2.64	2.64	2.46	2.58
6th day	-	-	-	-	-	-	-	-
9th day	0	0	0	0	0	0	0	0
12th day	0	0	0	0	0	0	0	0
15th day	4.14	4.08	2.07	3.43	0	2.07	2.14	1.64

Results expressed as units/ml culture filtrate

3. Race 2

Sample	20°C				30°C			
	R1	**R2**	**R3**	**Mean**	**R1**	**R2**	**R3**	**Mean**
3rd day	2.3	2.38	2.34	2.34	2.3	0	0	0.76
6th day	-	-	-	-	-	-	-	-
9th day	0	0	0	0	0	0	0	0
12th day	3.14	0	2.38	1.84	0	0	0	0
15th day	2.93	7.21	3.25	4.46	2.07	2.22	2.22	2.17

Results expressed as units/ml culture filtrate

4. V2

Sample	20°C				30°C			
	R1	**R2**	**R3**	**Mean**	**R1**	**R2**	**R3**	**Mean**
3rd day	0	0	0	0	0	0	0	0
6th day	2.83	0	2.38	1.74	3.14	2.73	3.14	3
9th day	9.51	7.46	13.93	10.3	2.93	2.93	2.93	2.93
12th day	9.19	6.96	4.14	6.76	0	0	0	0
15th day	15.45	14.93	14.93	15.1	7.73	0	0	2.58

Results expressed as units/ml culture filtrate

5. Jabalpur

Sample	20°C				30°C			
	R1	**R2**	**R3**	**Mean**	**R1**	**R2**	**R3**	**Mean**
3rd day	0	0	0	0	0	0	0	0
6th day	8.28	8.28	6.73	7.76	9.19	5.28	3.25	5.91
9th day	26	19.03	26	23.68	128	8	16	50.67
12th day	6.5	5.1	7.46	6.35	2.22	0	33.13	11.78
15th day	14.42	26	0	13.47	22.63	13.45	18.38	18.15

Results expressed as units/ml culture filtrate

APPENDIX IV

Influence of temperature on the growth of FOC 5, race1, race2, V2 and Jabalpur at 20^oC and 30^oC

1. **FOC 5**

Sample	20°C				30°C			
	R1	**R2**	**R3**	**Mean**	**R1**	**R2**	**R3**	**Mean**
3rd day	0	0	0	0	0	0	0	0
6th day	0.470	0.597	0.078	0.382	0.273	0.438	0.445	0.385
9th day	-	-	-	-	-	-	-	-
12th day	0.218	0.879	0.827	0.641	0.425	0.497	0.510	0.477
15th day	0.736	0.300	0.214	0.416	0.108	0.507	0.639	0.418

(Results expressed as g dry weight/100ml culture)

2. **Race 1**

Sample	20°C				30°C			
	R1	**R2**	**R3**	**Mean**	**R1**	**R2**	**R3**	**Mean**
3rd day	0	0	0	0	0	0	0	0
6th day	-	-	-	-	-	-	-	-
9th day	0.264	0.269	0.112	0.215	0.047	0.459	0.312	0.273
12th day	0.037	0.283	0.412	0.244	0.436	0.590	0.461	0.496
15th day	0.051	0.359	0.389	0.266	0.399	0.445	0.360	0.401

(Results expressed as g dry weight/100ml culture)

3. **Race 2**

Sample	20°C				30°C			
	R1	**R2**	**R3**	**Mean**	**R1**	**R2**	**R3**	**Mean**
3rd day	0	0	0	0	0	0	0	0
6th day	-	-	-	-	-	-	-	-
9th day	0.238	0.091	0.319	0.213	0.205	0.648	0.626	0.493
12th day	0.028	0.342	0.280	0.216	0.701	0.594	0.300	0.532
15th day	0.093	0.434	0.398	0.308	0.709	0.837	0.822	0.789

(Results expressed as g dry weight/100ml culture)

4. V2

Sample	20°C				30°C			
	R1	**R2**	**R3**	**Mean**	**R1**	**R2**	**R3**	**Mean**
3rd day	0	0	0	0	0	0	0	0
6th day	0.190	0.292	0.299	0.260	0.020	0.410	0.502	0.311
9th day	0.423	0.613	0.440	0.492	0.601	0.911	0.590	0.701
12th day	0.466	0.549	0.693	0.569	0.030	0.439	0.054	0.174
15th day	0.853	0.573	0.829	0.751	0.249	0.174	0.621	0.348

(Results expressed as g dry weight/100ml culture)

5. Jabalpur

Sample	20°C				30°C			
	R1	**R2**	**R3**	**Mean**	**R1**	**R2**	**R3**	**Mean**
3rd day	0	0	0	0	0.252	0.321	0.224	0.266
6th day	0.743	0.735	0.508	0.662	0.403	0.378	0.498	0.426
9th day	0.687	0.563	0.569	0.606	0.573	0.443	0.483	0.499
12th day	0.300	0.257	0.333	0.296	0.403	0.566	0.391	0.453
15th day	0.090	0.511	0.733	0.445	0.470	0.505	0.355	0.443

(Results expressed as g dry weight/100ml culture)

APPENDIX V

Toxic activity of ethyl acetate fraction of culture filtrates from cultures of FOC 5, race 1, race 2, V2 and Jabalpur grown at 20°C and 30°C

1. **FOC 5**

Sample	20°C				30°C			
	R1	**R2**	**R3**	**Mean**	**R1**	**R2**	**R3**	**Mean**
3rd day	2.61	0	0	0.87	0	2	2.59	1.53
6th day	44	0	25	23	2.1	2.4	1.47	1.99
9th day	-	-	-	-	-	-	-	-
12th day	145	95	90	110	15.6	5.88	0	7.16
15th day	56	36	40	44	6	8	6.4	6.8

