PARTIAL CHARACTERIZATION OF TOXIGENIC *Fusarium*

By

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Preface

The work described in this thesis was carried out by the author in the Department of Biotechnology, Faculty of Science, Engineering and the Built Environment, M.L. Sultan Campus, Durban Institute of Technology, under the supervision of Prof. B. Odhav and Dr R. Govinden.

This study represents the original work by the author and has not been submitted in any form to another tertiary institution. Where use was made of the work by others, it has been duly acknowledged in the text.

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List of Abbreviations

AOAC	-	Association of Official Analytical Chemists
BEA	-	Beauvericin
bp	-	Base pairs
CA	-	Cluster analysis
CHEF	-	Contour-clamped homogenous electric field electrophoresis
DAS	-	Diacetoxyscirpenol
DFA	-	Discriminant function analysis
DON	-	Deoxynivalenol
E	-	Eye infection
EDTA	-	Ethylene diamine tetraacetic acid
ELEM	-	Equine leukoencephalomalacia
ELISA	-	Enzyme-linked immunosorbant assay
EN	-	Electronic nose
FA	-	Fusaric acid
FB_1	-	Fumonisin B_1
FB_2	-	Fumonisin B_2
FB_3	-	Fumonisin B_3
FB_4	-	Fumonisin B_4
FC_1	-	Fumonisin C_1
FC_3	-	Fumonisin C_3
FUP	-	Fusaproliferin
FUS	-	Fusarenon
GC	-	Gas chromatography
GC-MS	-	Gas chromatography-mass spectrometry
HPLC	-	High performance liquid chromatography
IAC	-	Immunoaffinity chromatography
MON	-	Moniliformin
MRC	-	Medical Research Council

MS	-	Mass Spectrometry
MVOC	-	Microbial volatile organic compound
NIV	-	Nivalenol
OPA	-	o-pthaldialdehyde
OPC	-	Operon Technologies
PCA	-	Principle component analysis
PCR	-	Polymerase chain reaction
PFGE	-	Pulsed-field gel electrophoresis
PKS	-	Polyketide synthase
ppri	-	Plant Protection Research Institute
RAPD	-	Random amplified polymorphic DNA
RFLP	-	Restriction fragment length polymorphism
RIA	-	Radioimmunoassay
SAX	-	Strong anion exchange
SDA	-	Sabouraud dextrose agar
SDB	-	Sabouraud dextrose broth
T-2	-	T-2 toxin
TAE	-	Tris-acetate EDTA
TE	-	Tris-EDTA
TLC	-	Thin-layer chromatography
UBC	-	University of British Columbia
UV	-	Ultraviolet
UV-VIS	-	Ultraviolet-visible
ZON	-	Zearalenone

Abstract

Various methods have been developed for the analysis of *Fusarium* and its toxins. Advances in molecular biology can lead to efficient characterization of this group of fungi. This study was undertaken to examine random amplified polymorphic DNA, volatile compound production and hydrolytic enzyme production by 19 Fusarial isolates. These techniques were employed to assess their abilities in differentiating *Fusarium* species and *F. verticillioides* strains and extending the analysis to discriminate toxin producing capabilities amongst these fungi. Genetic variation of the isolates was determined with 17 randomly-chosen primers and cluster analysis was carried out by constructing dendrograms. The volatile compounds produced by these fungi were profiled using an electronic nose, and the hydrolytic enzymes produced were determined through substrate utilization. The clusters or profiles generated were correlated to the profiles of fumonisin B_1, moniliformin, fusaric acid and zearalenone production. Results indicated that all the techniques could differentiate species but none of them could conclusively differentiate various strains within a species. The random amplified polymorphic DNA technique was the only method that could differentiate between the Fumonisin B_1-producers and non-producers with three primers, namely, UBC 204, UBC 270 and UBC 284. No correlation existed for the production of volatile compounds as well as enzyme production with respect to toxins of the Fusaria. The results of this study showed that random amplified polymorphic DNA analysis, volatile profiles and enzymes can be applied to discriminate a number of *Fusarium* species, namely, *F. verticillioides*, *F. dimerum*, *F. solani*, *F. chlamydosporum* and *F. semitectum*. Further molecular characterization of *Fusarium* isolates using microsatellites, restriction fragment length polymorphisms and electrophoretic karyotyping may be explored to achieve intra-specie differentiation as well as to ascertain any correlation with the organism's toxin-producing ability.

CHAPTER ONE : INTRODUCTION AND LITERATURE REVIEW

1.1 BACKGROUND AND MOTIVATION

Interest in toxigenic *Fusarium* species is increasing worldwide due to the discovery of a growing number of naturally-occurring *Fusarium* mycotoxins that have practical importance as threats to human and animal health. There are 30 recognized species of *Fusarium* based on cultural and morphological characteristics. Many of these naturally-occurring *Fusaria* produce mycotoxins that have commercial implications and pose serious threats to human and animal health.

The genus *Fusarium* contains important mycotoxin-producing species that have been implicated in human diseases, such as alimentary toxic aleukia, Urov or Kashin-Beck disease, Akakabi-byo or scabby grain intoxication and oesophageal cancer. Many of these species have also been implicated in several animal diseases, including haemorrhagic, oestrogenic, emetic, feed refusal syndromes, fescue foot, degnala disease, mouldy sweet potato toxicosis, bean hulls poisoning and equine leukoencephalomalacia. There is compelling evidence implicating *Fusarium* and its mycotoxins in multiple contamination of animal feed and human food (D'Mello *et al.*, 1999). Many toxigenic species of *Fusarium* are also common phytopathogens, causing cereal crop diseases, which are difficult to control. Several of these diseases have been linked to different mycotoxins produced by different fusarial species. These species produce a variety of mycotoxins, such as fumonisins (Gelderblom *et al.*, 1988; Leslie *et al.*, 1992; Thiel *et al.*, 1991), zearalenone (ZON) [Rosenberg *et al.*, 1998],

trichothecenes (Marasas *et al.*, 1984), and deoxynivalenol (DON) [Childress *et al.*, 1990]. Other mycotoxins of significance are fusaric acid (FA) [Bacon *et al.*, 1996; Marasas *et al.*, 1984], moniliformin (MON) [Leslie *et al.*, 1996; Marasas *et al.*, 1984], beauvericin (BEA) [Logrieco *et al.*, 1998], fusarin C (Wiebe and Bjeldanes, 1981), and gibberellic acid (Tudzynski and Hölter, 1998).

Improvements in methods to prevent toxin formation rely on the ability to detect and quantify specific fungi and their mycotoxins. Many methods have been developed for toxin analysis with thin-layer chromatography (TLC) [qualitative] and high-pressure liquid chromatography (HPLC) [quantitative] now routine for most mycotoxins. Immunological procedures, e.g. enzyme-linked immunosorbent assay (ELISA), have also been successfully used to detect mycotoxins (Lee *et al.*, 1992). These procedures offer certain advantages in the detection of mycotoxins, however, advances in technology are required to identify the fungi prior to toxin production.

A variety of methods, based on DNA sequences and metabolite profiles such as volatile compounds and enzymes produced by fungi have been used to enable differentiation between genera, races, species and strains. Molecular techniques have supplemented traditional taxonomic methods with DNA-based tools with which to examine phylogenetics and systematics of fungi. For the Fusaria, restriction fragment length polymorphism (RFLP) analysis with DNA probes constructed from nuclear or mitochondrial regions or of the ribosomal DNA have been used (Manicom *et al.*, 1987; Kim *et al.*, 1993). Random amplified polymorphic DNA (RAPD) markers have also been applied to the study of genomic variation of various fungal species, to examine

population variability in *F. graminearum* and *F. solani* and to differentiate races of *F. oxysporum* (Donaldson *et al.*, 1995).

The volatile compounds produced by fungi during metabolism, gives a characteristic aroma profile which is detected by electronic nose (EN) technology. This has been used in fungal systematics (Borjessön *et al.*, 1990; Kaminski *et al.*, 1985; Keshri *et al.*, 2002; Tuma *et al.*, 1989).

Enzymes are also good early indicators of the activity of spoilage moulds (Magan, 1993). Studies completed in recent years provide evidence of this presumption. However, no studies have attempted to determine whether the production of extracellular enzymes would differentiate between toxin-producing and non-toxigenic *Fusarium* species. These techniques could lead to the differentiation and selection of toxin-producing and non-producing strains, which could ultimately develop into strategies to shut off toxin production in toxigenic strains.

1.1.1 Aims and Objectives

The Fusarial group of fungi are significant in that they impact human health, agriculture and the economy. Early identification and differentiation of this genus is essential in providing a useful and powerful tool for detecting inoculum sources and to aid in the development of disease management strategies.

Therefore, this study was, undertaken to characterize strain and specie variation within *Fusarium*, in relation to their toxin profiles, namely, fumonisin B_1 (FB_1), ZON, FA and MON. The emphasis was on the FB_1-producing Fusaria since they have serious implications on human health. The objectives of this study, were to differentiate *Fusarium* species and strains based on (i) genetic variation using RAPD's; (ii) the volatile compounds pattern generated by an EN system; and (iii) the production of hydrolytic enzymes.

1.1.2 Study Design

The first component of this study involved acquiring and maintaining the 15 *F. verticillioides* strains and four *Fusarium* species. This included the culturing of all Fusaria on Sabouraud dextrose agar (SDA) for working stocks as well as their preservation at –70°C for long-term storage. In addition, all isolates were verified by morphological characteristics and light microscopy.

Determination of the mycotoxin-producing potential of each of the *Fusarium* isolates formed the second phase of research. This was achieved by culturing the isolates on SDA and extracting and purifying the FB_1, ZON, FA and MON using appropriate procedures. The resulting toxins were quantified by HPLC with the use of commercial standards.

The third aspect of this study examined the production of RAPD profiles of each *Fusarium* isolate using 17 commercial arbitrary primers. This procedure involved the

isolation and quantification of Fusarial genomic DNA, amplifying the DNA on a thermal cycler and, thereafter subjecting the PCR product to electrophoresis. The fourth phase of this study was that of sensory evaluation. An EN was employed to establish any differences in the production of volatile compounds by each of the 19 *Fusarium* isolates. Finally, each *Fusarium* isolate was assessed for the production of four hydrolytic enzymes, viz., α-D-galactosidase, β-D-xylosidase, β-D-glucosidase and α-D-mannosidase. Each enzyme assay was carried out with the use of commercial substrates.

Results of the latter three aspects of work were analyzed and correlated to toxicity of the *Fusarium* isolates in order to determine if these methods could be used as a quick and easy mark of determining whether a *Fusarium* isolate is mycotoxigenic.

1.2 LITERATURE REVIEW

Taxonomy continues to be the cornerstone of any research or applications. It is of crucial importance in epidemiology and ecology to be able to identify microbial species and strains accurately so that management strategies can be designed as quickly as possible. A number of different phenotypic and genotypic (nucleic acid-based) methods are presently being employed for microbial identification and classification. Rapid identification and classification of microbes is normally carried out by studying the morphology, nutritional requirements, antibiotic resistance, isoenzyme comparisons and phage sensitivity (Welsh and McClelland, 1990).

1.2.1 Fusarial Classification

The form-genus *Fusarium* is the largest in the *Tuberculariaceae* and classified in the class Hyphomycetes (sub-division Deuteromycetes) [Hawksworth *et al.*, 1995]. There are 30 species of *Fusarium* that are most commonly recognized, but many additional species have been isolated and identified based on cultural and morphological characteristics. However, due to the varying culture conditions and the mutational possibilities of these species, not all scientists recognize them as unique (Seifert, 1996).

1.2.1.1 Traditional Methods of Classification

Knowledge of the taxonomy of *Fusarium* and the appropriate procedures for identification is basic to most studies of the genus. *Fusarium* taxonomy has long been

controversial and confusing, even for taxonomic specialists because of the use of different taxonomic systems (Seifert, 1996). Taxonomy is further complicated since the genus is notorious for their high species variability due to their genetic structure and also because environmental changes can easily cause changes in their morphology (Malloch, 1997). Correct identification of *Fusarium* is also becoming increasingly important due to the toxin-producing capabilities of some of these species, especially the fumonisin producers. Attempts have been made to classify *Fusarium* on the basis of soluble protein electrophoretic patterns (Glyn and Reid, 1969 - cited by Manicom *et al.*, 1987), zymograms (Scala *et al.*, 1981 - cited by Manicom *et al.*, 1987), immunoelectrograms (Abd-el-Rehim and Fadel, 1980) and monoclonal antibody reactions (Dietricht *et al.*, 1995).

Current taxonomic identification of *Fusarium* is based on micro- and macro-morphological characteristics, such as cultural morphologies including colony and colour characteristics on specific culture media, the size, shape, development of sexual and asexual spores, and spore-forming structures, and/or physiological characteristics such as the ability to utilize various compounds as nitrogen and carbon sources (Glass and Donaldson, 1995).

Identification of *Fusarium* and other filamentous moulds is based almost entirely on the structures bearing spores and on the spores themselves. The presently available identification manuals provide a series of synoptic keys (Nelson *et al.*, 1983), dichotomous keys (Booth, 1971), tabular keys (Burgess *et al.*, 1988) or no key at all (Gerlach and Nirenberg, 1982). The most common means of identifying moulds is by the use of a dichotomous *key*, a very clever device presenting a series of alternatives for

consideration. The dichotomous keys are designed to work like the mind of an experienced mycologist, eliminating the most common or most expected fungi first and relegating the less common ones to the end. These are composed of keys to several groups of genera. Group I contains the most commonly encountered genera, group II, those that are a little less common, and group III, those that are less common yet, and so on down to the end. All identifications should be checked against the appropriate description and illustration following the key. Fungi from one group may be identified incorrectly in the key to another group and only the description and illustration will reveal the mistake (Malloch, 1997).

Picture keys are arranged in the same manner as the dichotomous keys, that is, the first set of pictures illustrates the most commonly encountered moulds. Those in the second set are also common, but not quite as common as those in the first group. To use the keys, one must browse over the first group to see if the unknown specimen matches one of the pictures. If so, choose that picture for further information. If the fit is not very good, the application needs to be repeated. If the unknown is not in group I, one must go on to group II (Malloch, 1997).

Profiles of secondary metabolites together with physiological characteristics have been combined with morphological characteristics to create a more reliable fungal taxonomy. Besides confirming or disproving the identity of a particular fungal isolate, the metabolic profile also shows the potential of the isolate to produce mycotoxins and other biologically active compounds. Furthermore, profiling is used as a means to describe and characterize new taxonomic units and as a measure of response in

physiological experiments. This kind of information is very valuable when working with fungi associated with human consumption and environment. Metabolite profiling as a taxonomic tool has been applied to the genera like *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, *Paecilomyces*, *Trichoderma*, *Chaetomium*, *Eurotium*, *Stachybotrys* and *Stemphylium* (Mycology, 2003).

1.2.1.1.1 Morphological Characteristics of *Fusarium*

Fusarium cultures may be brightly-coloured but colony morphology and pigmentation are variable in culture and thus not useful in differentiating between species. Therefore, their main identifying characteristic is their colourless spores which are canoe-shaped in side view, have a distinct "foot cell" at the lower end, and are divided by several cross-walls. Members of this form-genus typically produce three types of conidia; macroconidia and microconidia, termed due to their sizes, and chlamydospores. The conidiophores are often clustered to form sporodochia and produce large pasty masses of spores from tapered phialides. Chlamydospores are thick-walled swellings along the filaments (Malloch, 1997). The important cultural characters of *Fusarium* species (Burgess *et al.*, 1988), which are used in conjunction with a *Fusarium* taxonomic key for identification, are outlined in TABLE 1.1.

TABLE 1.1 Cultural characteristics of *Fusarium* species and a description of each of the characters used in its identification

CHARACTERISTICS	*Fusarium* CHARACTERS
Shape of the macroconidium	A macroconidium may be stout or slender. It can be falcate or relatively straight. The walls can be parallel or curved and appear thick or thin. The number of septa usually varies. The apical cell can be short and blunt rounded) or hooked (beaked), papillate (dolphin-nosed), tapered to a point or very long (filamentous) or even curved or coiled.
Presence or absence of microconidia	Produced in the mycelium. Presence or absence of microconidia is an important diagnostic feature.
Shape and mode of formation of microconidia	Are mostly 1- or 2-celled, are 0-3 septate and are generally smaller than macroconidia. Are formed in chains or false-heads.
Nature of the conidiogenous cell bearing microconidia	Most *Fusarium* species produce monophialides. Other species produce polyphialides, in addition to monophialides.
Presence or absence of chlamydospores	The presence of chlamydospores is a useful criterion for differentiation between other fungal isolates.
Colony diameters on PDA	They can be extremely beneficial in distinguishing some populations.

1.2.1.2 Molecular Methods of Classification

1.2.1.2.1 Genetically-based Methods

The more recent advent of DNA-based methods has provided useful tools with which to study the systematics and phylogeny of fungi and to differentiate between species, formae speciales, races and strains. A variety of techniques, including rRNA sequences (Woese, 1986 - cited by Welsh and McClelland, 1990), strain-specific fluorescent

oligonucleotides (Amann *et al.*, 1990 - cited by Welsh and McClelland, 1990), and RFLP analysis of hybridization data with DNA probes (Kohn *et al.*, 1988), have been used.