Results expressed as units/ml culture filtrate

2. **Race 1**

Sample	20°C				30°C			
	R1	**R2**	**R3**	**Mean**	**R1**	**R2**	**R3**	**Mean**
3rd day	7.45	0.49	3.33	3.76	1.49	11.03	11.91	8.14
6th day	-	-	-	-	-	-	-	-
9th day	4	3.3	5.3	4.2	2.14	4.92	5.10	4.05
12th day	17.6	16	9.02	14.21	0	11.6	8.32	6.64
15th day	7.81	9.08	4.63	7.17	8.3	9.73	13.33	10.45

Results expressed as units/ml culture filtrate

3. **Race 2**

Sample	20°C				30°C			
	R1	**R2**	**R3**	**Mean**	**R1**	**R2**	**R3**	**Mean**
3rd day	5.15	6.02	2.95	4.71	1.55	7.06	6.73	5.11
6th day	-	-	-	-	-	-	-	-
9th day	0	2.14	2.55	1.56	3.03	0	5.28	2.77
12th day	9.17	5.13	5.84	6.71	9.29	4.92	6.34	6.85
15th day	4.94	8.35	6.11	6.47	9.72	9.05	7.61	8.8

Results expressed as units/ml culture filtrate

4. V2

Sample	20°C				30°C			
	R1	**R2**	**R3**	**Mean**	**R1**	**R2**	**R3**	**Mean**
3rd day	5.25	5.96	4.9	5.37	0.53	4.4	4.5	3.14
6th day	2.97	0.56	4.97	2.83	2.92	3.83	3.08	3.28
9th day	4.77	5.62	4.80	5.06	7.17	5.07	5.44	5.89
12th day	3.27	5.46	2.94	3.89	0.43	4.4	4.90	3.24
15th day	14.7	14.45	0.77	11.3	3.6	5.09	5.2	4.63

Results expressed as units/ml culture filtrate

5. Jabalpur

Sample	20°C				30°C			
	R1	**R2**	**R3**	**Mean**	**R1**	**R2**	**R3**	**Mean**
3rd day	6.96	8.17	8.74	8	4.86	5.08	5.17	4.90
6th day	18.94	17.66	12.9	16.5	10	6.20	6.35	7.52
9th day	16.95	9.5	9.39	11.95	17.5	9.72	13.10	13.44
12th day	5.6	5.40	5.40	5.47	12.8	7.90	13.9	11.53
15th day	18.35	3.43	22.6	14.8	27.95	19.6	18.95	22.17

Results expressed as units/ml culture filtrate

APPENDIX VI

Activity and mycelial dry weight of the ethyl acetate fractions from red and colourless cultures of FOC 5

Replication	Red cultures		Colourless cultures	
	Activity (units/ml)	Mycelia dry wt. (grams)	Activity (units/ml)	Mycelia dry wt. (grams)
R1	192	0.572	17.2	0.331
R2	156	0.657	9.9	0.325
R3	146	0.711	11.1	0.238

APPENDIX VII

Recovery of toxin activity from fractionation of partially purified preparations (ethyl acetate phase of culture filtrate) on solid phase extraction (5g, C_{18} end capped cartridge)

MeOH %	Percentage recovery					Mean and (Standard Deviation)
	R1	**R2**	**R3**	**R4**	**R5**	
Starting material	477,710 units (100%)	445,720 units (100%)	362,040 units (100%)	494,560 units (100%)	512,000 units (100%)	
Non-adsorb.	12.06%	12.10%	7.42%	5.82%	8.24%	9.13% (2.83)
10% MeOH	1.92%	3.71%	1.21%	0.87%	1.05%	1.75% (1.16)
20% MeOH	1.26%	1.56%	0%	0.87%	1.01%	0.94% (0.59)
30% MeOH	1.18%	1.46%	0%	0.93%	0%	0.71% (0.68)
40% MeOH	3.35%	0.96%	0%	1.86%	1.14%	1.46% (1.25)
50% MeOH	4.58%	1.03%	1.31%	3.24%	1.48%	2.33% (1.53)
60% MeOH	6.04%	2.72%	2.21%	6.47%	2.21%	3.93% (2.14)
70% MeOH	7.43%	8.54%	5.10%	6.70%	6.50%	6.84% (1.26)
80% MeOH	3.59%	13.90%	5.10%	6.70%	6.25%	7.11% (3.98)
90% MeOH	3.60%	5.83%	9.47%	6.04%	10.2%	7.03% (2.75)
100% MeOH	1.61%	2.21%	4.12%	3.35%	3.6%	2.98% (1.04)
Total recovery %	**46.6%**	**54.0%**	**35.9%**	**42.8%**	**41.6%**	**44.2%**

APPENDIX VIII

Recovery of toxin activity from fractionation of partially purified preparations (ethyl acetate phase of culture filtrate) on solid phase extraction (5g, Cyano cartridge)

Acetonitrile % (ACN %)	**Percentage recovery**					**Mean and (Standard Deviation)**
	R1	**R2**	**R3**	**R4**	**R5**	
Starting material	265,020 units (100%)	465,624 units (100%)	256,000 units (100%)	304,435 units (100%)	304,435 units (100%)	
Non-adsorb.	2.92%	4.23%	11.70%	5.26%	5.65%	5.95% (3.38)
10% ACN	2.25%	2.85%	4.70%	3.81%	3.72%	3.47% (0.94)
20% ACN	1.51%	2.52%	2.50%	2.81%	2.14%	2.30% (0.50)
30% ACN	6.04%	5.60%	6.30%	3.98%	3.72%	5.13% (1.97)
40% ACN	9.15%	13.30%	13.40%	11.26%	10.20%	11.46% (1.87)
50% ACN	12.07%	22.34%	17.70%	15.94%	15.40%	16.69% (3.76)
60% ACN	3.02%	4.90%	8.80%	2.13%	2.14%	4.20% (2.81)
70% ACN	2.13%	1.63%	5.80%	0%	0%	1.91% (2.37)
80% ACN	0%	0%	0%	0%	0%	0% (0.00)
90% ACN	0%	0%	0%	0%	0%	0% (0.00)
100% ACN	0%	0%	0%	0%	0%	0% (0.00)
Total recovery %	**39.08%**	**57.31%**	**70.90%**	**45.19%**	**42,97%**	**51.09%**

REFERENCES

Alam, S.S. (1989). Discovery, isolation and identification of two toxins involved in blight of chickpea caused by *Ascochyta rabiei* (pass.) Lab. Ph.D. Thesis. University College London, London.

Alam, S.S., Bilton, J.N., Slawin, A.M.Z., Williams, D.J., Sheppard, R.N and Strange, R.N. (1989). Chickpea blight: Production of the phytotoxins solanapyrones A and C by *Ascochyta rabiei*. *Phytochemistry*, **28**: 2627-2630.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) Basic Local Alignment Search Tool. *Journal of Molecular Biology*. **215**: 403-410.

Ansari, M.A., Rupela, O.P., Douaik, A., Gopalakrishnan, S. and Sharma, S.B. (2002). Effect of culture filtrates of *Pseudomonas striata*, *Trichoderma harzianum*, *T. viride* and *Aspergillus awamori* on egg hatch of *Meloidogyne javanica*. *International Journal of Nematology*, **12**: 131-136.

Armstrong, G.M. and Armstrong, J.K (1981). Formae speciales and races of *Fusarium oxysporum* causing wilt diseases. In. Nelson, P.E., Toussoun, T.A. and Cook, R.J. eds. Fusarium: disease, biology and taxonomy. University Park, PA, USA; State University Press, 391-399.

Awasthi, C.P., Abidi, A.B. and Chowdhury, A.R. (1991). Studies on the nutritional quality of different varieties of chickpea. *Indian Journal of Agricultural Research*, **25**: 21-26.

Barve, M.P., Haware, M.P., Sainani, M.N., Ranjekar, P.K. and Gupta, V.S. (2001). Potential of microsatellites to distinguish four races of *Fusarium oxysporum* f. sp. *ciceri* prevalent in India. *Theoretical Application of Genetics*, **102**: 138-147.

Behboudian, M.H., Ma, Q., Turner, N.C. and Palta, J.A. (2001). Reactions of chickpea to water stress: yield and seed composition. *Journal of the Science of Food and Agriculture*, **81**: 1288-1291.

Bell, A.A., Wheeler, M.H., Liu, J., Stipanovic, R.D., Puckhaber, L.S. and Orta, H. (2003). United States Department of Agriculture – Agricultural Research Service studies on polyketide toxins of *Fusarium oxysporum* f. sp. *vasinfectum*: potential targets for disease control. *Pest Management Science*, **59**: 736-747.

Booth, C. (1971). The genus *Fusarium*. Commonwealth. Mycological. Institute. Kew, Surrey, England.

Breiman, A. and Barash, I. (1981). Partial characterization of phytotoxic compounds in culture filtrates of *Phytophthora citrophthora*. *Phytopathologische Zeitschrift*, **102**: 1-9.

Brian. P.W., Dawkins, A.W., Grove, J.F., Hemming, H.G., Love, D. and Norris, L.F. (1961). Phytotoxic compounds produced by *Fusarium equiseti*. *Journal of Experimental Botany*, **12**: 1-12.

Cachinero, J.M., Hervas, A., Jimenez-Diaz, R.M. and Tena, M. (2002). Plant defence reactions against Fusarium wilt in chickpea induced by incompatible race 0 of *Fusarium oxysporum* f. sp. *ciceri* and non-host isolates of *F. oxysporum*. *Plant Pathology*, **51**: 765-776.

Catal, M. and Schilder, A.M.C. (2003). A PCR based method to distinguish *Eutypa lata* and *Eutypella vitis* from grapevine. Submitted (11-Nov-2003), Plant Pathology, Michigan State University, Wilson Road, East Lansing, MI 48823, USA.

Cawood, M.E., Gelderblom, W.C.A., Vleggaar, R., Behrend, Y., Thiel, P.G. and Marasas, W.F.O. (1991). Isolation of the Fumonisin mycotoxins: A quantitative approach. *Journal of Agricultural Food Chemistry*, **39**: 1958-1962.

Chattopadhyay, S.B. and Sen Gupta, P.K. (1967). Studies on wilt diseases of pulses. I: Variation and taxonomy of *Fusarium* species associated with wilt disease of pulses. *Indian Journal of Mycological Research*, **5**: 45-53.

Chauhan, S.K., Nene, Y.L., Johansen, C., Haware, M.P., Saxena, N.P., Singh, S.D., Sharma, S.B., Shararwat, K.L., Burford, J.R., Rupela, O.P., Kumar Rao, J.V.D.K., and Sitanathan, S. 1988. Effects of soil solarization on Fusarium wilt of pigeonpea and chickpea. *ICRISAT Research Bulletin*, 11-16.

Chen, Y.M and Strange, R.N (1994). Production of a proteinaceous phytotoxin by *Ascochyta rabiei* grown in expressed chickpea sap. *Plant Pathology,* **43:** 321-327.

Csinos, A. and Hendrix, J.W. (1977). Toxin production by *Phytophthora cryptogea* active on excised tobacco leaves. *Canadian Journal of Botany*, **55**: 1156-1162.