Direct sequencing of conserved regions such as nuclear and mitochondrial rRNA genes has proven to be valuable in studies of intra- and inter-family level relationships. However, these conserved regions may reveal insufficient variability to be useful in resolving relationships within species (Bruns *et al.*, 1992 - cited by Gordon and Okamoto, 1992). Studies on genetic relationships and phylogeny among *Fusarium* spp. have been conducted at the protein (Partridge, 1991) and DNA levels (Manicom *et al.*, 1990).

a) Random Amplified Polymorphic DNA Analysis

This technique relies on the presence of priming sites for a single primer on the genome in an inverted orientation and close enough to permit polymerase chain reaction (PCR) amplification. A PCR reaction is conducted with a single primer, usually nine or ten nucleotides in length. The sequence of the primer is arbitrary and computer programmes can be used to ensure no secondary structures will form. The primer finds homology in the DNA added to the PCR reaction and initiates extension. Since the primer sequence is random, the annealing temperature is low, typically 35-37°C, to ensure that the primer will anneal to the template DNA. After 30-40 cycles have been completed, the product is run on an agarose gel and viewed over an ultraviolet (UV) light source following ethidium bromide staining. The method of PCR works remarkably well in generating RAPD's which can be analyzed from differences in the sizes of bands on a gel

indicating sequence differences in the DNA of different strains or species (Foster *et al.*, 1993). No prior knowledge of the genome to be analyzed is required.

The accuracy of RAPD markers in predicting genetic relationships has been demonstrated in plant phylogenetic studies which provide a quantitative assessment of genetic relationships and similarities of genotypes at the sub-species level and which correspond to taxonomic systems based on morphological, genetic, and agronomic criteria which is consistent with established phenotypic schemes (Kazan *et al.*, 1993). Random amplified polymorphic DNA markers have been applied to the study of genomic variation of a number of fungal species (Crowhurst *et al.*, 1991; Ouellet and Seifert, 1993). This technique was used to examine population variability in *F. graminearum* (Ouellet and Seifert, 1993) and *F. solani* (Crowhurst *et al.*, 1991) and to differentiate races of *F. oxysporum* (Grajal-Martin *et al.*, 1993). In 1995, Voigt *et al.*, showed the RAPD technique to be efficient in distinguishing between different species of the genus *Fusarium.*

b) Other Molecular Methods

Another technique with powerful resolving ability for the rapid classification of fungal isolates is the use of RFLP's. Restriction fragment length polymorphism's can in theory, by suitable choice of a probe, be set to any particular taxonomic level, or used as markers for a trait, such as pathogenicity.

It is unlikely that a number of different fungi would have identical RFLP patterns for all amplification products, and therefore, this test could be used as a diagnostic tool

for the detection and identification of filamentous *Fusarium.* Restriction fragment length polymorphism analyses of nuclear or mitochondrial DNA have been employed to estimate the genetic diversity of *F. oxysporum* within and between formae speciales and among non-pathogenic strains (Gordon and Okomoto, 1992). Probes have been constructed from mitochondrial DNA to identify RFLP's among *F. oxysporum* formae speciales (Kistler *et al.*, 1987).

Electrophoretic karyotyping permits such studies in organisms in which classical cytological analyses are not practical, e.g., those lacking a known sexual reproductive phase since some cells from vegetative tissue can be used as a DNA source. Pulsed-field gel electrophoesis (PFGE) techniques have been used to construct karyotypes of various fungi, including several *Fusarium* spp. (Boehm *et al.,* 1994; Fekete *et al.*, 1993; Migheli *et al.,* 1993; Nagy and Hornok, 1994; Xu *et al.*, 1995). In most of these cases, however, investigators have focussed on only a single species and have not used this powerful tool to elucidate the relationships between related groups. In 1993, Fekete and colleagues described electrophoretic karyotypes for eight different *Fusarium* species, viz., *F. avenaceum, F. camptoceras, F. chlamydosporum, F. fusaroides, F. pallidoroseum, F. poae, F. sporotrichioides* and *F. tricinctum.*

1.2.1.2.2 Electronic Nose Technology

Microorganisms produce volatile compounds, some of which impart high flavour and odour impact although present at concentrations in the sub parts per billion range. Fungi commonly produce volatile compounds as they start colonizing nutrient-rich

substrates such as grain. In the 1970's, Kaminski *et al.* (1972; 1974; 1975) demonstrated that spoilage fungi produced volatiles, which were characteristic and different from those produced by bacteria or the seeds themselves. Linton and Wright (1993) reviewed the possible reasons for the production of fungal volatiles. Production of a volatile may be a way of removing inhibitory intermediates from the metabolism under unfavourable conditions. They also suggested that volatiles might have inhibitory effects on other fungi and act as self-regulators of growth and development.

Most of the early work on the detection of volatiles was done using gas chromatography (GC) and mass spectrometry (MS). For GC analysis, most workers have used a polar stationary phase for the separation of the fungal alcohols, aldehydes, ketones and esters. Kaminski *et al.* (1985; 1987) also developed a spectrophotometric method for the accurate quantification of volatile carbonyl compounds.

There has been interest in the potential of using the dominant odour volatiles produced by fungi, particularly 3-methyl-1-butanol, 1-octen-3-ol and 3-octanone, as indicators of spoilage in stored grain (Borjessön *et al.*, 1990; Kaminski *et al.*, 1985; Tuma *et al.*, 1989). Numerous *in vitro* studies have been carried out to determine the types of volatiles produced by grain spoilage fungi. A range of classes of volatile compounds including alcohols, carbonyls and hydrocarbons have been identified.

Studies on a number of fungal species revealed that specific volatiles such as 3-methylfuran are produced in similar amounts regardless of the fungal species and grain substrate used (Borjessön *et al.*, 1992). However, other volatile compounds,

especially terpenes, were both specie- and strain-specific (Borjessön *et al.*, 1989; 1993; Larsen, 1997; Larsen and Frisvad, 1995; Nilsson *et al.* 1996) e.g. *Aspergillus* species produced thujopsene, which was absent from the *Penicillium* cultures. *A. candidus* produced a monoterpene not found in the other fungi examined, suggesting that monoterpene profiles could be used to differentiate between fungi.

Volatile profiles can be used rather than individual volatile compounds to classify fungi at a species level, as a combination of volatiles is often unique to each species (Korpi *et al.*, 1998; Larsen, 1997; Larsen and Frisvad, 1994; Wilkins *et al.*, 1997). Larsen (1997) was able to classify *Penicillium* species within two days after inoculation and in a mixed culture of *P. roquefortii* and *P. commune*, with identification after three days. Larsen and Frisvad (1995) were also able to use these patterns to reclassify similar groups of *Penicillium* spp. into separate clusters based on the production of geosmin. Since it is clear that there is a range of characteristic volatile odours produced by fungi when colonizing grain, the question is whether there is any relationship between these patterns and the odour descriptors.

Recently, there has been much interest in several competing electronic devices for the sensing of food aroma. They are claimed to operate on principles that are similar, in some respects, to the human olfactory system (Mielle, 1996). Whilst not truly mimicking the natural olfactory system, EN's borrow from the mechanism of natural olfaction.

In this rapidly growing field, arrays of cross-reactive (non-specific) chemical sensors are coupled to pattern-recognition programs, paralleling the biological olfactory system, in which semi-selective olfactory receptors are combined with higher–order neural processing. In a similar way to the natural system, artificial noses are able to quantify and discriminate between odours. Hence, products with similar aroma generally result in similar sensor response patterns (similar "fingerprints"), whereas products with different aromas show differences in their patterns (different "fingerprints"). The interaction of volatiles with the array of sensors provokes a series of signals which are then processed by the computer via a pattern recognition program (Figure 1.1) [Ampuero and Bosset, 2003].

Figure 1.1 Principle of the electronic nose (Department of Electronic Science, 2003).

The potential applications for such a device span a wide range of areas, from bioprocess monitoring and medical diagnosis to breath analysis, and are likely to extend beyond the gas phase to liquid phase measurements. Although the most substantial commercial market is currently process and quality control in the food industry, artificial nose technology is now beginning to be applied in a number of other fields, such as environmental monitoring and medical diagnosis (Bartlett *et al.*, 1997).

The EN is an analytical instrument which electronically measures odours and aromas. It performs this measurement objectively and quickly – typically 10 minutes per sample. There is no need for complex solvent extractions or special columns as used with chromatography techniques. The EN is also fast, easy to use and operates at a low cost per sample. The data obtained can be analyzed by non-linear mapping techniques such as Sammon mapping where clusters formed, give a simple visual representation of how similar or different the test aromas are (GammaTec, 1998). The main advantage of this instrument is that in a matter of seconds, it delivers objective, reproducible aroma discrimination with sensitivity comparable to the human nose for most applications.

This new technology enables the on-line detection of a drift or a defect in a process or in a discrete product, but in most cases does not identify either the nature or the origin of this defect (Mielle, 1996). It has a range of potential applications in the food and other industries, and more may emerge in the future.

While EN systems are qualitative, volatile production patterns can be specific for different species. Bacterial species can be differentiated based on their volatile production patterns (Gibson *et al.*, 1997), although much less information is available on food spoilage moulds (Keshri *et al.*, 1998; Magan *et al.*, 2000).

Filer *et al.* (2001) screened twelve fungal species, including *F. solani*, for more than 150 microbial volatile organic compounds (MVOC's) and concluded that each specie had a defined MVOC profile which may be subjected to considerable modification in response to external factors such as cultivation. The objective of Greene-McDowelle

and colleagues in 2001 was to utilize the volatile compounds found in the head-space of rice and fungi to develop a rapid, instrumental method to detect and identify damaged rice as a result of fungal contamination.

Potential exists for distinguishing between species of fungi based on characteristic volatile patterns, which may be important when key spoilage fungi may be responsible for the production of harmful mycotoxins. It may be possible to use EN systems to try and distinguish between grain colonized by mycotoxigenic and non-mycotoxigenic species, and this area requires further investigation.

1.2.1.2.3 Enzyme Profiling

Previously, quantitative enzyme assays for specific hydrolytic enzymes produced by spoilage fungi, including *Fusarium* spp., have been shown to be good early indicators of growth prior to visible moulding (Jain *et al.*, 1991; Magan, 1993; Marin *et al.*, 1998). However, none of these studies evaluated whether extracellular enzyme production could be used to differentiate between non-toxic and toxigenic strains of individual species. In 2000, Keshri and Magan investigated this with two *Fusarium* species, viz., *F. proliferatum* and *F. verticillioides* using seven extracelluar enzymes, viz., β-fucosidase, α-galactosidase, β-glucosidase, α-mannosidase, β-xylosidase, α-glucosaminidase and β-glucosaminidase. Results attained from the study revealed that a number of hydrolytic enzymes are important in the initial colonization of a substrate, however, they were not such good early indicators of fungal activity. Since

this analysis was limited to short incubation times, it may be of interest to research enzyme production after visible growth and toxin production has occurred.

1.2.2 *Fusarium* Species and their Toxins

Several *Fusarium* species have a worldwide distribution and have been found to occur in nature in various environments. Not all Fusaria are pathogenic but *Fusarium* is best known as a plant pathogen. A number of *Fusarium* species are parasitic, causing wilting of the host plant (Alexopoulos and Mims, 1979). Fusarial mycotoxins are usually produced while the crops are still in the field. They have been recovered from hosts as diverse as bananas, figs, mango, pine, rice, sorghum, and sugarcane (Leslie, 1995). Several *Fusarium* species that occur worldwide on cereals as causal agents of 'head blight' (scab) of small grain cereals and 'ear rot' of maize, are capable of accumulating several mycotoxins of relevant impact to human and animal health, in infected kernels (Bottalico, 1998). These toxins, if produced, remain until the grains are stored, processed, and used as food or feed. *Fusarium* species have also been isolated from samples of soil, running water, insects, seeds and roots from most plants (Hagedorn, 2004). Warm soil temperatures tend to favour *Fusarium* diseases and they are commonly found in soil, and dead or living plants where they often cause plant disease. Comparisons of data from worldwide surveys associate high levels of *Fusarium* infection and fumonisins with drier, warmer climates. The relatively warm tropical highlands of Western Kenya thus appear to provide suitable conditions for the production of fumonisins (Shephard *et al.*, 1996).

The mycotoxins more frequently encountered in ear blight of wheat and other grain cereals in Europe, proved to be DON and ZON, produced by *F. graminearum* and *F. culmorum*, succeeding from southern (warm) and northern (cold) European areas, respectively (Lew *et al.*, 1997). ZON has been shown to occur in almost every agricultural product and a variety of food-grade grains and foods have been found to contain this mycotoxin including corn and corn products, breakfast cereals, corn beer, wheat flour, bread and walnuts and in animal feed products. Depending on climatic, harvest and storage conditions, the levels of ZON found in maize and maize products are between 1 and 2 900 µg/kg (Krska and Josephs, 2001). Moniliformin produced by *F. avenaceum*, and T-2 toxin (T-2) and diacetoxyscirpenol (DAS) produced by *F. sporotrichioides* and *F. poae* were found in central to north-eastern Europe countries (Bottalico, 1998). Two surveys of several types of cereal grain, mixed livestock, and poultry feed indicated that FA is a natural contaminant of these food and feed grains (Porter *et al.*, 1995; Smith and Sousadias, 1993).

As of 1984, at least 20 toxins have been described from about 30 species of *Fusarium* (Moss and Smith, 1984). The main groups of *Fusarium* toxins commonly recognized are: trichothecene (Dube, 1983), including T-2, DAS, DON, fusarenone (FUS), nivalenol (NIV), ZON (Bottalico *et al.*, 1989; Visconti *et al.*, 1989) and fumonisins (Shephard *et al.*, 1996), in particular, fumonisin B_1 (FB_1). In addition, MON (Leslie *et al.*, 1996), BEA (Gupta *et al.*, 1991), FA (Yabuta *et al.*, 1937 – cited by Bacon *et al.*, 1996) and fusaproliferin (FUP) [Ritieni *et al.*, 1997 – cited by Fotso *et al.*, 2002] were also found in *Fusarium* infected cereal ears, as emerging problems (Bottalico, 1998). Some of the major fusarial toxins and their effects are listed in TABLE 1.2.

TABLE 1.2 The major fusarial mycotoxins and their effects

Fusarium TOXIN	TOXIC EFFECTS IN HUMANS AND ANIMALS	REFERENCE
Fumonisin B_1	Equine leukoencephalomalacia, Porcine pulmonary oedema. Associated with human oesophageal cancer.	Marasas (1995) Rheeder *et al.* (1992) Munkvold and Desjardins (1997)
Fusaric acid	Affects brain and pineal neurotransmitters. Hypotensive reaction in dogs and cats.	Porter *et al.* (1990; 1995)
Moniliformin	Decreases performance and immune function of broiler chicks.	Li *et al.* (2000)
Deoxynivalenol	Nausea, vomiting, visual disturbances, chills, vertigo and feed refusal in animals. Acute gastrointestinal illness in humans.	Childress *et al.* (1990) Luo (1988) Bhat *et al.* (1989)
Zearalenone	Immunosuppressor, strong inhibitor of protein synthesis in mammalian cells.	Rotter *et al.* (1994)

1.2.2.1 Fumonisins

Fumonisins are an important group of *Fusarium* mycotoxins produced primarily by *F. verticillioides.* However, *F. proliferatum* (Keller and Sullivan, 1960 – cited by Chulze *et al.*, 1998), *F. napiforme* (Nelson *et al.*, 1992) and *F. nygamai* (Thiel *et al.*, 1991) together with *Gibberella fujikuroi* (Desjardins *et al.*, 1997) are also known to produce these toxins. At least 12 fumonisins and structurally-related analogues have been recognized and are categorized into five groups: A, B, C, P and H (Musser and Plattner, 1997). However, of these only the B-series have been confirmed as natural

products, with FB_1 usually being the most abundant (Sweeney and Dobson, 1999). The following sections will focus mainly on the B-series, in particular, FB_1.

The B-series toxins are identical in structure except for the number and position of hydroxyls along their linear carbon backbone. Their basic chemical structure is a C-20 diester of propane-1,2,3-tricarboxylic acid and a pentahydroxyeicosane containing primary amino group (Figure 1.2) [Sigma-Aldrich - South Africa, 2004].

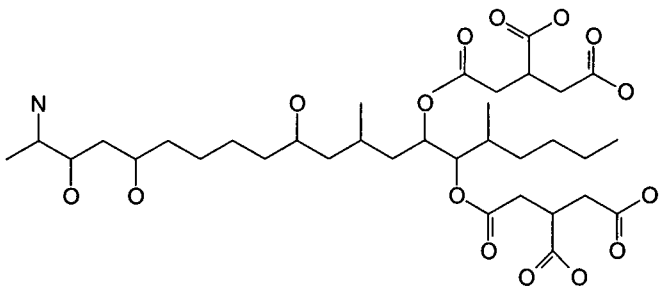
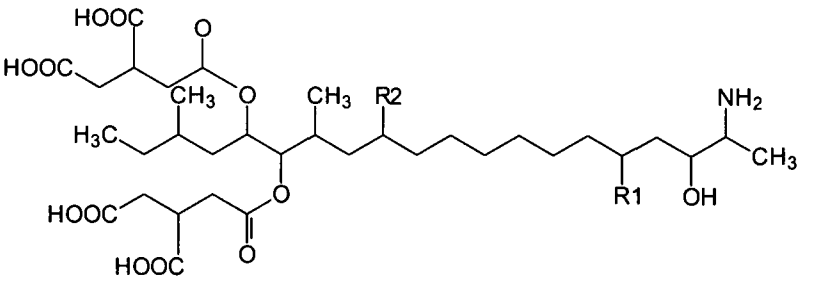


Figure 1.2 Structure of fumonisin B_1.

Fumonisin B_2 (FB_2) is the C-10 deoxy analogue of FB_1, in which the corresponding stereogenic units on the eicosane backbone have the same configuration (Figure 1.3). The full stereochemistry of fumonisin B_3 (FB_3) and fumonisin B_4 (FB_4) is unknown, but the amino terminal of FB_3 has the same absolute configuration as that of FB_1 (Hartl and Humpf, 1998). They have a similar structure to sphingosine, which forms the backbone of sphingolipids (Pitt and Hocking, 1997).



FB_1 : R1= OH; R2 = OH; R3= OH;
FB_2 : R1= H; R2 = OH; R3= OH;
FB_3 : R1= OH; R2 = OH; R3= H;
FB_4 : R1= H; R2 = OH; R3= H.

Figure 1.3 Structure of fumonisin B_1, fumonisin B_2, fumonisin B_3 and fumonisin B_4.

Two new fumonisins, hydroxylated fumonisin C_1 (FC_1) and C_3 (FC_3) have been isolated from cultures of *F. oxysporum.* Both compounds are structurally similar except for an additional hydroxy group at C-3 of hydroxylated FC_1. Fumonisin C_3 is structurally similar to FB_3, except that the C-1 terminal methyl group is missing (Seo-Jeong *et al.*, 1996).

1.2.2.1.1 Toxicity of Fumonisins

The severity and effects of fumonisins vary widely in different species. Horses, the most sensitive species known for fulminant toxicity, succumb to equine leukoencephalomalacia (ELEM) [Riley *et al.*, 1997]. The South African research group which was the first to identify and characterize the fumonisins was also the first to

demonstrate that pure FB_1 is able to produce ELEM in a horse (Marasas *et al.*, 1988). Fumonisin B_1 is hepatotoxic and hepatocarcinogenic in rats (Gelderblom *et al.*, 1988) and, in addition, has been shown to inhibit sphingolipid biosynthesis, which has been linked as a contributing factor in both its toxicity and carcinogenicity (Riley *et al.*, 1996). Fumonisin B_1 has also been associated with an increased risk of human oesophageal cancer in central regions of South Africa (Sydenham *et al.*, 1990) and China (Chu and Li, 1994).

The toxicological profiles of FB_2 and FB_3, as far as the toxicity data are available, are very similar to FB_1. Various chemical derivatives of fumonisins have been tested in a number of biological test systems to gain insight into structure-activity relationship. The free amino group appears to play a specific role in the biological activity of FB_1 (WHO Technical Report Series, 2002).

1.2.2.1.2 Biosynthesis of Fumonisins

Interest in fumonisins is due primarily to the discovery that they are potent inhibitors of sphingolipid biosynthesis and that they can impair animal health. Understanding fumonisin biosynthesis and its regulation should facilitate the development of measures to control fumonisin contamination. Numerous studies of the genetics and molecular biology of *G. fujikuroi* mating population S have been undertaken in an effort to decipher fumonisin biosynthesis (Desjardins *et al.*, 1992; Desjardins *et al.*, 1996a; Desjardins *et al.*, 1996b).

Fumonisins are structurally similar to long-chain base sphingosine, a constituent of various sphingolipids that play a role in membrane function. Wang *et al.* (1991) have reported that FB_1 is a specific receptor of sphingosine biosynthesis and have suggested that this inhibition may be the basis for fumonisin toxicity. Fumonisin B_1 inhibits ceramide synthase, the enzyme responsible for the acylation of sphingonine in the *de novo* biosynthetic pathway for sphingolipids. Fumonisins are believed to be synthesized through the condensation of the amino acid alanine to an acetate-derived precursor (Branham and Plattner, 1993). It is well established that sphingosine is biosynthesized from palmityl-coenzyme A and serine. If fumonisins are synthesized in a similar manner, then alanine would be expected to combine with linoloyl-coenzyme A, and methyl transfer, hydroxylation and esterification would follow (Wang *et al.*, 1991).

Biochemical analyses indicate that fumonisins are a product of polyketide biosynthesis. Virtually nothing is known about the molecular genetics of fumonisin production. Classical genetic studies with natural variants and laboratory mutants of *G. fujikuroi* mating population A have identified four closely-linked loci, *fum1*, *fum2, fum3* and *fum4*, which constitute a gene cluster and which are involved in fumonisin biosynthesis (Desjardins *et al.*, 1992; 1996b; Plattner *et al.*, 1996). In 1995, Xu *et al.* described the karyotype using contour-clamped homogenous electric field gel electrophoresis (CHEF) and identified 12 chromosomes ranging in size from 0.7 to 10 Mb with a total genome size of approximately 46 Mb. Marker-based mapping has localized the *fum1* locus to a region of the largest chromosome (chromosome 1) of 12 chromosomes of *G. fujikuroi* (Xu and Leslie, 1993; Xu *et al.*, 1995). Strains defective at the *fum1* locus do not

produce fumonisins (Desjardins *et al.*, 1992) while strains defective at *fum2* lack the ability to hydroxylate C-10 of the fumonisin backbone and, therefore, produce FB_2 and FB_4, but not FB_1 or FB_3 (Desjardins *et al.*, 1996a). Similarly, in a *fum3*$^-$ mutant, neither FB_1 nor FB_2 could form, because *fum3* controls hydroxylation of C-5 and conversion of FB_4 to FB_2 and of FB_3 to FB_1. *Fum4* represents a single strain that shows reduced fumonisin production and appears to be closely-related (4.3% recombination) to *fum1*, although not allelic. *Fum3* and *fum4* affect the hydroxylation of FB_1 at C-10 and C-5, respectively, and do not affect the overall level of fumonisin production. Both *fum3* and *fum4* are closely-linked to *fum1*.

Tentative estimations suggest that *fum4* and *fum2* are situated 250 kb and 360 kb from *fum1*, respectively, however, gene order has not been elucidated (Sweeney and Dobson, 1999). The physical distance between *fum1* and *fum4* can be estimated as approximately 150 kb (Plattner *et al.*, 1996). In a study carried out in 2001, Seo *et al.* identified four more *F. verticilliodes* genes (*fum6*, *fum7*, *fum8*, *fum9*) adjacent to *fum5*. Gene disruption analysis revealed that *fum6* and *fum8* are required for fumonisin production and Northern blot analysis revealed that expression of all four recently identified genes is correlated with fumonisin production. Nucleotide sequence analysis indicated that the predicted *fum6* translation product is most similar to cytochrome P450 monooxygenase-P450 reductase fusion proteins and the predicted products of *fum7*, *fum8* and *fum9* are most similar to type III alcohol dehydrogenase, class-II-alpha-aminotransferases, and dioxygenases, respectively. Together, these data are consistent with *fum5* through to *fum9* as being part of a fumonisin biosynthetic gene cluster. It would not be unexpected if this were so, since genes that control the biosynthesis of

sterigmatocystin and aflatoxins by *Aspergillus* and of trichothecenes by *Fusarium* species are also organized in complex clusters (Brown *et al.*, 1996; Desjardins *et al.*, 1996a; Yu *et al.*, 1995).

The number and order of the steps of the biosynthetic pathway that lead to the oxygenation of the acetate-derived backbone and subsequent esterification are unknown. Fumonisin biosynthetic enzymes might be able to aminate, methylate, hydroxylate and esterify stearic acid to yield FB_1.

1.2.2.2 Zearalenone

Zearalenone, estrogenic toxin or F-2 toxin derives its name from *G. zeae* and is described chemically as a phenolic acid lactone produced by a variety of *Fusarium* species growing on corn. The most important producing species are *F. graminearum*, *F. roseum*, *F. tricinctum*, *F. oxysporum*, *F. culmorum*, *F. crookwellense* and *F. verticillioides*; *F. roseum* being the most potent producer. These fungi require a period of low temperatures (12-14°C) for significant toxin production (Dube, 1983). In a study carried out by Ryu and Bullerman (1999), ZON was found to be most stable at pH 4 and the greatest losses of toxin occurred at 175°C.

In cereals and animal feeds, closely-related compounds or conjugated products are also known to occur. Some of these are considered to be more potent oestrogens than the parent compound. Because of these complex interactions, some of these ZON-related

chemicals may enter the food chain in animal products such as meat and milk (Leatherhead Food International, 2003a).

Zearalenone is a sex-hormone of *Fusarium* and its most important effect is on the reproductive system. It is known to cause hypoestrogenism in pigs, and to a lesser extent, in cattle and poultry. Symptoms include vulva swelling, decreased fertility and abortion in females, and decreased libido and enlargement of mammary glands in males. The oestrogenic effects are accompanied by diarrhoea, weight gain and haemorrhagia (Dube, 1983). Animal studies show that ZON is fairly rapidly absorbed following oral administration and can be metabolized by intestinal tissue in pigs and possibly also in humans with the formation of α- and β-zearalenols, which are subsequently conjugated with glucuronic acid. The proportions of these various products have been shown to vary considerably between species (Leatherhead Food International, 2003a).

Although the association between ZON exposure and human diseases remains speculative, it was considered as a possible causative agent in the outbreaks of precocious pubertal changes in thousands of young children in Puerto Rico. It has also been suggested to have a possible involvement in human cervical cancer (Kuiper-Goodman *et al.*, 1987).

The ZON biosynthetic pathway has been investigated using radioactive potential precursors (Steele *et al.*, 1974 - cited by Hidy *et al.*, 1977). Since the structure of ZON (Figure 1.4) [Sigma-Aldrich - South Africa, 2004] suggests a condensation of nine acetate units, precursors such as acetate, diethyl malonate, senecioate, shikimate, and

DL-mevalonic-2-lactone have been tested using cultures of *G. zeae* grown on solid substrates. Only acetate and diethyl malonate were incorporated readily into ZON, indicating that ZON is synthesized via the polyketide pathway (Hidy *et al.*, 1977).

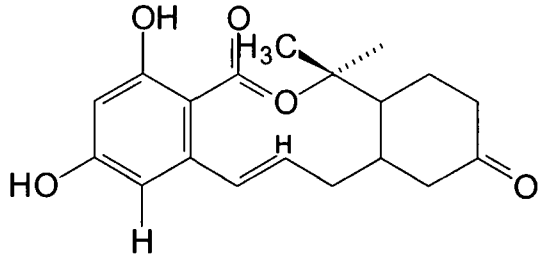


Figure 1.4 Structure of zearalenone.

1.2.2.3 Fusaric acid

Fusaric acid (Figure 1.5) [Sigma-Aldrich - South Africa, 2004] was one of the first fungal metabolites implicated in the pathogenesis of tomato wilt symptoms caused by *F. oxysporum* f. sp. *lycopersiei* Schlect. Emend. Snyd & Hans (Gaumann, 1957 – cited by Bacon *et al.*, 1996). Fusaric acid is probably one of the most widely-distributed mycotoxins produced by strains in the genus *Fusarium* (Bacon *et al.*, 1995). In a study carried out in 1996, Bacon and his colleagues reported on the ability of 13 *Fusarium* species, including six *G. fujikuroi* mating populations, to produce FA when cultured on autoclaved corn. These fungi, namely, *F. oxysporum*, *F. sambucinum*, *F. napiforme* and *F. solani* are widely-distributed on both grain and non-grain hosts. In addition to the suggested role in plant pathogenesis, FA is potentially toxic to animals; mildly toxic to mice (Hidaka *et al.*, 1969), and has several important pharmacological properties (Porter *et al.*, 1990; 1995) in that both brain and pineal neurotransmitters and metabolites are

affected. The physiological effect of FA is a drop in blood pressure. This toxin is known to augment the overall toxicity of other mycotoxins in terms of both animal and plant toxicities. It acts with vomitoxin to reduce feed intake, trigger muscle degeneration and cause lethargy (Smith *et al.*, 1994). Thus, the major importance of FA to animal toxicity may be synergistic interactions with other naturally-occurring mycotoxins.

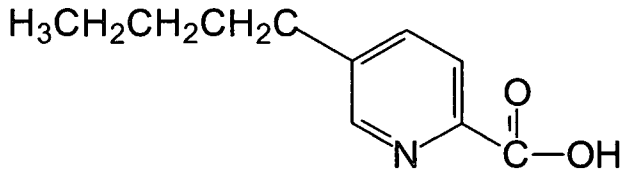


Figure 1.5 Structure of fusaric acid.

1.2.2.4 Moniliformin

Moniliformin is a fungal metabolite produced by many *Fusarium* moulds which commonly contaminate cereal grains, including corn, rye, oats and wheat. A number of cultures of *F. verticillioides* from different sources in Mozambique, Namibia and South Africa have been reported to produce MON (Rabie *et al.*, 1982). There have, however, been conflicting reports on the production of MON by *F. verticillioides*. Several authors (Allen *et al.*, 1981; Rabie *et al.*, 1982) report on the production of the metabolite while others (Marasas *et al.*, 1979; Thiel *et al.*, 1982) report no production.

Moniliformin is an ionic compound and occurs as the sodium or potassium salt of 1-hydroxycyclobut-1-ene-3,4-dione (Figure 1.6) [Sigma-Aldrich - South Africa, 2004]. It is soluble in water as a polar solvent and the free acid decomposes at 150 -153°C without melting (Leatherhead Food International, 2003b).

This metabolite is extremely toxic to many animals such as ducklings, rats, mice, chickens and swine. It is cardiotoxic and some scientists suggested that the toxin might be an aetiological factor in a heart disease called Keshan Disease (Yu *et al.*, 1995). Moniliformin, produced by *F. avenaceum*, also causes acute degenerative lesions in the myocardium, the symptoms being similar to those for arsenic poisoning (Smith *et al.*, 1994). In a few studies, it was found that MON caused chromosomal aberrations. Phytotoxic effects have also been observed (Leatherhead Food International, 2003b). Gutema *et al.*, in 2001, showed the co-occurrence of fumonisins and MON in food-grade corn and corn-based products which indicates a risk of simultaneous exposure of consumers to both toxins. It is a potent inhibitor of mitochondrial pyruvate and ketoglutarate oxidation and acts as an inhibitor of the tricarboxylic acid cycle in intermediary metabolism (Smith *et al.*, 1994).

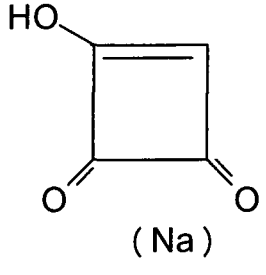


Figure 1.6 Structure of moniliformin.

1.2.3 Methods for the Detection of Mycotoxins

Analytical methods for mycotoxin analyses are based on many factors and no one technique is suitable for all applications. The initial considerations in analytical schemes include the chemical nature of the mycotoxin of interest, the molecular weight and the functional groups. These factors determine the volatility and solubility properties of the toxin and influence the analytical approach selected for a specific toxin or group of toxins. The sample matrix to be extracted for mycotoxins also influences the extraction and method selection. Current analytical procedures are usually based on extraction into a solvent followed by a partial purification step and quantification.

Some of the techniques used widely in laboratories are TLC, HPLC, immunoaffinity chromatography (IAC) and GC. Thin-layer chromatography was the first means of detecting mycotoxins and is still routinely performed. The advent of HPLC has allowed the quantification of several mycotoxins. These include patulin in apple juice (Brackett and Marth, 1979; Buchanan and Sommer, 1978; Leuenberger *et al.*, 1978; Ware, 1975 – cited by Lee *et al.*, 1992), ochratoxin, penicillanic acid, and ZON (Ware and Thorpe, 1978).

Analytical determinations of mycotoxins have traditionally been delegated to chemists. More recently, biologists have developed analytical methods based on immunological assays. Special polyclonal and monoclonal antibodies have been raised against mycotoxin-protein conjugates. These antibodies have been incorporated into novel columns for sample cleanup. The toxins are removed from sample extracts by

antibodies, washed and then removed from the antibodies. Antibodies are also used in radioimmunoassays (RIA's) and ELISA's. These procedures are common in clinical laboratories and were proposed for the screening of mycotoxins. Radioimmunoassays basically involve the incubation of a specific antibody with a radiolabelled toxin. Precipitation with a second antibody removes the toxin-antibody complex from solution. This method has been applied to aflatoxins (Groopman *et al.*, 1984), T-2, ZON (Kruger *et al.*, 1999; Park and Chu, 1996) and ochratoxin (Bisson *et al.*, 1994). The advantages of the immunochemical methods are their ease of use and the short time required for analysis, however, the immunochemical kits detect the mycotoxin indirectly and thus are more prone to error. Radioimmunoassays require labelling the toxins with tritium which is expensive and difficult. The toxin also gradually degrades and must be repurified.

The literature outlined above reveals that important *Fusarium* species and strains are currently being investigated in various areas related to their physiology and molecular biology. Research on the improvement of taxonomic classification systems have been attempted to differentiate *Fusarium* species and strains based on RAPD's or RFLP's and only one paper described the use of the electronic nose and enzyme profiles to differentiate this group of organisms (Keshri and Magan, 2000).

CHAPTER TWO : MATERIALS AND METHODS

2.1 OVERVIEW

All the isolates were initially verified by observing their respective cultural and microscopic characteristics. Their FB_1, ZON, FA and MON profiles were then determined by HPLC. Discrimination between the strains and species was attempted by, (i) ascertaining their genetic variation using RAPD analysis, (ii) generating aroma profiles using the electronic nose to examine the volatile compounds produced during growth of the Fusarial isolates, and (iii) determining the hydrolytic enzyme profiles of each isolate using substrate specificity.

2.2 *Fusarium* ISOLATES

Nineteen *Fusarium* isolates were used in this study. Seven *F. verticillioides* isolates were obtained from the Plant Pathology Research Institute (ppri) and six from the Medical Research Council (MRC, Pretoria). Four *Fusarium* species and two *F. verticillioides* strains were isolated from Fusarial keratitis eye infections from a previous study (Naicker, 2001). The strain designation and origin/source of each of the 19 *Fusarium* isolates are outlined below in TABLE 2.1.