Cubero, J.I. (1987). Morphology of chickpea. In: The chickpea, pp. 35-66 (Eds. M.C. Saxena and K.B. Singh). CAB International, Wallingford, U.K. and ICARDA, Aleppo, Syria.

Daub, M.E. (1986). Tissue culture and the selection of resistance to pathogens. *Annual Review of Phytopathology*, **24**: 159-186.

De Cal, A., Garcia-Lepe. R., Pascual, S. and Melgarejo, P. (1999). Effects of timing and method of application of *Penicillium oxalicum* on efficacy and duration of control of Fusarium wilt of tomato. *Plant Pathology*, **48**: 260-266.

De Cal, A. and Melgarejo, P. (2001). Repeated application of *Penicillium oxalicum* prolongs biocontrol of Fusarium wilt of tomato plants. *European Journal of Plant Pathology*, **107**: 805-811.

Desjardins, A.E., Gardner, H.W. and Weltring, K.M. (1992). Detoxification of sesquiterpene phytoalexins by *Gibberella pulicaris* (*Fusarium sambucinum*) and its importance for virulence on potato-tubers. *Journal of Industrial Microbiology*, **9**: 201-211.

Desjardins, A.E. *et al*. (1995). Genetic analysis of fumonisin production and virulence of *Gibberella fujikuroi* mating population A (*Fusarium moniliforme*) on maize (*Zea mays*) seedlings. *Applied and Environmental Microbiology*, **61**: 79-86.

Dhawan, K., Malhotra, S., Dahiya, B.S. and Singh, D. (1991). Seed protein fractions and amino acid composition in gram (*Cicer arietinum* L.). *Plant Foods for Human Nutrition*, **41**: 225-232.

Discostanzo, A., Johnston, L.W.H. and Murphy, M. (1996). A review of the effects of molds and mycotoxins in ruminants. *Professional Animal Science*, **12**:138-150.

Di-Pietro, A., Madrid, M.P., Caracuel, Z., Delgado-Jarana, J. and Roncero, M.I.G. (2003). *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. *Molecular Plant Pathology*, **4**: 315-325.

Di-Vito, M., Singh, K.B., Greco, N. and Saxena, M.C. (1996). Sources of resistance to cyst nematode in cultivated and wild *Cicer* species. *Genetic Resources and Crop Evolution,* **43**: 103-107.

Drummond, J and Pinnock, D.E. (1990). Aflatoxin production by entomopathogenic isolates of *Aspergillus parasiticus* and *A. flavus*. *Journal of Invertebrate Pathology*, **55**: 332-336.

Dutta, S.K., Nema, V.K. and Bhardwaj, R.K. (1985). Physical properties of gram. *Journal of Agricultural Engineering Research*, **39**: 259-268.

Earley, E.D. (1978). Phytotoxin studies with plant cells and protoplasts. Pages 363-372. In: Frontiers of plant tissue cultures. Proceedings of the 4th International Congress of plant tissue and cell culture, Calgary, Alberta, (T.A. Thorpe, ed.).

Edwards, C and Hammond, K. (1994). Cartridges fuel improvement in flash chromatography. *Laboratory Equipment Digest*, **December 1994**, 19-20.

Edwards, C., Lawton. L.A., Coyle, S.M and Ross, P. (1996 A). Laboratory scale purification of microcystins using flash chromatography and reversed phase high-performance liquid chromatography. *Journal of Chromatography A*, **734**: 163-173.

Edwards, C., Lawton. L.A., Coyle, S.M and Ross, P. (1996). Automated purification of microcystins. *Journal of Chromatography A*, **734**: 175-182.

FAOSTAT (2001). Statistical data on agricultural production. In *Crop Protection Compendium*, **2002**. *CAB international*, Wallingford, Oxon OX10 8DE, UK.

Food and Agriculture Organization (FAO). Quarterly Bulletin of Statistics. 5:50, *FAO*. **1988**. Rome, Italy.

Fravel, D., Olivain, C. and Alabouvette, C. (2003). *Fusarium oxysporum* and its bio-control. *New Phytologist*, **157**: 493-502.

Freeman, S., Zveibil, A., Vintal, H. and Maymon, M. (2002). Isolation of non-pathogenic mutants of *Fusarium oxysporum* f. sp. *melonis* for biological control of Fusarium wilt in cucurbits. *Phytopathology*, **92**:164-168.

Gardes, M., White, T. J., Fortin, J. A., Bruns, T. D., And Taylor, J. W. (1991) Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. *Canadian Journal of Botany*, **69**: 180-190.
Gengenbach, B.G., Green, C.E. and Donovan, C.M. (1977). Inheritance of selected pathotoxin resistance in maize plants regenerated from cell culture. *Proceedings of Natural Academy of Science, USA*, **74**: 5113-5117.

Greco, N. (1987). Nematodes and their control. In: The Chickpea, pp. 271-281. (Eds. M.C. Saxena and K.B. Singh). CAB International, Wallingford, U.K., and ICARDA, Aleppo, Syria

Grewal, J.S. and Pal. M. (1970). Fungal diseases of gram and arhar. *Proceedings of* 4th *Workshop of Pulse Crops*, Ludhiana, 169-170.

Halila, M.H. (1994). The biology and control of wilt of chickpea caused by *Fusarium oxysporum* f. sp. *ciceri*. PhD thesis. University College London.

Halila, M.H., Gridley, H. and Houdiard, P. (1984). Sources of resistance to Fusarium wilt in kabuli chickpeas. *International Chickpea Newsletter*, **10**: 13-14.