TABLE 2.1 Strain designation and origin of *Fusarium* isolates used in this study

***Fusarium* ISOLATE**	**STRAIN DESIGNATION**	**ORIGIN/SOURCE**
F. dimerum	E21	Eye infections
F. solani	E1398	Eye infections
F. chlamydosporum	E1364	Eye infections
F. semitectum	E1822	Eye infections
F. verticillioides	ppri 1059	Ex *Zea mays*, Elandshoogte, KZN
F. verticillioides	E110	Eye infections
F. verticillioides	ppri 5085	Ex poisonous chicken meal, Christiana mills, North West
F. verticillioides	M8335	Not available
F. verticillioides	E18	Eye infections
F. verticillioides	M3125	Not available
F. verticillioides	ppri 1065	Ex *Zea mays*, Settlers, Limpopo
F. verticillioides	M7390	Not available
F. verticillioides	ppri 5084	Ex poisonous chicken meal, De Aar mills, Northern Cape
F. verticillioides	M3120	Not available
F. verticillioides	ppri 6525	Ex *Zea mays*, ARC-Grain Crops Institute, Potchefstroom, North West
F. verticillioides	M5550	Not available
F. verticillioides	ppri 6019	Ex Fodder, Onderstepoort
F. verticillioides	M3674	Not available
F. verticillioides	ppri 5146	Ex rhizosphere of carnations, Potchefstroom, North West

PPRI - Plant Pathology Research Institute
M - Medical Research Council
E - Eye infection

2.3 GROWTH AND MAINTENANCE OF *Fusarium* ISOLATES

Sabouraud dextrose agar (SDA, Oxoid) was used as the growth medium for all Fusarial isolates and was prepared according to the manufacturer's instructions. Working stocks of all cultures were grown on SDA plates and incubated at 25°C for three weeks and stored at 4°C. Every month these isolates were sub-cultured from the original and incubated and stored as described above. For long-term storage of cultures, Fusaria were maintained on SDA slants at 25°C and also freeze-dried with liquid nitrogen and stored at –70°C.

2.4 MYCOTOXIN ANALYSIS

2.4.1 Preparation of Fungal Spore Suspensions

Each *Fusarium* strain was inoculated onto SDA plates and incubated at 25°C until sporulation. After approximately three weeks, 10 ml of sterile distilled water was used to wash off the spores from the agar plate and the spore suspension was stored in sterile test tubes. The number of spores in the suspension was enumerated using a haemocytometer (Neubauer, Germany) [The counting chamber consists of 25 big squares each containing 16 small squares therein. The count per millilitre was obtained by multiplying the average count of spores within five big squares (80 small squares) by a dilution factor of 2.5×10^5]. The spore concentration was standardized to 1.6×10^4 spores/ml and was inoculated into 200 ml of Sabouraud dextrose broth (SDB, Oxoid) which was prepared according to the manufacturer's instructions. Uninocluated

broths served as negative controls. Incubation conditions were 25°C for 25 days to assess toxin production. The predominant mycotoxins, viz., FB_1, ZON, FA and MON produced by the 19 *Fusarium* isolates were then analyzed by HPLC. All experiments were carried out in duplicate.

2.4.2 Extraction and Purification of Mycotoxins

2.4.2.1 Fumonisin B_1

Fumonisin B_1 was extracted from SDB according to the method by Sydenham *et al.* (1992). After 25 days of incubation at 25°C on an orbital shaker (New Brunswick Scientific, USA) at 130 rpm, 5 ml of culture from SDB was removed and added to centrifuge tubes (Corning) containing 20 ml methanol:water (3:1; v/v). After gentle mixing, the solution was centrifuged at 12 300 × *g* (Eppendorf, Germany) for 15 min and the supernatant filtered through a Whatman No. 4 filter. The filtered extract was passed through Strong Anion Exchange (SAX) cartridges (Bond Elut LRC, Varian Inc., USA) that were previously conditioned with 5 ml of methanol and then 5 ml of methanol:water (3:1). Five millilitres of methanol:water (3:1), followed by 5 ml methanol was then used to wash the cartridge and remove contaminants. A flow rate of 2 ml/min was maintained through the cartridge. The purified toxins were eluted with 10 ml 0.5% glacial acetic acid:methanol in a ratio of 0.5:99.5 (v/v) and collected in a suitable vial. The purified toxin was evaporated to dryness under a gentle stream of nitrogen gas at 50°C using an evaporating unit (Reacti-Vap, Pierce, Europe). Dried samples were stored at 4°C until analysis of FB_1 by HPLC.

2.4.2.2 Zearalenone

Zearalenone extraction was performed after 25 days according to the method of Howell and Taylor (1981) with modifications. Fifty millilitres of SDB was placed in a 500 ml round-bottomed flask to which 250 ml chloroform and 25 ml deionized water was added. Flasks were shaken for 30 min on an orbital shaker set at 150 rpm (New Brunswick Scientific, USA). The extract was filtered through 10 g Celite 545 filter aid in Whatman No. 1 filter paper. The filtrate was then collected and evaporated to 10 ml using a rotary evaporator (Büchi Rotavapor RE 120, Switzerland). Purification of the ZON extract was achieved with Sep-Pak cartridges (Waters Corporation, Ireland). Cartridges were pre-treated with two 0.5 ml aliquots of toluene, followed by 10 ml of sample. Ten millilitres of toluene was used to wash the cartridge and the purified ZON extract was collected with 10 ml of toluene:acetone (95:5), evaporated to dryness under nitrogen gas and stored at 4°C for HPLC analysis.

2.4.2.3 Fusaric acid

Fusaric acid was extracted by liquid-liquid extraction according to the protocol described by Smith and Sousadias (1993) with modifications. Five millilitres of SDB was extracted after 25 days of incubation with *Fusarium* cultures and shaken thoroughly to a uniform consistency with 25 ml of methanol:1% KH_2PO_4 (50:50; v/v) [An equal volume of 1% KH_2PO_4 was added to methanol and the pH adjusted to 3.0 using 0.1 M HCl]. This mixture was centrifuged at 12 300 × *g* for 20 min at ambient temperature. The supernatant was removed and the pH was adjusted to pH 3 using

2 M HCl. The toxin-containing extract was added to 20 ml dichloromethane and the acidified supernatant was extracted sequentially three times with 20 ml dichloromethane. The dichloromethane extracts were pooled and concentrated by reducing the volume to 10 ml using a rotary evaporator. The dichloromethane extract was then extracted twice with 5 ml 5% $NaHCO_3$. The dichloromethane extract was discarded and the $NaHCO_3$ solutions were pooled. The pH of the $NaHCO_3$ extract was adjusted to 3.0 with 5 M HCl and the solution was thereafter extracted twice with dichloromethane. The dichloromethane extracts were combined and removed *in vacuo* at 40°C using a rotary evaporator. The resulting residue was stored at 4°C for HPLC analysis.

2.4.2.4 Moniliformin

Moniliformin was extracted according to the method of Thiel (1990) with modifications. After 25 days of incubation, 10 ml of inoculated SDB was extracted with 20 ml of methanol:water (3:1) on an orbital shaker set at 100 rpm for 2 h. The slurry was filtered through Whatman No. 4 filter paper. Bond Elut (SAX) resin cartridges were used for the purification of MON. Cartridges were pre-conditioned with 1 ml of methanol followed by the application of 1 ml methanol:water (3:1). Two millilitres of filtered extract was passed through the column at a flow rate of 1 ml per minute. The cartridge was washed with 1 ml methanol:water (3:1), followed by 1 ml methanol. The final purified eluate was collected in a clean vial with 2 ml of 1% HCl in methanol. The samples were dried under a stream of nitrogen and stored at 4°C until required for HPLC analysis.

2.4.3 Analysis of Mycotoxins by High Pressure Liquid Chromatography

Fumonisin B_1, ZON, FA and MON extracts were analyzed by HPLC (Merck-Hitachi Model D-7000, Japan) using a 5 μm Lichrospher Reverse Phase–RP-18 (Merck Clevenot, Darmsadt) column (4 × 250 mm in diameter) with an M45 pump. Fumonisin B_1, ZON, FA and MON standards were purchased from Sigma Chemicals and diluted in methanol. The following concentrations (μg/ml) were prepared for detection by HPLC :

FB_1 : 300, 200, 100, 50, 10, 5, 0.5, 0.4, 0.2, 0.1, 0.08, 0.05 μg/ml

ZON : 100, 50, 20, 10, 1, 0.5, 0.2, 0.1 μg/ml

FA : 100, 50, 20, 10, 1, 0.5, 0.1, 0.05 μg/ml

MON : 100, 50, 20, 10, 1, 0.5, 0.1, 0.05 μg/ml

The lowest concentration that produced a reproducible peak area that was three times the signal of the noise level was considered the detection limit for that toxin (Ravindranath, 1989). All samples were analyzed in duplicate.

2.4.3.1 Fumonisin B_1

High performance liquid chromatography analysis of FB_1 was carried out according to the method of Thiel *et al.* (1993). The purified toxin was reconstituted in 2 ml methanol and 50 μl was transferred to a vial to which 450 μl o-pthaldialdehyde (OPA) [40 mg OPA was dissolved in 1 ml MeOH to which 5 ml $B_4Na_2O_7$ (3.8 g in

100 ml) and 50 µl 2-mercaptoethanol was added] was added. Immediately thereafter (within 1 min of adding the OPA), 50 µl was injected into the HPLC system. The mobile phase was methanol:0.1 M NaH_2PO_4 (80:20) [15.6 g $Na_2H_2PO_4$ was dissolved in deionized water and the pH was adjusted to 3.3 with H_3PO_4, then made up to 1 L]. Fluorescent detection was set at 335 nm (excitation) and 440 (emission) with the flow rate at 1.2 ml/min. The concentration of FB_1 in the injection volume and reconstituted volume was calculated from the peak areas using a calibration curve with the following equation:

$$y = mx + c$$

Where, y = concentration of injected and reconstituted toxin (µg/ml)

m = gradient in linear equation

x = peak area of toxin

c = 0 (constant)

2.4.3.2 Zearalenone

Zearalenone was quantified according to the method by Adler *et al.* (1995) under reversed-phase conditions. The mobile phase was methanol:water (70:30; v/v) and samples were detected at wavelengths of 274 nm (excitation) and 450 nm (emission) with a fluorescent detector. Samples and standards were reconstituted in 2 ml of

methanol, and a 50 µl injection volume was used. The final concentration of ZON was calculated as outlined in 2.4.3.1.

2.4.3.3 Fusaric acid

Fusaric acid was analyzed according to the method of Venter and Steyn (1998) under reversed-phase conditions. The mobile phase consisted of 40% methanol in 60% of an aqueous solution of 0.62 mM Na_2EDTA and 2% H_3PO_4 (A 0.062 mM Na_2EDTA solution was prepared by diluting 0.231 g Na_2EDTA to 980 ml deionized water. The solution was made up to mark in a 1 L with 20 ml H_3PO_4). The system was stabilized at a flow rate of 1.0 ml/min. Peaks were detected at a fixed wavelength of 254 nm using a Merck L7400 UV lamp. Samples were reconstituted in 2 ml methanol and 50 µl was injected into the HPLC. The final concentration of FA was calculated as outlined in 2.4.3.1.

2.4.3.4 Moniliformin

Moniliformin was analyzed according to the method of Thiel (1990). Dried sample extracts were reconstituted in 2 ml methanol and 40 µl was injected into the system. The mobile phase consisted of deionized water:acetonitrile:ion-paired reagent (9:1:0.1; v/v/v) [The solution was made up by adding 9 ml of deionized water to 1 ml of acetonitrile and 0.1 ml of the ion-paired reagent (2.4 ml tetrabutylammonium hydroxide in 5 ml of 1.1 M KH_2PO_4.)]. Aliquots were analyzed on a reversed phase HPLC

column under UV detection at 229 nm and the system was stabilized at a flow rate of 1.0 ml/min. The final concentration of MON was calculated as outlined in 2.4.3.1.

2.4.4 Recovery Studies

Extraction efficiency for each method was investigated by determining the recovery of FB_1, ZON, FA and MON standards by spiking SDB with a concentration of 50 μg/ml of each toxin. Experiments were performed in duplicate and the percentage recovery was calculated using the following equation (Ravindranath, 1989):

$$\% \text{ Recovery} = \frac{C_{calculated} - C_{untreated}}{C_{initial\ spiked}} \times f \times 100$$

Where,

$C_{calculated}$ = detectable concentration of toxin (μg/ml) from the spiked sample,

$C_{untreated}$ = detectable concentration of toxin (μg/ml) from the untreated sample,

$C_{initial\ spiked}$ = initial concentration of toxin (μg/ml) used to spike the sample, and

f = dilution factor where f = 1 for the liquid-liquid extraction, and f = 5 for the SAX cartridge extraction method.

2.5 RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS

Random polymorphic DNA analysis was carried out with 17 arbitrary chosen primers to determine variations in the banding patterns of the *Fusarium* isolates. Chromosomal DNA was extracted, quantified and tested with 17 primers in a PCR reaction.

2.5.1 Extraction of Genomic DNA

Isolation of genomic DNA was as per the method by Raeder and Broda (1985) with modifications. Since mycelial growth was less confluent in SDB, Alberts broth was used as an alternative for the production of Fusarial growth for RAPD analysis. Two hundred millilitres of Alberts broth (Alberts *et al.*, 1994) [27 g glucose, 0.6 g KH_2PO_4, 1.05 g $(NH_4)_2$ SO_4, 0.09 g $MgSO_4$, 0.12 g $CaCl_2$, 0.0048 g $MnSO_4$ (anhydrous)] at pH 4.5 was inoculated with the *Fusarium* culture. This was incubated at 25°C on an orbital shaker set at 100 rpm for 10 days. The mycelium was then harvested through sterile Whatman No. 1 filter paper under vacuum and dried. The mycelium was recovered and frozen by plunging into a mortar containing liquid nitrogen and ground to a fine powder with a chilled pestle. The ground mycelium was transferred to an eppendorf tube and weighed to 0.005 g. Extraction buffer (200 mM Tris-HCl [pH 8.5], 250 mM NaCl, 25 mM EDTA and 0.5% SDS [added fresh], was added to 55 ml of deionized water) was added in a ratio of 1 ml to 0.1 g mycelium and mixed gently ensuring most of the frozen paste was dissolved. Impurities were removed by adding 0.7 volume of phenol and mixed gently for 2 min followed by the addition of 0.3 volume of isoamylalcohol:chloroform (1:24) and mixed for a further 2 min. The

mixture was then centrifuged at 5 320 × *g* (Biofuge, Heraeus, Germany) for 10 min. The upper aqueous supernatant was then transferred to another tube and incubated with 1/20 volume of RNase A at 37°C for 20-30 min to remove RNA. Extraction was carried out with one volume of isoamylalcohol:chloroform (1:24) and centrifuged at 5 320 × *g* for 5 min. The upper aqueous layer was transferred to a clean tube to which was added 2× volume of ice-cold 100% ethanol (due to the high molecular weight, DNA precipitates). The DNA suspension was then centrifuged at 5 320 × *g* for 5 min. The supernatant was decanted and the DNA was washed with 500 µl of ice cold 70% ethanol. This was placed on ice for 5 min, and then centrifuged at 5 320 × *g* for 5 min. The supernatant was decanted and all excess liquid was removed. Sterile Tris-EDTA (TE) buffer, (100 µl of 10 mM Tris-HCl at pH 8.0, 40 µl of 0.001 M EDTA at pH 8.0, 19.86 ml sterile deionized water) was added in the ratio of 0.2 ml/0.1 g mycelium for dissolution of the DNA pellet. Once dissolved, the DNA suspension was stored at – 20°C until required.

2.5.1.1 Quantification of Concentrated Genomic DNA

DNA was quantified with a UV-VIS spectrophotometer (Cary, Varian, Australia) [Ausubel, 1994]. A 1:100 dilution of the concentrated DNA suspension was made up in TE buffer and the absorbance thereof was read at 260 nm and 280 nm. Tris-EDTA buffer was used as the blank. At 260 nm, an absorbance of 1 OD corresponds to 50 ng/µl of DNA, therefore, the concentration of isolated DNA was calculated accordingly. The purity of DNA was determined by absorbance ratios at 260 nm / 280 nm.

2.5.2 Random Amplified Polymorphic DNA Analysis

2.5.2.1 Primers used in this Study

Amplification reactions were performed with a set of 17 arbitrary primers. Seven of these were purchased from Operon Technologies (OPC) and ten primers from the University of British Columbia (UBC). Primers were chosen based on their 10-mer sequences and GC content. The primers, their respective sequences, GC content and melting temperatures are listed in TABLE 2.2.

TABLE 2.2 RAPD 10-mer primers, their respective sequences, GC content and melting temperature

PRIMER	SEQUENCE	GC CONTENT (%)	MELTING TEMPERATURE (°C)
OPC 1	5'-GAT GAC CGC C-3'	70	34
OPC 2	5'-GAA CGG ACT C-3'	60	32
OPC 3	5'-GTC CGG ACG A-3'	70	34
OPC 4	5'-ACC TGG CCA C-3'	70	34
OPC 6	5'-TGT CAT CCC C-3'	60	32
OPC 7	5'-TGC GTG CTT G-3'	60	32
OPC 8	5'-GTT GCC AGC C-3'	70	34
UBC 204	5'-TTC GGG CCG T-3'	70	34
UBC 229	5'-CCA CCC AGA G-3'	70	34
UBC 230	5'-CGT CGC CCA T-3'	70	34
UBC 246	5'-TAT GGT CCG G-3'	60	32
UBC 270	5'-TGC GCG CGG G-3'	90	38
UBC 284	5'-CAG GCG CAC A-3'	70	34
UBC 285	5'-GGG CGC CTA G-3'	80	36
UBC 286	5'-CGG AGC CGG C-3'	90	38
UBC 295	5'-CGC GTT CCT G-3'	70	34
UBC 300	5'-GGC TAG GGC G-3'	80	36

OPC - Operon Technologies UBC - University of British Columbia

2.5.2.2 Amplification Reactions

The DNA from the 19 Fusaria was subjected to a PCR reaction with each of the 17 primers individually. Each PCR reaction of 50 µl contained 1 U *Taq* polymerase (Roche), 0.2 µM primer, 100 ng/µl template DNA, 100 µM dNTP mix [Boehringer Mannheim], 1.5 µl $MgCl_2$ (Roche) and deioinized water. A reaction omitting the template DNA represented the negative control. Forty-five amplification cycles were performed on a thermal cycler (Techne, England) which was programmed for: denaturation at 92°C for 1 min, annealing at 34°C and 32°C for 1 min for primers with melting temperatures above 32°C and below 32°C, respectively, and extension at 72°C for 2 min.