Halila, M.H. and Harrabi, M. (1990). Breeding for dual resistance to *Ascochyta* and wilt diseases in chickpea. In: Options Mediterraneennes, serie A: Seminaires Mediterraneens No 9 (M.C. Saxena, J.I. Cubero and J. Wery, eds.). *Proceedings of the International workshop on present status and future prospectus of chickpea crop production and improvement in the Mediterranean countries*. ECC/CIHEAM/ICARDA, 11-13 July 1988, Zaragoza, Spain, 163-166.

Halila, M.H. and Strange, R.N. (1996). Identification of the causal agent of wilt of chickpea in Tunisia as *Fusarium oxysporum* f. sp. *ciceri* race 0. *Phytopathology Mediterranean*, **35**: 67-74.

Hamid, K. and Strange, R.N. (1997). An easy method for isolating the solanopyrone toxins from culture filtrates of *Ascochyta rabiei*. *International Chickpea and Pigeonpea newsletter*, **4**: 20-22.

Hamid, K and Strange, R.N (2000). Phytotoxicity of solanopyrones A and B produced by the chickpea pathogen *Ascochyta rabiei* (Pass.) Labr. and the apparent metabolism of

solanapyrone A by chickpea tissues. *Physiological and Molecular Plant Pathology*, **56**: 235-244.

Hamid, K., Alam, S.S., Muhammad, H. and Strange, R.N. (2001). Phytotoxins of *Ascochyta rabiei* and *Fusarium oxysporum* f. sp. *ciceri* affecting chickpea. 4th *European conference on grain legumes*, 2001, Cracow. 62-63.

Harman, G.E., Chet, I. and Baker, R. (1981). Factors affecting *Trichoderma hamatum* applied to seeds as a bio-control agent. *Phytopathology*, **71**: 569-572.

Harvas, A., Trapero-Casas, J.L. and Jimenez-Diaz, R.M. (1995). Induced resistance against Fusarium wilt of chickpea by non-pathogenic races of *Fusarium oxysporum* f. sp. *ciceris* and non-pathogenic isolates of *F. oxysporum*. *Plant Disease*, **79**: 1110-6.

Haware, M.P. (1990). Fusarium wilt and other important disease of chickpea in the Mediterranean area. *Options Mediterraneennes – Serie Seminaires*, **9**: 61-64.

Haware, M.P., Jimenez Diaz, R.M., Amin, K.S., Philips, J.C. and Halila, H. (1990). Integrated management of wilt and root rots of chickpea. In: Chickpea in the nineties: Proc. 2nd Int. Workshop of Chickpea Improve. ICRISAT center, Patancheru, India, *ICRISAT Publication*, 129-133.

Haware, M.P., Nene, Y.L. and Rajeswari, R. (1978). Eradication of *Fusarium oxysporum* f. sp. *ciceri* transmitted in chickpea seed. *Phytopathology*, **68**: 1364- 1367.

Haware, M.P. and Nene, Y.L. 1982. Races of *Fusarium oxysporum* f. sp. *ciceri*. *Plant Disease*, **66**: 809-810.

Haware, M.P. and Nene, Y.L. 1982a. Symptomless carriers of the chickpea wilt. *Plant Disease*, **66**: 250-251.

Haware, M.P., Nene, Y.L. and Mathur, S.B. 1986. Seed-borne diseases of chickpea. *Technical bulletin No.1*.ISP. Copenhagen. ICRISAT, India.

Haware, M.P., Nene, Y.L., Pundir, R.P.S. and Rao, N.J. (1992). Screening of world chickpea germplasm for resistance to Fusarium wilt. *Field Crops Res*earch, **30**: 147-154.

Hayashi, N., Tanabe, K., Tsuge,T., Nishimura, S., Kohmoto, K. and Otani, H. (1990). Determination of host-selective toxin production during spore germination of *Alternaria alternata* by Hight Performance Liquid Chromatography. *Phytopathology*, **80**: 1088-1091.

Hoitink, H.A.J., Pelletier, R.L., and Coulson, J.G. (1966). Toxaemia of halo blight of beans. *Phytopathology*, **56**: 1062-1065.

Hopmans, E.C. and Murphy, P.A. (1993). Detection of fumonisins B1, B2 and B3 and hydrolysed fumonisin B1 in corn-containing foods. *Journal of Agricultural Food Chemistry*, **41**: 1655-1658.

Horn, N.M. and Reddy, S.V. (1996). Survey of chickpea (*Cicer arietinum* L.) for chickpea stunt disease and associated viruses in India and Pakistan. *Plant Disease*, **80**: 286-290.

Hussein, H.S. and Brasel, J.M. (2001). Toxicity, metabolism and impact of mycotoxins on humans and animals. *Toxicology*, **167**: 101-134.

ICRISAT (1985). *Annual report* 1984. ICRISAT, Patancheru, 502 324, India.

ICRISAT (1995). Growing chickpea in Bangladesh and Eastern India, India. ISBN 92-9066-254-9, Order code: GAE 032. ICRISAT, Patancheru, 502 324.

ICRISAT (1997). Growing chickpea in India, ISBN 92-9066-364-2, Order code: GAE 033, ICRISAT, Patancheru, 502 324, India.

Jalali, B.L., and Chand, H. (1991). Chickpea wilt. In: Mukhopadhya, A.N., Chaube, H.S., Singh. U.S. and Kumar, J. (Eds.) Diseases of International Importance, Vol.1, Prentice Hall, New Jersey, USA.

Jimenez-Diaz, R.M., and Trapero-Casas, A. (1985). Use of fungicide treatments and host resistance to control the wilt and root rot complex of chickpeas. *Plant Disease*, **69**: 591-595.

Jimenez-Diaz, R.M., Trapero-Casas, A. and Cabrera de la Colina, J. (1989). Races of *Fusarium oxysporum* f. sp. *ciceri* infecting chickpeas in Southern Spain. In: Tjamos, E.C., Beckman, C.H. (eds.). Vascular wilt diseases of plants. *NATO ASI series H*, **28**: 515-520.

Jodha, N.S., and Rao, S.K.V. (1987). Chickpea: World importance and distribution. Pages 1-10. In: The chickpea (M.C. Saxena and K. B. Singh, eds.). CAB International, Oxon, UK.

Joffe, A.Z. (1986). Phytotoxic effect of *Fusarium* species. In Fusarium species: Their Biology and Toxicology. John Wiley & sons, New York, Chichester, Brisbane, Toronto and Singapore, 173-224.

Kantar, F., Elkoca, E., Ogutcu, H and Algur, O.F. (2003). Chickpea yields in relation to *Rhizobium* inoculation from wild chickpea at high altitudes. *Journal of Agronomy & Crop Science*, **189**: 291-297.

Kaur, G., Singh, U.S. and Garg, G.K. (1987). Mode of action of toxin isolated from *Fusarium oxysporum* f. sp. *ciceri*. *Indian Phytopathology*, **40**: 76-84.

Khan, M.A. (1990). Production and utility of chickpea in Pakistan. *Progressive Farming*, **19**: 28-33.

Knoche, H.W and Duvick, J.P. (1987). The role of fungal toxins in plant disease. In: Fungal infection of plants, Symposium of the British Mycological society. Edited by G.F. Pegg & Peter G. Ayres, Cambridge University Press, 158-192.

Lagana, A., Curini, R., D'Ascenzo, G., De Leva, L., Faberi, A. and Pastorini, E. (2003). Liquid chromatography/tandem mass spectrometry for the identification and determination of trichothecenes in maize. *Rapid Communications in Mass Spectrometry*, **17**: 1037-1043.

Larkin, R.P. and Fravel, D.R. (2002). Effects of varying environmental conditions on biological control of Fusarium wilt of tomato by non-pathogenic *Fusarium* spp. *Phytopathology*, **92**: 1160-1166.

Latif, Z and Strange, R.N. (1993). Production of the phytotoxins, solanopyrones A and C and cytochalasin D among nine isolates of *Ascochyta rabiei*. *Plant Pathology,* **42**: 172-180.

Lifschitz, R., Windham, M.T. and Baker, R. (1986). Mechanisms of biological control of pre-emergence damping-off of pea by seed treatment with *Trichoderma* spp. *Phytopathology*, **76**: 720-725.

Loeffler, W. *et. al*. (1986). Antifungal effects of bacilysin and fengymycin from *Bacillus subtilis* F-29-3; a comparison with activities of other *Bacillus* antibiotics. *Journal of phytopathology-Phytopathologische Zeitschrift*, **115**: 204-213.

L'vova, L.S., Sedova, I.B., Kizlenko, O.I. and Tutel'yan, V.A. (2003). Production of fumonisins by *Fusarium moniliforme* strains isolated from corn grain. *Applied Biochemistry and Microbiology*, **39**: 197-201.

Malik, B.A. and Tufail, M. (1981). Chickpea production in Pakistan. In: Proceeding of the workshop on Ascochyta blight and winter sowing of chickpeas, pp. 229-235. (Eds. M.C. Saxena and K.B. Singh). 1984. Martinus Nijhoff / Dr W. Junk Publishers, The Hague, Netherlands.

Maloy, O.C. (1993). Resistance. In: Plant disease control, 279-302. John Wiley and Sons, INC.

Mandeel, Q. (1996). Integration of biological and chemical control of Fusarium wilt of radish. *Journal of Plant Diseases and Protection*, **103**: 610-619.

Mateo, J.J., Llorens, A., Mateo, R. and Jimenez, M. (2001). Critical study of and improvements in chromatographic methods for the analysis of type B trichothecenes. *Journal of Chromatography A*, **918**: 99-212.

Milat, M.L. and Blein, J.P. (1995). *Cercospora beticola* toxins III. Purification, thin-layer and high-performance liquid chromatographic analyses. Journal of chromatography A, **699**: 277-283.

Mitchell, R.E. (1979). Bean halo blight: comparison of phaseolotoxin and N-phosphoglutamate. *Physiological Plant Pathology*, **14**: 119-128.

Moretti, A., Belisario, A., Tafuri, A., Ritieni, A., Corazza, L. and Logrieco, A. (2002). Production of beauvericin by different races of *Fusarium oxysporum* f. sp. *melonis*, the Fusarium wilt agent of muskmelon. *European Journal of Plant Pathology*, **108**: 661-666.

Moss, M.O. (1991). The environmental factors controlling mycotoxin formation. In: Smith, J.E., Anderson, R.A. (Eds.), Mycotoxins and Animal foods. CRC Press, Boca Raton, FL, 37-56.

Nelson, P.E. (1981). Life cycle and epidemiology of *Fusarium oxysporum*. In Fungal wilt diseases of plants. Mace, M.E., Bell, A.A. and Beckman, C.H. (Eds.), Academic press, London, 51-80.

Nene, Y.L, Haware, M.P. and Reddy, M.V. (1978). Diagnosis of some wilt-like disorders of chickpea (*Cicer arietinum* L.). *ICRISAT Information Bulletin*, **3**: 44.

Nene, Y. L. (1980). Diseases of chickpea. Proceedings, of International Workshop on Chickpea Improvement. ICRISAT, 28 Feb.02, March 1979, Hyderabad, A.P. India.

Nene, Y.L (1985). Opportunities for research on diseases of pulse crops. *Indian Phytopathology*, **38**: 1-10.