Prior to analysis, the PCR reactions were optimized for repeatability and reproducibility. For reproducibility, reactions were carried out using DNA from a single Fusarial isolate (*F. verticillioides* E110) and five different primers (OPC 1, OPC 3, OPC 4, OPC 6 and OPC 7). For repeatability, *F. verticillioides* E110 was amplified with primer OPC 1 five times over five days. Band sizes were determined by a gel documentation system (SynGene, USA) in the Department of Biochemistry, University of Durban-Westville, by approximating it to a commercial molecular weight marker of comparable size, i.e. marker VI (Roche). This reaction was subsequently used as the positive control and as the marker (due to lack of stock of commercial molecular weight marker) in all further analyses to determine migration of the DNA.

Ten microlitres of the resulting amplifications was homogenized with 2 µl gel loading buffer (8 g sucrose [40%] and 50 mg bromophenol [0.25%] was dissolved in 20 ml deionized water) and resolved on a 1.2% agarose gel with 1× TAE buffer [Tris-Acetate EDTA] (50 × TAE at pH >8 was prepared with 242 g Tris base, 57.1 ml glacial acetic acid and 37.2 g EDTA (disodium salt). Electrophoresis was carried out at 100 V for approximately 2 hrs and DNA was visualized under UV light after staining with ethidium bromide (0.5 µg/ml) [50 mg ethidium bromide was dissolved in 100 ml deionized water. A dilution of 1:1 000 was necessary to make up a 0.5 µg/ml solution for staining gels. The solution is light sensitive]. The gel image was then captured on Scion, an imaging program (Scion Corporation, Maryland). This image was used to generate a dendrogram using GelCompar (Applied Maths, BVBA, Belgium), a gel documentation system at the Department of Medical Microbiology, Nelson R. Mandela School of Medicine. This program, based on normalization and background subtraction, was used to determine the levels of genetic similarity between RAPD patterns. Each relevant band size required for analysis, was determined by plotting a graph of DNA fragment size (bp) versus distance of migration by each fragment. A best-fit straight line was drawn and the appropriate band size was established by extrapolation.

2.6 ANALYSIS OF VOLATILE COMPOUNDS

The production of volatile compounds by each *Fusarium* isolate was determined using an EN. Experiments were carried out in duplicate.

2.6.1 Inoculum Preparation

Forty millilitres of SDA was prepared in 100 ml Schott bottles. The bottles were modified to contain an inlet and outlet valve for sampling of the head-space. Each bottle was inoculated with 1×10^5 spores/ml of each *Fusarium* isolate, which was spread over the agar surface using a surface-sterilized bent Pasteur pipette. Uninoculated agar served as controls. The bottles were incubated at 25°C for 25 days and the head-space was sampled for volatile compound production. The inoculated agar were also used to analyze for the production and activities of α-D-galactosidase, β-D-xylosidase, β-D-glucosidase, and α-D-mannosidase.

2.6.2 Volatile Compound Detection

An Aromascanner™ A32/8S (GammaTec, ENG (Pty) Ltd, United Kingdom) EN system incorporating 32 polymer sensors (Figure 2.1) was used to sample the head-space from each bottle in a 25°C constant temperature room. Sampling parameters were set as listed in TABLE 2.3. The normalized divergence and absorption data were analyzed using the program xISTAT. Principle Component Analysis (PCA), Discriminate Function Analysis (DFA) and Cluster Analysis (CA) were applied to classify and differentiate the fungal species according to the method supplied in the operating manual (GammaTec, 1998).

Light microscopic features that assisted in verification of the above Fusarial isolates were observed using the tape technique. The features observed for each isolate are listed in TABLE 3.1.

TABLE 3.1 : Microscopic features of the 19 *Fusarium* isolates used in this study

***Fusarium* ISOLATE**	**MICROSCOPIC CHARACTERISTICS**
F. verticillioides strains 5085, 6525, 5084, 1059, 6019, 5146, 1065, 3674, 7390, 8335, 5550, 3120, 3125, 1059, E18	Branched monophialides Presence of micronidia in chains Macroconidia sparsely-produced
F. solani E1398	Long slender monophialides Ellipsoidal microconidia Presence of macroconidia
F. chlamydosporum E1364	Rough-walled chlamydospores Spindle-shaped microconidia
F. semitectum E1822	Branched monophialides
F. dimerum E21	Small macroconidia Hooked apical cells and notched basal cells

3.2 PROFILE OF TOXIN PRODUCTION

3.2.1 Method Optimization

Prior to determining levels of FB_1, ZON, FA, and MON produced by each of the 19 *Fusarium* isolates, the toxin extraction methods were evaluated. The extraction efficiency from SDB spiked with 10 μg/ml, 20 μg/ml, 20 μg/ml and 20 μg/ml of

Figure 2.1 The AromaScanner A32/8S used in this study to analyze volatile compounds.

TABLE 2.3 Sampling parameters optimized for the detection of volatile compounds

SAMPLING	
Detection threshold	0.7
Integration threshold	5 seconds
Time per sample	290 seconds
VALVE SEQUENCE	
Reference	40 seconds
Sample	120 seconds
Wash	10 seconds
Reference	120 seconds
Carrier gas	Nitrogen

2.7 ANALYSIS OF ENZYME PRODUCTION

The activities of four extracellular enzymes (α-D-galactosidase, β-D-xylosidase, β-D-glucosidase, and α-D-mannosidase) were assayed. Samples that were tested for the production of volatile compounds were also used in the analyses of enzyme production.

2.7.1 Extraction of Enzymes

Enzyme extractions were carried out according to the method of Keshri and Magan (2000) with modifications. Eight millilitres of 0.01 M KH_2PO_4 extraction buffer (pH 7.2) [0.272 g of KH_2PO_4 was dissolved in 200 ml of deionized water] was added to each bottle containing *Fusarium* culture. The mycelium including some of the agar was scraped off using a sterile blade and placed in 50 ml centrifuge tubes. The tubes were shaken at 120 rpm on an orbital shaker (New Brunswick Scientific, USA) for 1 h at 4°C. The washings were decanted into 1.5 ml plastic Eppendorf tubes and centrifuged for 10 min at 2 050 × *g*. The supernatant was removed and stored at –20°C for the determination of enzyme activities.

2.7.2 Enzyme Activities

Four hydrolytic enzyme activities (α-D-galactosidase, β-D-xylosidase, β-D-glucosidase, and α-D-mannosidase) were assayed using ρ-nitrophenyl substrates (Sigma) according to the method of Herr *et al.* (1978). TABLE 2.4 illustrates the substrate and buffer concentrations used for each enzyme assay.

TABLE 2.4 The enzymes, their respective substrates and buffers used for assays

ENZYME	SUBSTRATE CONCENTRATION	BUFFER
α-D-galactosidase	4.0 mM 4-nitrophenyl-α-D-galactopyranoside	0.05 M citrate (pH 6.5)
β-D-xylosidase	3.0 mM 4-nitrophenyl-β-D-xylosidase	0.05 M citrate (pH 6.5)
β-D-glucosidase	4.0 mM 4-nitrophenyl-β-D-glucopyranoside	0.05 M citrate (pH 6.5)
α-D-mannosidase	2.0 mM 4-nitrophenyl-α-D-mannopyranoside	0.05 M citrate (pH 6.5)

2.7.2.1 α-D-Galactosidase

α-D-Galactosidase was determined by measuring the release of ρ-nitrophenol from 4-nitrophenyl-α-D-galactopyranoside (4 mg/ml, Sigma) in 0.05 M citrate buffer (pH 6.5) [10.51 g of citric acid was added to 1 L of deionized water] over 10 min at 50°C. The assay mixture consisted of 125 µl crude enzyme, 125 µl substrate and 250 µl citrate buffer. The reaction was terminated by the addition of 1 ml of a 2% solution of 1 M Na_2CO_3 (1.06 g Na_2CO_3 was added to 10 ml of deionized water). Absorbance was measured spectrophotometrically at 405 nm. The enzyme activity was calculated using an extinction coefficient of 18.5 $cm^2/\mu mol$. One unit of α-D-galactosidase activity was determined as the amount of enzyme which liberated 1 µmol of product per second.

2.7.2.2 β-D-Xylosidase

β-D-Xylosidase activity was measured as described for α-D-galactosidase (section 2.7.2.1) using 4-nitrophenyl-β-D-xylosidase (3.0 mg/ml, Sigma) as the substrate.

2.7.2.3 β-D-Glucosidase

β-D-Glucosidase activity was measured as described for α-D-galactosidase (section 2.7.2.1) using 4-nitrophenyl-β-D-glucopyranoside (4.0 mg/ml, Sigma) as the substrate.

2.7.2.4 α-D-Mannosidase

α-D-Mannosidase activity was measured as described for α-D-galactosidase (section 2.7.2.1) using 4-nitrophenyl-α-D-mannopyranoside (2.0 mg/ml, Sigma) as the substrate.

CHAPTER THREE : RESULTS

3.1 TAXONOMIC CHARACTERISTICS OF *Fusarium* ISOLATES

The *Fusarium* species and strains used in this study were verified by the observation of colony morphology, colour characteristics and their respective microscopic features. *F. verticillioides, F. solani*, *F. chlamydosporum*, *F. dimerum* and *F. semitectum* were confirmed by characteristic colony morphologies from cultures grown on SDA as well as their respective microscopic features (Figure 3.1 [a-e]). *F. verticillioides* had a distinctive cottony, white floccose mycelium with a purple tinge and violet undersurface (Figure 3.1 [a]). *F. solani* was identified by the production of white to cream mycelial growth with abundant green exudates (Figure 3.1 [b]). After maturation, *F. chlamydosporum* had a distinct white mycelial growth with its surface gradually becoming tan to brown. The undersurface also appeared tan (Figure 3.1 [c]). Mycelium growth in *F. dimerum* was scarce and yeast-like. The production of confluent, orange sporodochia coloured the surface while the undersurface appeared orange to tan (Figure 3.1 [d]). *F. semitectum* had a white to cream cottony mycelia with yellow to tan exudates. The undersurface of this specie appeared tan to brown (Figure 3.1 [e]).

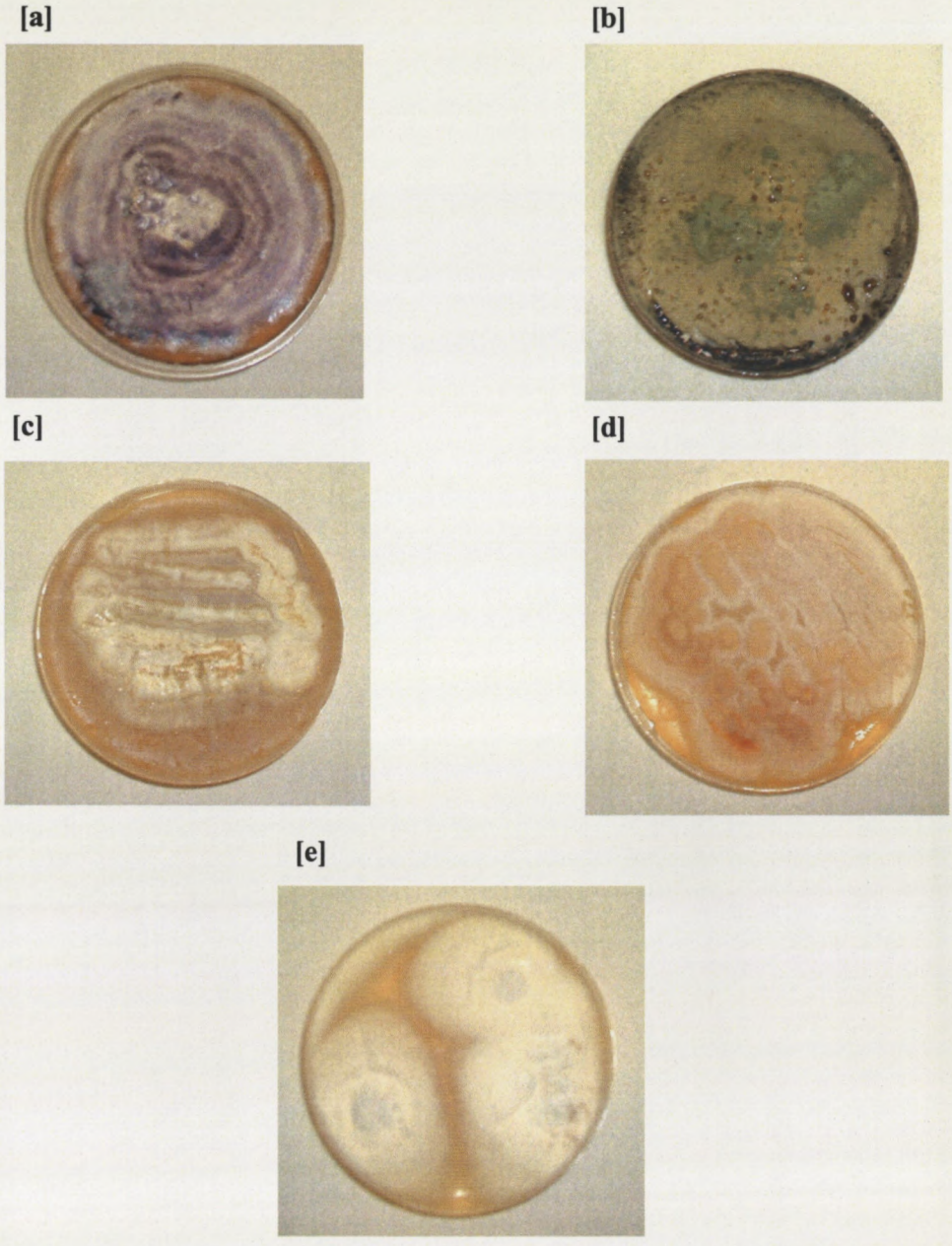


Figure 3.1 Mycelial formation and colony pigmentation of the five *Fusarium* species [a] *F. verticillioides* ppri 1059, [b] *F. solani* E1398, [c] *F. chlamydosporum* E1364, [d] *F. dimerum* E21 and, [e] *F. semitectum* E1822 cultured on SDA.

commercially purchased FB_1, ZON, FA and MON, , and standards at n=2, was 72.4%, 83%, 84.5% and 84.8%, respectively. The sensitivity of the HPLC systems for mycotoxin quantification was also determined by ascertaining toxin detection limits using commercial standards. The detection limits for FB_1, ZON, FA and MON with the tested methods were established at 0.01μg/ml, 0.1 μg/ml, 0.05 μg/ml and 0.1 μg/ml, respectively.

3.2.2 Detection of Mycotoxins from *Fusarium* Isolates

The retention times for FB_1, ZON, FA and MON were close to those of the commercial standards. However, any slight variation in the retention times between each of the extracted toxins and the standards was investigated by spiking the extracted compounds with the respective standards. An increase in the resulting peak confirmed the retention time of the extracted toxin. The peak obtained for 50 μg/ml of FB_1 standard represents an elution time of 7.60 min (Figure 3.2[a]). Figure 3.2 [b] shows a peak obtained from one of the purified extracts (*F. verticillioides* E18), which when compared to the standard, eluted at a similar retention time of 7.51 min. Figure 3.2 [c] shows a chromatogram obtained from an uninoculated SDB sample which served as the control.

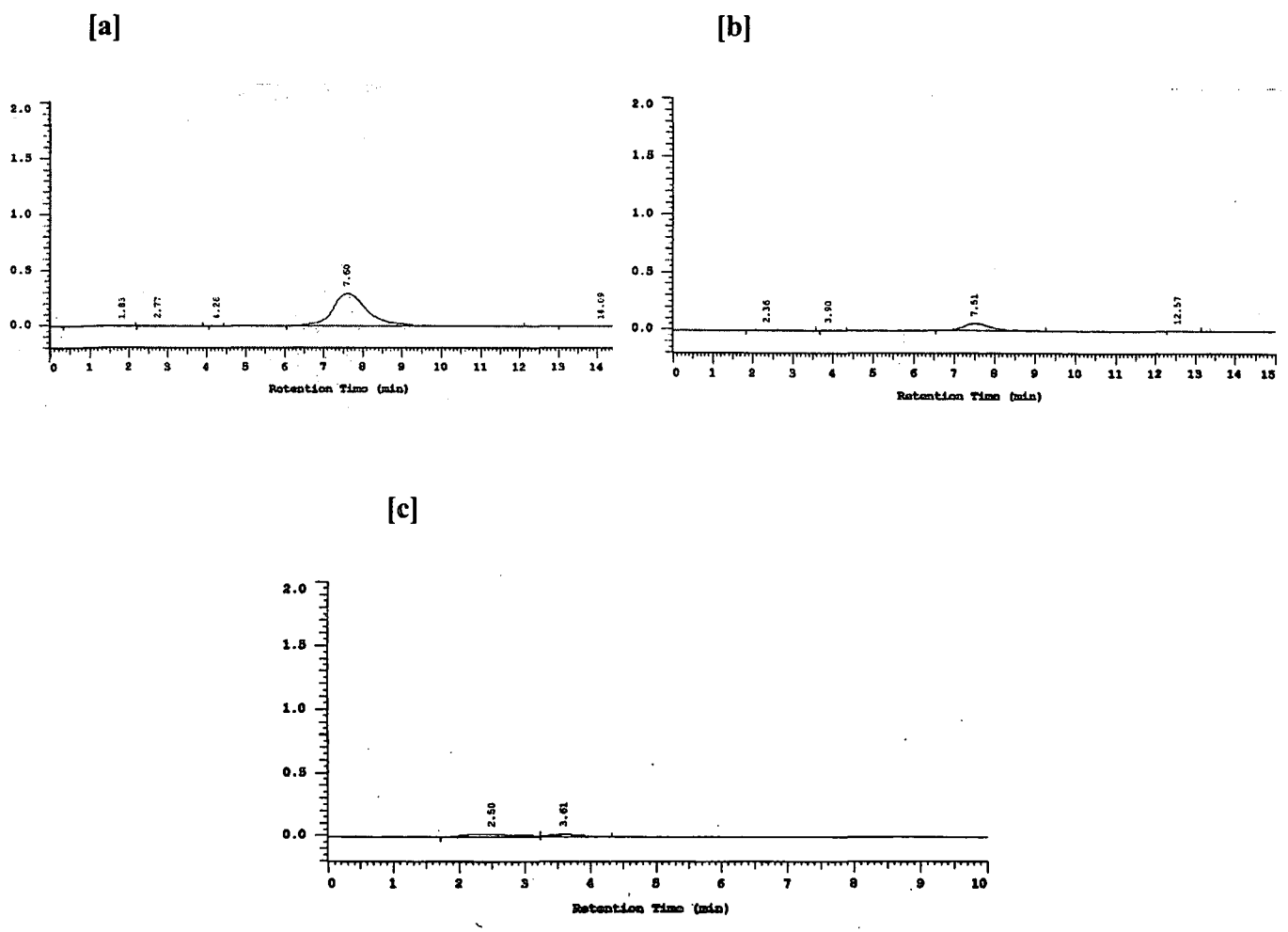
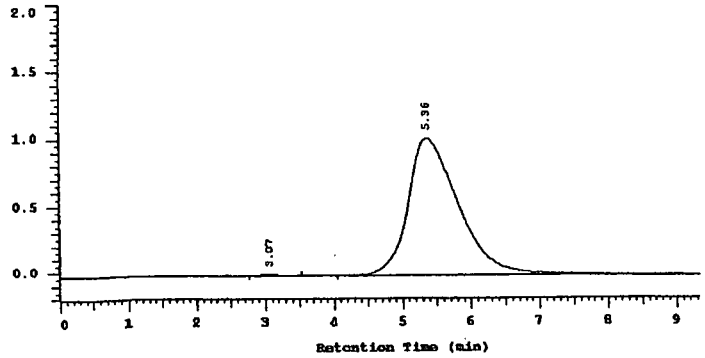


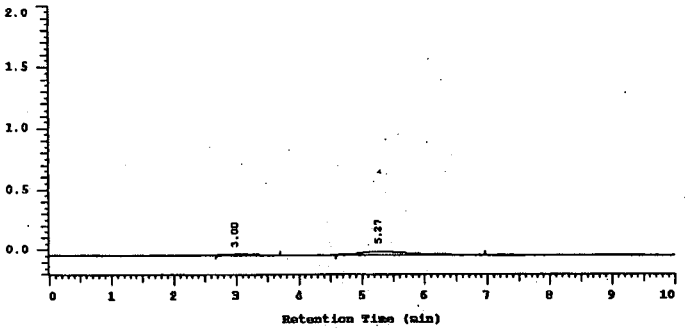
Figure 3.2 HPLC chromatograms of fumonisin B_1 for [a] 50 μg/ml fumonisin B_1 standard, [b] *F. verticillioides* E18 and, [c] control (uninoculated broth).

Upon injection of 20 μg/ml of ZON standard, a peak was obtained at a retention time of 5.36 min (Figure 3.3 [a]). Similarly, peaks were obtained for the purified ZON extracts as depicted in Figure 3.3 [b]. Figure 3.3 [c] demonstrates the control which is a sample of uninoculated SDB.

[a]



[b]



[c]

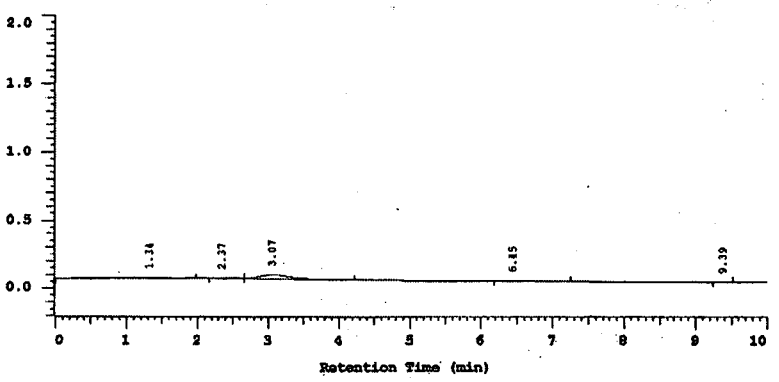


Figure 3.3 HPLC chromatograms of zearalenone for [a] 20 μg/ml zearalenone standard, [b] *F. verticillioides* 5084 and, [c] control (uninoculated broth).

A peak retention time of 2.47 min denotes the elution of a 3.5 μg/ml FA standard (Figure 3.4 [a]), and compares to the extracted toxins which eluted at a similar retention time of 2.88 min (Figure 3.4 [b]). Figure 3.4 [c] shows a control of uninoculated broth.

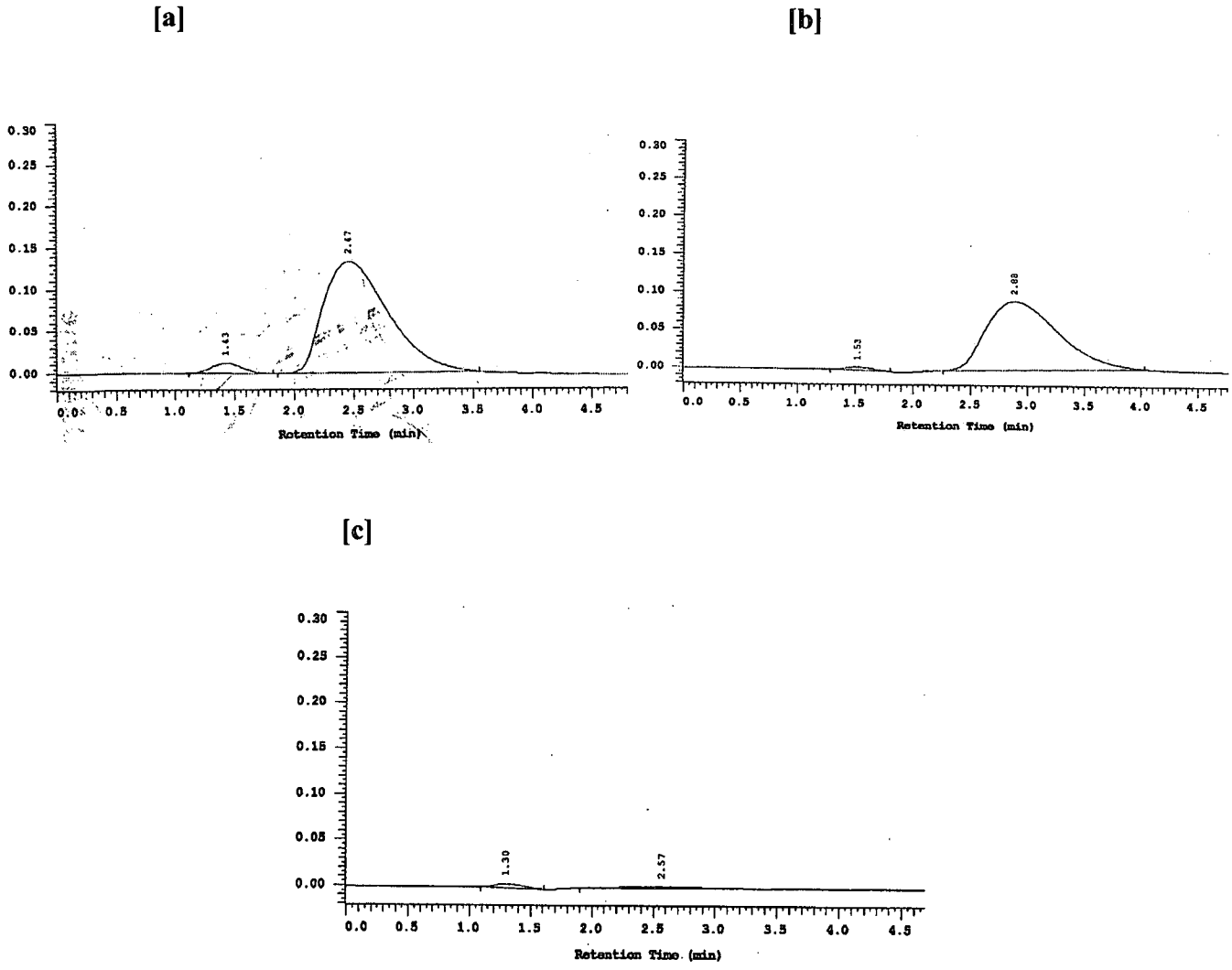


Figure 3.4 HPLC chromatograms of fusaric acid for [a] 3.5 μg/ml fusaric acid standard, [b] *F. solani* and, [c] control (uninoculated broth).

Injection of 100 μg/ml MON standard into the HPLC system revealed a peak at a retention time of 1.87 min (Figure 3.5 [a]). Likewise, peaks were obtained for the purified extracts of *Fusarium* isolates as depicted by *F. verticillioides* 6019 shown below in Figure 3.5 [b]. Figure 3.5 [c] illustrates a chromatogram obtained from an uninoculated SDB sample which served as the control.

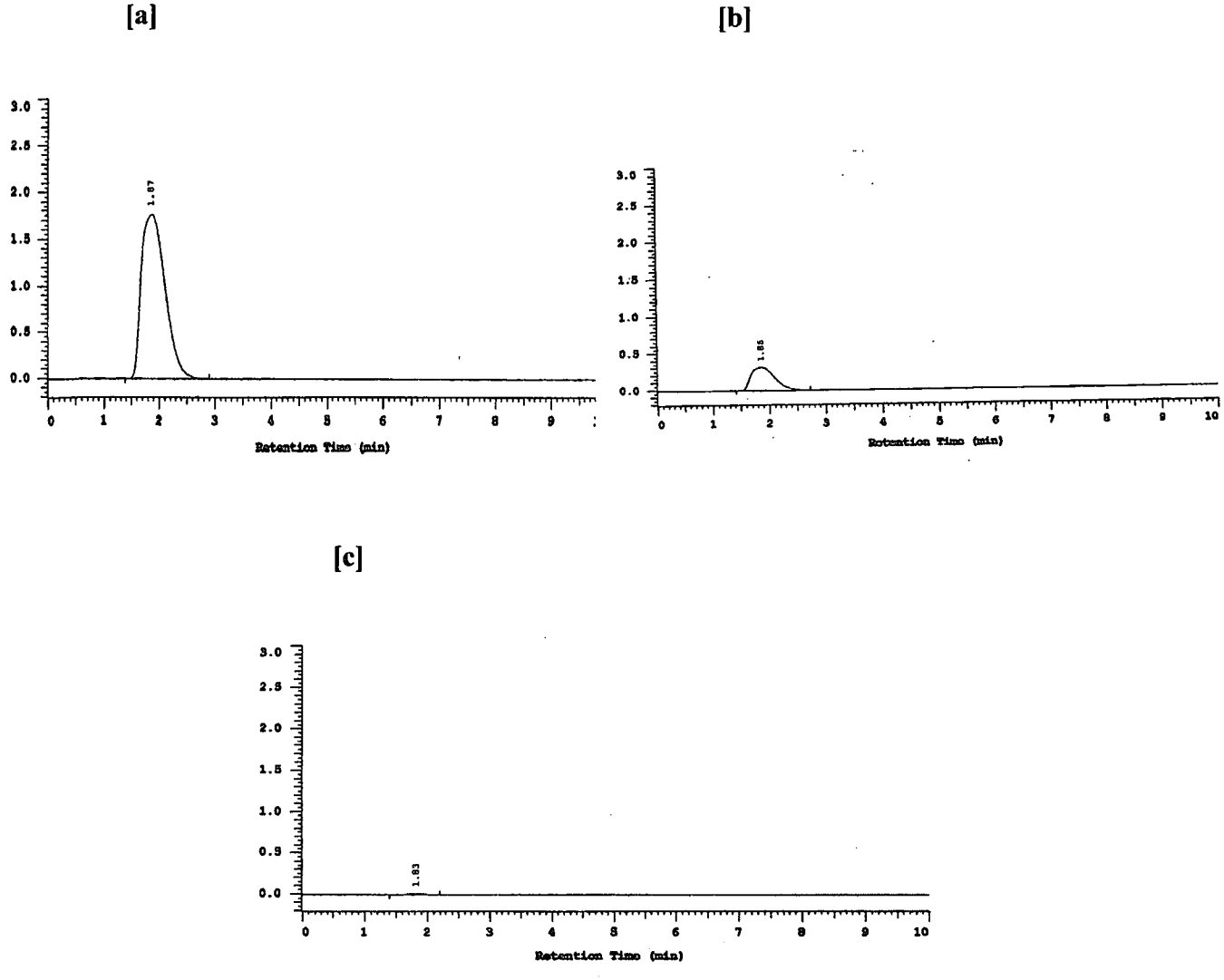


Figure 3.5 HPLC chromatograms of moniliformin for [a] 100 μg/ml moniliformin standard, [b] *F. verticillioides* 6019 and, [c] control (uninoculated broth).

The 19 *Fusarium* isolates produced different toxins and different levels of the four toxins (TABLE 3.2). Toxin levels were classified into three ranges of toxin levels, i.e. high, medium and low producers of toxins. From the table, it is evident that there are two high FB_1-producers, viz., *F. verticillioides* 3674 with a concentration of 7.06 μg/ml

and *F. verticillioides* E18 which produced 8.14 μg/ml of FB_1. The majority of the isolates were low FB_1-producers, where the concentrations ranged from 0.25-1.63 μg/ml. There was only one medium producer which was *F. verticillioides* 5085. Fumonisin B_1 was not detected in any of the other four species.

Levels of ZON produced by Fusarial species and strains were very low and ranged from 0.14 μg/ml to 0.81 μg/ml. Of the 19 isolates, only four were capable of producing ZON. None of the other 15 isolates were able to produce ZON.

Two strains of *F. verticillioides*, viz., *F. verticillioides* 6525 and *F. verticillioides* 5146 produced high levels of FA at concentrations of 9.01 μg/ml and 11.27 μg/ml, respectively. The majority of the strains were medium producers of FA. *F. verticillioides* 6019, *F. verticillioides* 1059 and *F. verticillioides* 5550 produced low levels of FA. Only one isolate, i.e. *F. verticillioides* 3674 did not produce FA.

The levels of MON were generally much higher for most of the isolates than for the other three toxins. All strains and species produced MON in the highest concentration, except for *F. verticillioides* 5146, *F. verticillioides* E18, *F. verticillioides* 5084, *F. verticillioides* 3120 and *F. verticillioides* 6525 which produced a higher level of FA, FB_1, FA, FA and FA, respectively. *F. verticillioides* 6019, *F. verticillioides* 3674, *F. verticillioides* 3125 and *F. verticillioides* E110 as well as *F. semitectum* were identified as high MON-producers with levels reaching up to 33.71 μg/ml.

TABLE 3.2 Average mycotoxin concentrations (µg/ml) of fumonisin B_1, zearalenone, fusaric acid and moniliformin produced by the 19 *Fusarium* species and strains

***Fusarium* SPECIES AND STRAINS**	**MYCOTOXIN CONCENTRATION (µg/ml)**			
	FB_1	**ZON**	**FA**	**MON**
***F. dimerum* E21**	ND	ND	0.26 (M)	8.19 (M)
***F. solani* E1398**	ND	ND	2.39 (M)	7.53 (M)
***F. chlamydosporum* E1364**	ND	ND	1.55 (M)	9.41 (M)
***F. semitectum* E1822**	ND	ND	1.75 (M)	33.71 (H)
***F. verticillioides* 1059**	0.25 (L)	ND	1.06 (L)	6.80 (L)
***F. verticillioides* E110**	0.43 (L)	ND	2.16 (M)	24.40 (H)
***F. verticillioides* 5085**	4.46 (M)	ND	2.41 (M)	8.81 (M)
***F. verticillioides* 8335**	1.63 (L)	ND	1.80 (M)	10.93 (M)
***F. verticillioides* E18**	8.14 (H)	ND	2.08 (M)	13.96 (M)
***F. verticillioides* 3125**	0.83 (L)	0.14 (L)	2.87 (M)	16.97 (H)
***F. verticillioides* 1065**	0.29 (L)	0.81 (L)	2.39 (M)	11.20 (M)
***F. verticillioides* 7390**	0.28 (L)	0.16 (L)	2.94 (M)	13.26 (M)
***F. verticillioides* 5084**	0.45 (L)	0.25 (L)	2.53 (M)	2.57 (L)
***F. verticillioides* 3120**	0.53 (L)	ND	2.33 (M)	5.64 (L)
***F. verticillioides* 6525**	0.30 (L)	ND	9.01 (H)	12.44 (M)
***F. verticillioides* 5550**	0.58 (L)	ND	1.21 (L)	5.45 (L)
***F. verticillioides* 6019**	0.72 (L)	ND	0.11 (L)	23.26 (H)
***F. verticillioides* 3674**	7.06 (H)	ND	ND	19.51 (H)
***F. verticillioides* 5146**	0.37 (L)	ND	11.27 (H)	8.50 (M)

ND - Not Detected

n = 2

	HIGH (H)	MEDIUM (M)	LOW (L)
FB_1	>4.0 µg/ml	2.0-4.0 µg/ml	<2.0 µg/ml
ZON	>3.0 µg/ml	1.0-3.0 µg/ml	<1.0 µg/ml
FA	>4.0 µg/ml	1.5-4.0 µg/ml	<1.5 µg/ml
MON	>15.0 µg/ml	7.0-15 µg/ml	<7.0 µg/ml

3.3 GENETIC PROFILE OF *Fusarium* ISOLATES

Reproducibility of the RAPD technique was demonstrated when an amplification reaction of *F. verticillioides* E110 with primer OPC 1 was repeated a number of times under unchanged conditions at different times. The consistent banding patterns confirm that the RAPD technique was repeatedly achievable under the set conditions (Figure 3.6). The size of each band was determined using a gel documentation system by approximating it to a commercial molecular weight marker of comparable size, i.e. marker VI. These sizes are represented in base pairs on the left of the figure below. This characterized reaction was then used as the positive control as well as the marker for all future amplifications.