Nene, Y.L. and Reddy, M.V. (1987). Chickpea diseases and their control. In: The chickpea, pp. 233-270. (Eds. M.C. Saxena and K.B. Singh). CAB International, Wallingford, U.K. and ICARDA, Aleppo, Syria.

Nene, Y.L., Haware, M.P., Reddy, N.M.V., Philips, J.C., Castro, E.L., Kotasthane, S.R., Gupta, O., Singh, G., Shukia, P., and Sah, R.P. (1989). Identification of broad and stable resistance to wilt and root-rots in chickpea. *Indian Phytopathology*, **42**: 499-505

Nirenberg, H.I. and O'Donnell, K. (1998). New *Fusarium* species and combination within the *Gibberella fujikuroi* species complex. Mycologia, **90**: 434-458.

O'Donnell, K. and Cigelnik, E. (1997). Two divergent intragenomic rDNA ITS 2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution*, **7**: 103-116.

Park, J.J., Smallery, E.B. and Chu, F.S. (1996). Natural occurrence of *Fusarium* mycotoxins in field samples from the 1992 Wisconsin corn crop. *Applied Environmental Microbiology*, **62**:1642-1648.

Penyalver, R., Vicedo, B. and Lopez, M.M. (2000). Use of genetically engineered *Agrobacterium* strain K 1026 for biological control of crown gall. *European Journal of Plant Pathology*, **106**: 801-810.

Phelps, D.C. *et al*. (1990). Immunoassay for naphthazarin phytotoxin produced by *Fusarium solani*. *Phytopathology*, **80**: 298-302.

Phillips, J.C. (1988). A distinct race of chickpea wilt in California. *International Chickpea Newsletter*, **18**: 19-21.

Pligh, M and Rudnicki, R.M. (1979). Studies on the toxins of *Phytophthora cactorum* pathogenic to apple trees. *Phytopathologische Zeitschrift*, **74**: 270-278.

Poling, S.M. and Plattner, R.D. (1996). Rapid purification of Fumonisins B_3 and B_4 with solid phase extraction columns. *Journal of Agricultural Food Chemistry*, **44**: 2792-2796.

Poling, S.M and Plattner, R.D. (1999). Rapid purification of Fumonisins and their hydrolysis products with solid-phase extraction columns. *Journal of Agricultural Food Chemistry*, **47**: 2344-2349.

Puhalla, J.E. (1985). Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. *Canadian Journal of Botany*, **63**: 179-183.

Qiagen (2002). QIAquick spin handbook, *Qiagen*, July 2002

Radova, Z., Holadova, K. and Hajslova, J. (1998). Comparison of two clean-up principles for determination of trichothecenes in grain extract. *Journal of Chromatography A*, **829**: 259-267.

Rao, S. and Padmaja, M. (2000). Selection of chickpea cell lines resistant to culture filtrate of *Fusarium oxysporum* f. sp. *ciceri*. *Phytomorphology*, **50**: 41-46.

Rangaswamy, G. and Prasad, N.N. (1960). A bacterial disease of *Cicer arietinum* L. *Indian Phytopathology*, **12**: 172-175.

Saxena, M.C.1989. Current status and prospectus of Kabuli chickpea production. Pages 1-10. In: Disease resistance of chickpea (K.B. Singh and M.C. Saxena, eds.). ICARDA, 6-8 March 1989, Aleppo, Syria.
Schaafsma, A.W., Nicol, R.W., Saward. M.E., Sinha, R.C., Reid, L.M. and Rottinghaus, G. (1998). Analysis of *Fusarium* toxins in maize and wheat using thin layer chromatography. *Mycopathologia*, **142**: 107-113.

Scheffer, R.P. (1983). Toxins as chemical determinant of plant disease. Pages 1-40. In: Toxins and plant pathogenesis (J.M. Dally and B.J. Deverall. Eds.). Sydney Academic Press.

Scott, P.M. and Lawrence, G.A. (1996). Determination of hydrolysed fumonisin B1 in alkali processed corn foods. *Food Addition and Contamination*, **13**: 823-832.

Shohet, S and Strange, R.N. (1989). Use of isolated cells and protoplasts to detect phytotoxic activity in cultures of *Phytophthora drechsleri* f. sp. *cajani. Physiological and Molecular Plant Pathology*, **34**: 345-359.

Singh, S.D., Girish, A.G., Rupela, O.P., Gopalakrishnan, S., Anitha, K. and Rao, P.J.M. (2003). Synergism between *Pseudomonas fluorescens* Migula and Thiram for the control of collar rot of chickpea. *Indian Journal of Plant Protection*, **31**: 40-42.

Singh, K.B. and Ocampo, B. (1997). Exploitation of wild *Cicer* species for yield improvement in chickpea. *Theoretical Application of Genetics*, **95**: 418-423.

Singh, U.P., and Singh, R.B. (1984). Effect of date of sowing on the incidence of Sclerotinia stem rot and wilt of gram (*Cicer arietinum* L.) *Phytopatho Logische Zeischrift: Journal of Phytopathology*, **109**: 254-260.

Singh, U. (1985). Nutritional quality of chickpea (*Cicer arietinum* L.): current status and future research needs. *Qualitas Plantarum Plant Foods for Human Nutrition*, **35**: 339-351.

Snyder, W.C. and Hansen, H.N. (1945). The species concept in *Fusarium* with reference to discolour and other sections. *American Journal of Botany*, **32**: 657-666.

Snyder, W.C. and Toussoun, T.A. (1965). Current status of taxonomy in *Fusarium* species and in their perfect stages. *Phytopathology*, **55**: 83-837.

Strange, R.N. (1993). Plant disease control – Towards environmentally acceptable methods. Chapman & Hall, London.

Strange, R.N. (2003). Introduction to Plant Pathology. John Wiley & Sons Ltd., Chichester, England.

Tekeoglu, M., Tullu A., Kaiser, W.J. and Muehlbauer, F. J. (2000). Inheritance and linkage of two genes that confer resistance to *Fusarium* wilt in chickpea. *Crop Science*, **40**: 1247-1251.

Thangavelu, R., Palaniswami, A., Ramakrishnan, G., Doraiswami, S. Muthukrishnan, S. and Velazhahan, R. (2001). Involvement of fusaric acid detoxification by *Pseudomonas fluorescens* strain Pf10 in the biological control of Fusarium wilt of banana caused by *Fusarium oxysporum* f. sp. *cubense*. *Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz – Journal of Plant Diseases and Protection*. **108**: 433-445.

Thomashow, L.S. and Weller, D.M. (1990). Role of antibiotics and siderophores in biocontrol of take-all disease of wheat. *Plant and Soil*, **129**: 93-99.

Toussoun, T.A. and Nelson, P.E. (1968). Fusarium: A pictorial guide to the identification of Fusarium species according to the taxonomic system of Snyder and Hansen. University Park: Pennsylvania State University Press.

Toussoun, T.A. and Nelson, P.E. (1975). Variation and speciation in the fusaria. *Annual Review of Phytopathology*, **13**: 71-82.

Toussoun, T.A. and Nelson, P.E. (1976). Fusarium: A pictorial guide to the identification of Fusarium species according to the taxonomic system of Snyder and Hansen, 2nd ed. University Park: Pennsylvania State University Press.

Trapero-Casas, A. and Jimenez-Diaz, R.M. (1985). Fungal wilt and root rot diseases of chickpea in southern Spain. *Phytopathology*, **75**: 1146-1151.

Turner, J.G. and Mitchell, R.E. (1985). Association between symptom development and inhibition of ornithine carbamoyltransferase in bean-leaves treated with phaseolotoxin. *Plant Physiology*, **79**: 468-473.

Van der Maesen, L.J. (1972). *Cicer* L., a monograph of the genus, with special reference to the chickpea (*Cicer arietinum* L.) its ecology and cultivation. Medelingen Landbowhogeschool Wageningen 72-10. Wageningen, Netherlands: H.Veenman and Zonen, N.V. 342.

Van der Maesen, L.J. (1987). Origin, history and taxonomy of chickpea. Pages 11-34. In: The chickpea (M.C. Saxena and K.B. Singh, eds.). CAB International, Oxon, UK.

Van Driesche, R.G. and Bellows, Jr., T.S (1996). Biological Control, 1-20.

Van Loon, L.C., Bakker, P.A.H.M. and Pieterse, C.M. (1998). Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology*, **36**: 453-83.

Vessonder, RF. and Hesseltine, C.W.(1981). Metabolites of *Fusarium*. Pages 350 –364. In: Fusarium: Diseases, Biology and Taxonomy (P.E. Nelson, T.A. Toussou and R.J. Cook, Eds.) PSU Press. University Park.

Wang, Y.Y., Whitehead, M., Zhu, Y.Y., Burns, A., Hockling, T., Ruan, X.Y., and Kong, Q. (2003). *Fusarium oxysporum* f. sp. *vanillae* isolate ML-1-1 internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2 sequences. Submitted (10-Sep-2003), Department of Plant Pathology, College of Plant Protection, Yunnan Agricultural University, Long Tou Street, Kunming, Yunnan 650201, P.R. China.

Weltzien, H.C. (1989). Role of host-plant resistance in integrated disease management. Pages 144-154. In: Disease resistance breeding in chickpea (K.B. Singh and M.C. Saxena, eds.). ICARDA. 6-8 March 1989. Aleppo, Syria.

Westerlund, F.V., Campbell, R.N. Jr. and Kimble, K.A. 1974. Fungal root rots and wilt of chickpea in California, 64: 432-436Williams, P.C. and Singh, U., (1987). Nutritional quality and the evaluation of quality in breeding programmes. In: The Chickpea, pp. 329-356 (Eds.

M.C. Saxena and K.B. Singh). CAB International, Walling ford, U.K. and ICARDA, Aleppo, Syria.

White T. J., Bruns T., Lee S. and Taylor J., (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M. A., Gelfand D.H., Sninsky J. J., White, T. J. (eds.). PCR protocols: A Guide to Methods and Applications, pp 315-322. Academic Press, New York.

Wilkes, J.G. and Sutherland, J.B. (1998). Sample preparation and high-resolution separation of mycotoxins possessing carboxyl groups. *Journal of Chromatography B*, **717**: 135-156.

Windels, C.E., (1992). Fusarium. In: Methods of research on soil borne phytopathogenic fungi. St. Paul, M.N. USA. Eds. Singleton L.L., Mihail, J.D. and Rush, C.M. American Phytopathological Society, 115-128.

Wolpert, T.J., Dunkle, L.D. and Ciuffetti, L. M. (2002). Host-selective toxins and avirulence determinants: what is in a name? *Annual Review of Phytopathology*, **40**: 251-85.

Woo, S., Fogliano, V., Scala, F. and Lorito, M. (2002). Synergism between fungal enzymes and bacterial antibiotics may enhance biocontrol. Anttonie Van Leeuwenhoek *International Journal of General and Molecular Microbiology*, **81**: 353-356.

Yoder, O.C. (1980). Toxins in pathogenesis. *Annual Review of Phytopathology* **18**: 103-129.

Zulet, M.A. and Martinez, J.A. (1985). Corrective role of chickpea intake on a dietary induced model of hypercholesterolemia. *Plant Foods for Human Nutrition* **48**: 269-277.