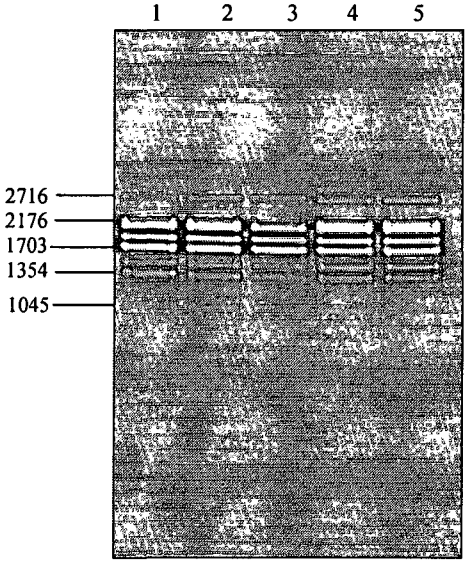


Figure 3.6 Agarose gel exhibiting the reproducibility of the RAPD technique when *F. verticillioides* E110 was amplified with primer OPC 1 under identical experimental conditions (lanes 1-5).

Each of the 17 primers was tested with the 19 isolates of the *Fusarium* species and strains to determine genetic variation. Of the 17 primers tested, only three, viz., primers UBC 204, UBC 270 and UBC 284 produced discernible results. Figures 3.7 – 3.9 demonstrate these banding patterns obtained on agarose gels together with their respective dendrograms. The remaining 14 primers did not produce decisive results and their gels and corresponding dendrograms are presented in the Appendix A.

Banding patterns of *Fusarium* isolates obtained with primer UBC 204 are depicted in Figure 3.7. This primer forms a 100% cluster between *F. verticillioides* 1059, *F. verticillioides* 5085, *F. verticillioides* 5084 and *F. verticillioides* 5550 (lanes 7, 9, 15, and 18, respectively), however, the bands of ~1 050 bp has a much higher intensity in *F. verticillioides* 1059 than the other three strains. A cluster also forms between the above four strains and *F. verticillioides* 6019 (lane 19) at 92% genetic similarity with a one band difference. *F. verticillioides* 3125 and *F. verticillioides* 3674 form a third group of 92% similarity with *F. verticillioides* 3125 lacking the first band (~1 898 bp). *F. verticillioides* strains, excluding only *F. verticillioides* E18, have bands 2 and 3 (~1 050 bp and ~841 bp) in common and also of high intensity. All species were found to have unique banding profiles.

F. verticillioides 1065 and *F. verticillioides* 6525 clustered together at a similarity of 100% with primer UBC 270 (Figure 3.8). *F. verticillioides* 5084 is also related to the above cluster at 92.5% but lacks the last band. *F. verticillioides* 3674 and *F. verticillioides* 5550 formed a group with 100% similarity. Banding intensities of both clusters were identical. *F. verticillioides* 5550 was found to be 80% similar to

F. verticillioides 5084 by lacking the fourth band of ~1 141 bp. However, the intensities of all bands were equivalent. All 15 *F. verticillioides* strains were noted to have four bands (~1 908 bp, ~1 464 bp, ~1 200 bp and ~576 bp) in common, except *F. verticillioides* 8335 and *F. verticillioides* E18. *F. verticillioides* 8335 has four distinct bands but all four show size shifts compared to the other 14 *F. verticillioides* strains. Primer UBC 270 produced unique profiles for all the other *Fusarium* species.

Primer UBC 284 clustered *F. verticillioides* 7390 and *F. verticillioides* 5084 at 100% genetic similarity, however, the ~1 365 bp band had a much higher intensity for *F. verticillioides* 5084 (Figure 3.9). Both strains had a high intensity for the ~2 245 bp band. A second cluster formed between *F. verticillioides* 7390, *F. verticillioides* 5084 and *F. verticillioides* 5085 with 94.1% resemblance. *F. verticillioides* 5085 lacked the third band (~2 245 bp) and has a high intensity ~2 365 bp band. *F. verticillioides* 5085 also appeared to be similar to *F. verticillioides* 8335 but lacked the ~700 bp band. *F. verticillioides* E110 was found to be related to *F. verticillioides* 5085 at 80% and lacked the band of ~2 800 bp. This primer produced a distinctive banding pattern for the *F. verticillioides* strains. There were six bands (~2 245 bp, ~1 685 bp, ~1 365 bp, ~1 190 bp, ~480 bp and ~155 bp) that are identical for 12 *F. verticillioides* strains, except *F. verticillioides* E18, *F. verticillioides* 3125 and *F. verticillioides* 5146 (lanes 11, 12 and 21, respectively). *F. verticillioides* 1065 (lane 13) is lacking the first two high molecular weight bands common for the other 11 strains. This lane shows a high degree of background and could indicate degradation of the lighter molecular weight DNA. Primer UBC 284 produced unique banding patterns for the five *Fusarium* species tested, allowing for species differentiation. Conclusions can not be made

regarding *F. verticillioides* 3125 and *F. verticillioides* 5146 since no DNA is visible in those lanes.

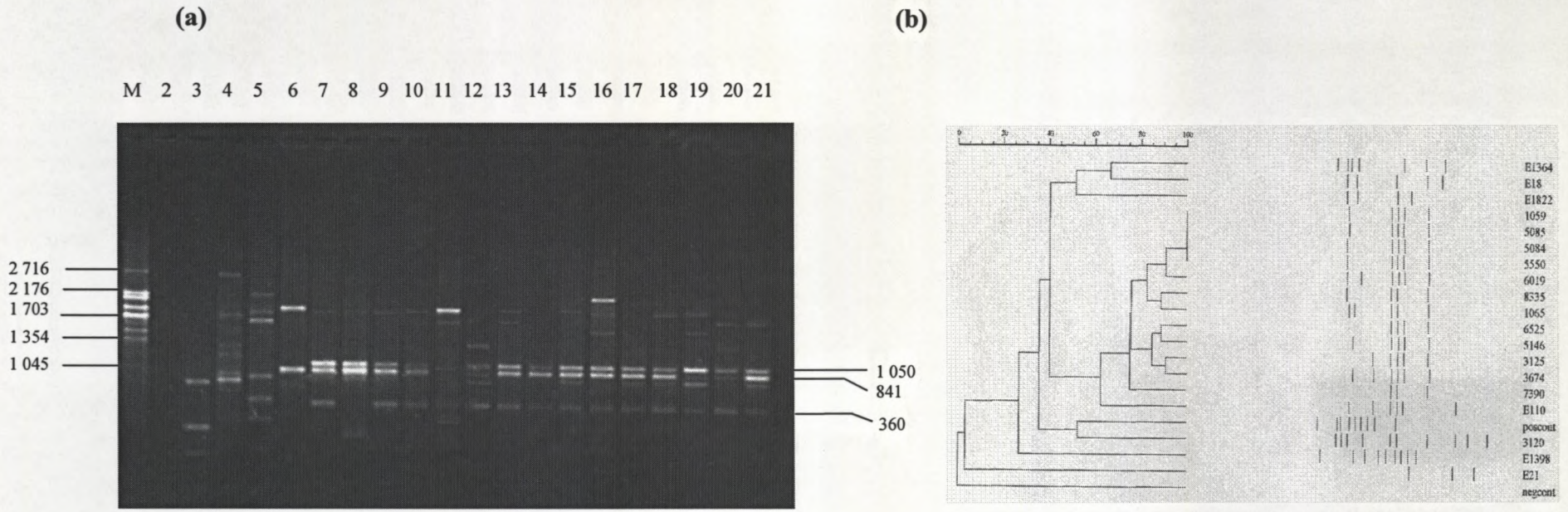


Figure 3.7 Agarose gel (a) and dendrogram (b) of 19 *Fusarium* isolates amplified by primer UBC 204. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* specie, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

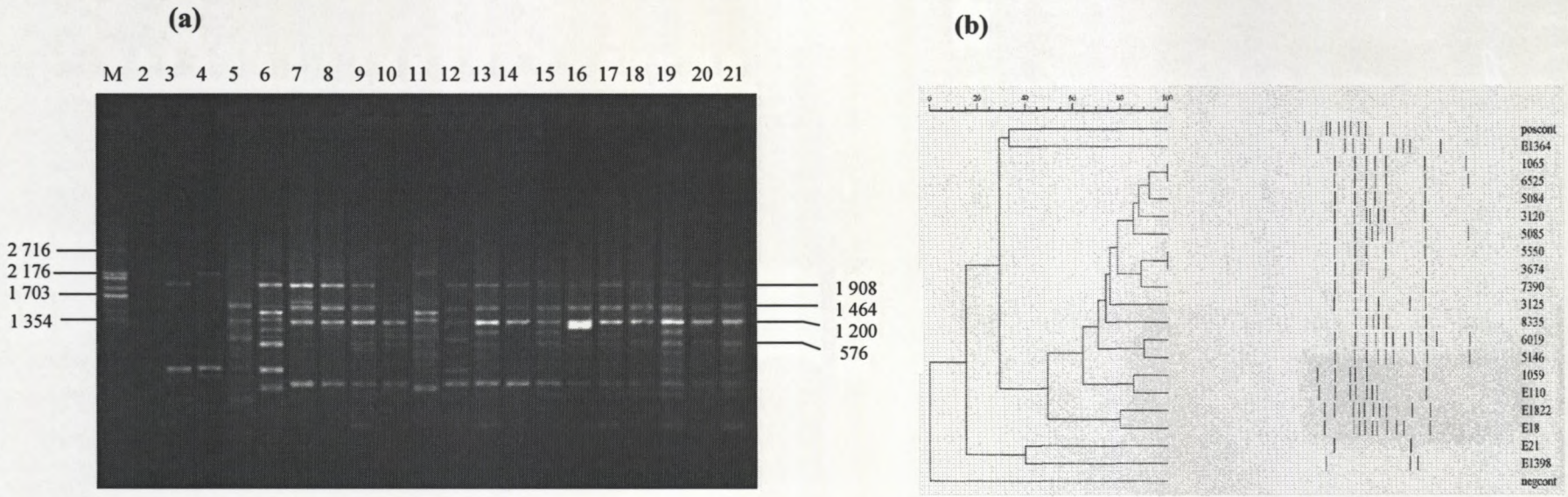


Figure 3.8 Agarose gel (a) and dendrogram (b) of 19 *Fusarium* isolates amplified by primer UBC 270. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

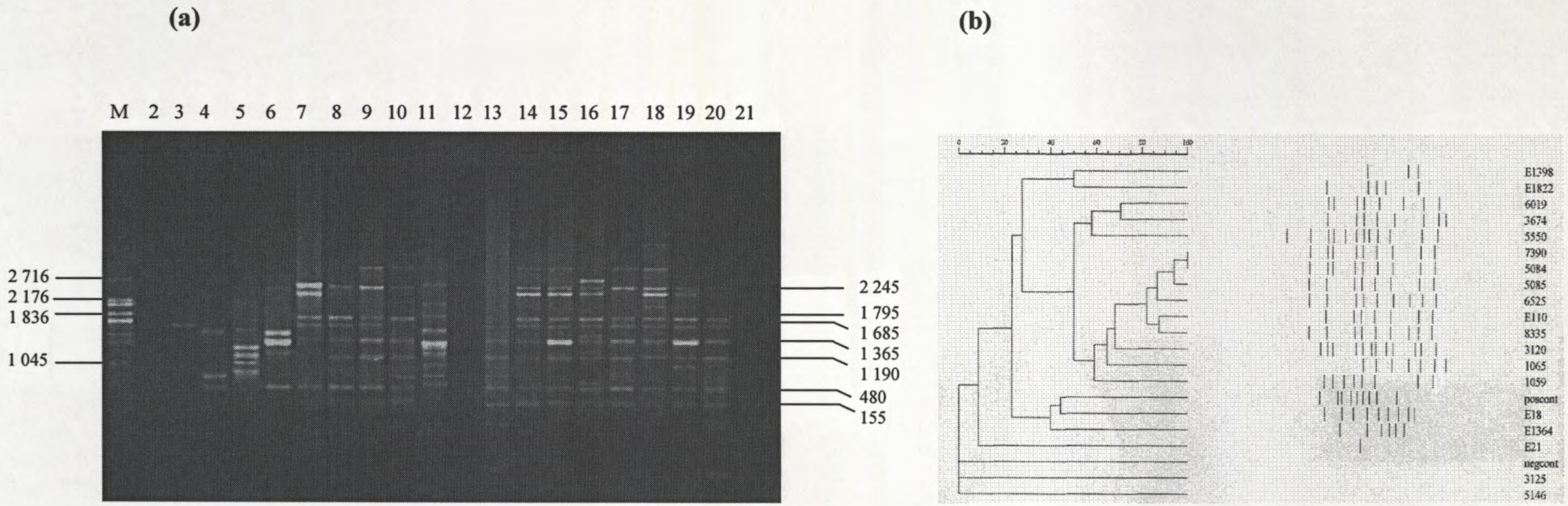


Figure 3.9 Agarose gel (a) and dendrogram (b) of 19 *Fusarium* isolates amplified by primer UBC 284. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 indicate DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

Primers OPC 3, OPC 4 and OPC 8 produced different banding patterns for *F. semitectum*, *F. chlamydosporum* and *F. dimerum*. However, based on the dendrograms, some similarities exist among the *Fusarium* species i.e. in the range of 45%-60% (Figures A3, A4 and A7).

Based on the individual dendrograms, the clustering of *Fusarium* species and strains formed by the different primers, is summarized in TABLE 3.3. Percentage similarity was used to identify identical and similar isolates. Strains were classified as identical when 100% similarity was observed and similar when there was a similarity of 95%. No clustering formed amongst the four different *Fusarium* species, viz., *F. dimerum*, *F. solani*, *F. chlamydosporum* and *F. semitectum* with any of the tested primers.

No clusters were obtained for seven primers which included primers OPC 1, OPC 2, OPC 7, OPC 8, UBC 285, UBC 286 and UBC 300. Primer OPC 3 shows a 100% similarity between *F. verticillioides* 1065 and *F. verticillioides* 6525 (Figure A3), as well as a 100% similarity between *F. verticillioides* E110 and *F. verticillioides* 5085. Primer OPC 4 produced a 95% similarity between *F. verticillioides* 5085 and *F. verticillioides* 1065 (Figure A4). Primer OPC 6 formed a cluster of 100% similarity with *F. verticillioides* 5550 and *F. verticillioides* 3674 and a second cluster, also at 100%, with *F. verticillioides* 1059 and *F. verticillioides* 6525 (Figure A5). Primer UBC 229 produced a 100% cluster grouping *F. verticillioides* 3120, *F. verticillioides* 5550 and *F. verticillioides* 6019 (Figure A8). A 100% cluster was formed between *F. verticillioides* 1065 and *F. verticillioides* 7390 with primer UBC 230 (Figure A9). Primer UBC 246 produced a four strain cluster at 100% with *F. verticillioides* 8335,

F. verticillioides 1065, *F. verticillioides* 5084 and *F. verticillioides* 5146 (Figure A10). A second 100% cluster was also produced by this primer which grouped *F. verticillioides* 1059, *F. verticillioides* E110, *F. verticillioides* 5085 and *F. verticillioides* 6525. Cluster formation between *F. verticillioides* 1065 and *F. verticillioides* 6525 (100%) was produced by a second primer, viz., UBC 270. This primer also formed another cluster at 100%. *F. verticillioides* 7390 and *F. verticillioides* 5084 came together at 100% similarity with amplification by primer UBC 284. Primer UBC 295 grouped *F. verticillioides* 6019 and *F. verticillioides* 5146 together at 100% (Figure A13). From TABLE 3.3 below, only two primers, i.e. OPC 3 and UBC 270 clustered *F. verticillioides* 6525 and *F. verticillioides* 1065 together. In addition, only two primers, viz., UBC 270 and OPC 6, clustered *F. verticillioides* 5550 and *F. verticillioides* 3674 together.

Also, two primers, OPC 6 and UBC 246 clustered *F. verticillioides* 1059 and *F. verticillioides* 6525 together as part of a group. *F. verticillioides* 1059 also came together with *F. verticillioides* 5085 by primers UBC 204 and UBC 246. *F. verticillioides* E110 and *F. verticillioides* 5085 formed part of a cluster by two primers, viz., OPC 3 and UBC 246. From TABLE 3.3, it was observed that for all *F. verticillioides* strains except *F. verticillioides* 5550, *F. verticillioides* E18 and *F. verticillioides* 3125, different primers produced different clusters. *F. verticillioides* E18 and *F. verticillioides* 3125 produced unique banding patterns with all other strains with all other primers. *F. verticillioides* 5550 formed four different patterns with four different primers (OPC 6, UBC 204, UBC 229 and UBC 270) as well as four clusters with six different strains (*F. verticillioides* 3674, *F. verticillioides* 1059,

F. verticillioides 5085, *F. verticillioides* 5084, *F. verticillioides* 6019 and *F. verticillioides* 3120). However, only *F. verticillioides* 5550 and *F. verticillioides* 6525 were clustered together more than once by two primers.

TABLE 3.3 Summary of the clustering between different species and strains of *Fusarium*

PRIMER	CLUSTER FORMATION (% SIMILARITY)
OPC 1	NO CLUSTERS
OPC 2	NO CLUSTERS
OPC 3	1065/6525 (100%) E110/5085 (100%)
OPC 4	5085/1065 (95%)
OPC 6	5550/3674 (100%) 1059/6525 (100%)
OPC 7	NO CLUSTERS
OPC 8	NO CLUSTERS
UBC 204	1059/5085/5084/5550 (100%)
UBC 229	3120/5550/6019 (100%)
UBC 230	1065/7390 (100%)
UBC 246	8335/1065/5084/5146 (100%) 1059/E110/5085/6525 (100%)
UBC 270	1065/6525 (100%) 5550/3674 (100%)
UBC 284	7390/5084 (100%)
UBC 285	NO CLUSTERS
UBC 286	NO CLUSTERS
UBC 295	6019/5146 (100%)
UBC 300	NO CLUSTERS

3.3.1 Correlation of RAPD Profiles with Toxin Production

Random amplified polymorphic DNA profiles showed no correlation for ZON, FA or MON. However, profiles observed with three of the primers, viz., UBC 204, UBC 270 and UBC 284 could be correlated with FB_1 production. Primer UBC 204 produced three distinct bands (a doublet and a singlet) of ~1 050 bp, ~842 bp and ~360 bp that were constantly present for 14 of the 15 FB_1-producing *F. verticillioides* strains (Figure 3.7). Similarly, primer UBC 270 produced four bands of ~1 908 bp, ~1 464 bp, ~1 200 bp and ~576 bp in 14 of the 15 *F. verticillioides* strains (Figure 3.8). For both these primers, the exception was *F. verticillioides* E18. Figure 3.9 (primer UBC 284) shows six bands (~2 245 bp, ~1 685 bp, ~1 410 bp, ~1 190 bp, ~480 bp and ~155 bp) present in all but two *F. verticillioides* strains. *F. verticillioides* E18 is the exception with a different banding pattern. *F. verticillioides* 1065 only displays four of the six bands with the two high molecular weight bands appearing to have degraded as demonstrated by the presence of a high background.

Primer UBC 284 also produced discernible RAPD profiles in relation to FB_1 production. Figure 3.9 shows five bands which could be useful in identifying FB_1-producing *F. verticillioides* strains. The bands of ~1 685 bp, ~1 410 bp, ~1 190 bp, ~480 bp and ~155 bp could be related to FB_1 production since all FB_1-producing *F. verticillioides* strains, except E18 had these bands in common.

From TABLE 3.4, it can be seen that there were varying levels of the four mycotoxins produced for each of the different clusters formed with the range of primers tested.

F. verticillioides 1065 and *F. verticillioides* 6525 clustered together at 92.5% and 100% with primers OPC 1 and OPC 3, respectively. Primer UBC 270 also clustered these strains at 100%. These isolates varied in their ZON and FA levels, however, they have similar MON and FB_1 levels. *F. verticillioides* 7390 and *F. verticillioides* 1065 have different ZON levels but were very similar in their production of the other toxins even at a 60% similarity. Primer OPC 3 grouped together *F. verticillioides* E110 with *F. verticillioides* 5085 at 100%. However, the only similar levels of toxins produced were that of ZON and FA. ZON was not detected and FA was moderately produced. *F. verticillioides* 5084 and *F. verticillioides* 6019 formed a 92.5% cluster with the only similarity being their levels of FB_1. *F. verticillioides* E110, *F. verticillioides* 5085 and *F. verticillioides* 1059 did not produce ZON and all strains possessed different toxin profiles for FA, FB_1 and MON.

The cluster between *F. verticillioides* 7390 and *F. verticillioides* 6019 formed by OPC 4, only had similar FB_1 levels which were low. The concentrations of the other three toxins varied and no similarities were detected. A second cluster (*F. verticillioides* 3125 and *F. verticillioides* 5084) formed where both strains produced similar FB_1, ZON and FA concentrations. With the clustering of *F. verticillioides* 5085, *F. verticillioides* 1065 and *F. verticillioides* E110, the levels of FB_1, ZON and also FA were found to be similar. With respect to their ZON levels, both *F. verticillioides* 5085 and *F. verticillioides* 1065 produced comparable concentrations. The 82.5% cluster between *F. verticillioides* 5085 and *F. verticillioides* 8335 showed similarities between the levels of FA and MON. Both did not produce ZON but they differed in their production of FB_1. Cluster formation between *F. verticillioides* 6019 and

F. verticillioides 5550 occurred at 82.5%. The same toxins were also produced, however, the concentrations of MON varied, while their levels of FB_1 and FA were similar.

F. verticillioides 6525 and *F. verticillioides* 5550 produced the same toxins although were only identical in their ZON production and similar for FB_1. *F. verticillioides* 3674 and *F. verticillioides* 3125 clustered together at 92% but only produced similar MON levels. Primer UBC 204 produced a cluster consisting of five *F. verticillioides* strains which were found to be 92% similar. All strains, except *F. verticillioides* 5085, were similar in the level of FB_1 produced and all strains were similar in their ZON levels, except *F. verticillioides* 5084. The production of FA and MON varied.

F. verticillioides 5550 and *F. verticillioides* 3674 clustered at 100% with UBC 270 and both strains did not produce ZON. Both strains also produced very different FB_1 and MON levels, in addition to their different FA concentrations. *F. verticillioides* 7390 and *F. verticillioides* 5084 also clustered at 100% with three of the mycotoxins (FB_1, ZON and FA) producing similar levels. Primer UBC 285 clustered *F. verticillioides* 3674 and *F. verticillioides* 5146 at 87.2%. No similarities existed regarding toxin production or their concentrations, except for ZON not being produced by either of the strains.

TABLE 3.4 Correlation between cluster formation for each primer and mycotoxin levels

PRIMER	CLUSTER	PERCENTAGE SIMILARITY	TOXIN LEVELS			
			FB_1	ZON	FA	MON
OPC 1	1065	92.5	L	H	M	M
	6525		L	ND	H	M
	7390	60	L	L	M	M
	1065		L	H	M	M
OPC 3	1065	100	L	H	M	M
	6525		L	ND	H	M
	E110	100	M	ND	M	M
	5085		L	ND	M	H
	5084	92.5	L	L	M	L
	6019		L	ND	L	H
	E110	93	M	ND	M	M
	5085		L	ND	M	H
	1059		L	ND	L	L
OPC 4	7390	94.1	L	L	M	M
	6019		L	ND	L	H
	3125	93.9	L	L	M	H
	5084		L	L	M	L
	5085	95	M	ND	M	M
	1065		L	H	M	M
	5085	92	M	ND	M	M
	1065		L	H	M	M
	E110		L	ND	M	H

L - low toxin production
M - medium toxin production
H - high toxin production

TABLE 3.4 Correlation between cluster formation for each primer and mycotoxin levels (cont.)

PRIMER	CLUSTER	PERCENTAGE SIMILARITY	TOXIN LEVELS			
			FB_1	ZON	FA	MON
	5085	82.5	M	ND	M	M
	8335		L	ND	M	M
OPC 7	6019	82.5	L	ND	L	H
	5550		L	ND	L	L
	6525	85	L	ND	H	M
OPC 8	5550		L	ND	L	L
	1059		L	ND	L	L
	5085		M	ND	M	M
	5084	100	L	L	M	L
	5550		L	ND	L	L
	3674	92	H	ND	ND	H
UBC 204	3125		L	M	M	H
	1059		L	ND	L	L
	5085		M	ND	M	M
	5084	92	L	L	M	L
	5550		L	ND	L	L
	6019		L	ND	L	H
	1065	100	L	H	M	M
	6525		L	ND	H	M
UBC 270	5550	100	L	ND	L	L
	3674		H	ND	ND	H
UBC 284	7390	100	L	L	M	M
	5084		L	L	M	L
UBC 285	3674	87.2	H	ND	ND	H
	5146		L	ND	H	M
UBC 300	5084	80	L	L	M	L
	6525		L	ND	H	M

L - low toxin production
M - medium toxin production
H - high toxin production

3.4 VOLATILE COMPOUND PROFILE PRODUCED BY *Fusarium* ISOLATES

The AromaScanner was used to qualitatively determine the production of volatiles. A typical profile that was obtained upon aroma sampling is depicted in Figure 3.10. The profile shows a curve with an initial reference period by sampling of the head-space of the fungal specimen. Thereafter, there is a second wash step followed by a final reference stage. Computer analysis thereafter normalized the data and provided a quality value.

In addition to obtaining a quality value, a Sammon map is drawn which allows for improved visual differentiation. A characteristic map showing the clustering of *F. verticillioides* E18, *F. verticillioides* 3120, *F. verticillioides* 6019, *F. verticillioides* 5146 and *F. verticillioides* 5085 is illustrated in Figure 3.11. The results were analyzed using CA and strains were classified as significantly different (quality value >2.1) or significantly similar (quality value <2.1) [TABLE 3.5].

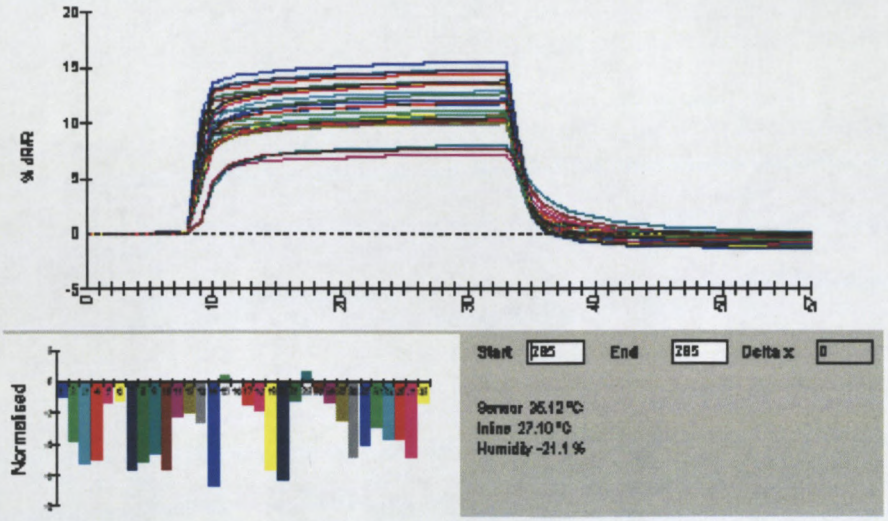


Figure 3.10 Typical aroma scan profile.

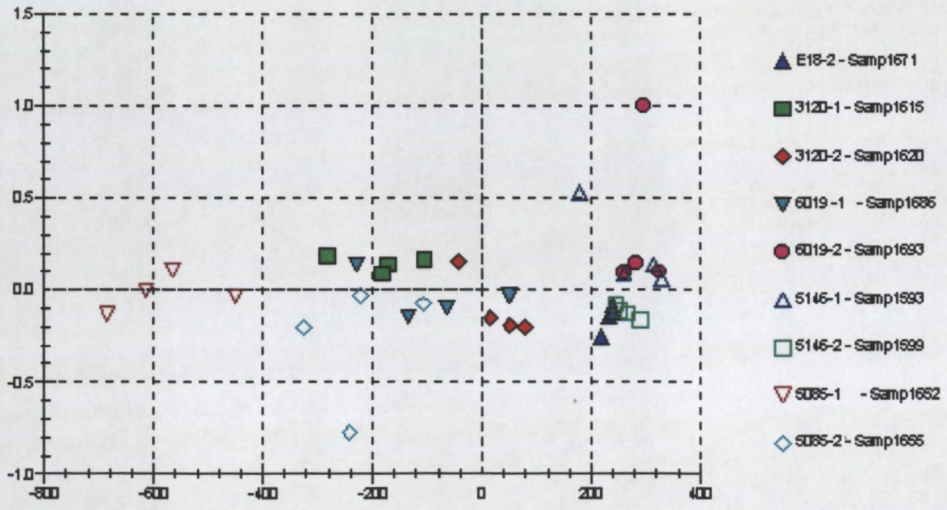


Figure 3.11 Typical Sammon map showing differences between *F. verticillioides* E18, *F. verticillioides* 3120, *F. verticillioides* 6019, *F. verticillioides* 5146 and *F. verticillioides* 5085.

The quality value obtained for *F. verticillioides* 8335, *F. verticillioides* 7390 and *F. verticillioides* 1059 with *F. verticillioides* 5085 was less than 2.1 which implied that there was no significant difference between these strains. One can see that *F. verticillioides* 3674 was similar to *F. verticillioides* 5550 and *F. verticillioides* 3125. *F. verticillioides* 5550, in turn, was similar to *F. verticillioides* 3674, *F. verticillioides* 3125 and *F. verticillioides* 6019, while *F. verticillioides* 3125 was found to be similar to *F. verticillioides* 3674, *F. verticillioides* 5550 and *F. verticillioides* 5084. *F. verticillioides* 6019, on the other hand, was only similar to *F. verticillioides* 5550.

A grouping occurred between *F. verticillioides* 8335 and *F. verticillioides* 5085 as well as *F. verticillioides* 7390 and *F. verticillioides* 5085. *F. verticillioides* 1059 was found to have some degree of similarity with *F. verticillioides* 5084 and *F. verticillioides* 5085, while *F. verticillioides* 5084 had a quality value less than 2.1 when compared with *F. verticillioides* 1059 and *F. verticillioides* 3125.

TABLE 3.5 Differentiation of *Fusarium* species and strains based on quality values

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y
2		Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3			N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
4				Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
5					N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
6						N	Y	N	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y
7							N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
8								N	N	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y
9									N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
10										Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
11											N	Y	Y	N	Y	Y	N	Y	N	Y
12												N	Y	Y	Y	Y	Y	Y	Y	Y
13													Y	Y	Y	Y	Y	Y	Y	Y
14														N	Y	Y	Y	Y	Y	Y
15															Y	Y	Y	Y	Y	Y
16																N	Y	Y	Y	Y
17																	Y	N	N	Y
18																		N	Y	Y
19																			N	Y
20																				N

Y - (Yes) Significant difference (>2.1) N - (No) No significant difference (<2.1)

1-Control
2-*F. dimerum*
3-*F. solani*
4-*F. chlamydosporum*
5-*F. semitectum*
6-*F. verticillioides* 1059
7-*F. verticillioides* E110
8-*F. verticillioides* 5085
9-*F. verticillioides* 8335
10-*F. verticillioides* E18
11-*F. verticillioides* 3125
12-*F. verticillioides* 1065
13-*F. verticillioides* 7390
14-*F. verticillioides* 5084
15-*F. verticilioides* 3120
16-*F. verticillioides* 6525
17-*F. verticillioides* 5550
18-*F. verticillioides* 6019
19-*F. verticillioides* 3674
20-*F. verticillioides* 5146

3.4.1 Correlation of Volatile Compound Profile with Toxin Production

The production of volatile compounds was correlated to the levels and production of FB_1, ZON, FA and MON. TABLE 3.6 summarizes the clustering between strains that have a quality value less than 2.1. *F. verticillioides* 3674 and *F. verticillioides* 5550 formed a cluster, however, their levels of toxin production varied. There was no correlation between their levels of toxins produced and their volatile profiles, except for the absence of ZON production in both strains. *F. verticillioides* 3674 and *F. verticillioides* 3125 were grouped together but only their high MON levels were similar. *F. verticillioides* 5550 also came together with *F. verticillioides* 3125 with both strains producing low levels of FB_1. Fusaric acid, ZON and MON were produced differently by both these strains. *F. verticillioides* 6019 and *F. verticillioides* 5550 as a cluster with similar low levels of FA and FB_1. ZON was also not detected in both these isolates. *F. verticillioides* 5084 and *F. verticillioides* 1059 to be similar, however, their toxin profiles showed their relatedness in only their low FB_1 and MON levels. *F. verticillioides* 5084 had a quality value of less than 2.1 when compared with *F. verticillioides* 3125. These strains were similar in their low FB_1 and ZON levels while FA was produced moderately. *F. verticillioides* 5085 and *F. verticillioides* 1059 to toxin production, it was found that only ZON production was identical due to its absence. There was a partial correlation between *F. verticillioides* 5085 and *F. verticillioides* 8335, where ZON was not produced, and FA and MON were produced at medium levels. *F. verticillioides* 5085 produced a high concentration of FB_1, while *F. verticillioides* 8335 was only capable of producing this toxin at low levels. A cluster between *F. verticillioides* 5085 and *F. verticillioides* 7390. Both strains were medium

producers of FA and MON but differed in their FB_1 and ZON levels. It is evident that none of the species were grouped together by the analysis of volatile production.

TABLE 3.6 Correlation between volatile compound clusters and mycotoxin levels of each Fusarial isolate

CLUSTER	TOXIN LEVELS			
	FB_1	ZON	FA	MON
F. verticillioides 3674	H	ND	ND	H
F. verticillioides 5550	L	ND	L	L
F. verticillioides 3674	H	ND	ND	H
F. verticillioides 3125	L	L	M	H
F. verticillioides 5550	L	ND	L	L
F. verticillioides 3125	L	L	M	H
F. verticillioides 6019	L	ND	L	H
F. verticillioides 5550	L	ND	L	L
F. verticillioides 5084	L	L	M	L
F. verticillioides 1059	L	ND	L	L
F. verticillioides 5084	L	L	M	L
F. verticillioides 3125	L	L	M	H
F. verticillioides 5085	L	ND	M	M
F. verticillioides 1059	L	ND	L	L
F. verticillioides 5085	H	ND	M	M
F. verticillioides 8335	L	ND	M	M
F. verticillioides 5085	H	ND	M	M
F. verticillioides 7390	L	L	M	M

3.5 ENZYME PROFILES OF FUSARIAL ISOLATES

Four hydrolytic enzyme activities, viz., α-D-galactosidase β-D-xylosidase, β-D-glucosidase, and α-D-mannosidase were assayed using the respective ρ-nitrophenyl substrates. TABLE 3.7 illustrates the activities of each of the four hydrolytic enzymes produced by the *Fusarium* isolates.

It can be seen that *F. solani* and *F. verticillioides* 3120 had the highest galactosidase activity. Most of the isolates appear to have had similar α-D-galactosidase activities within the range of 0.4784 nkat/ml and 1.0238 nkat/ml, with the exception of *F. verticillioides* 1059, *F. verticillioides* E18, *F. verticillioides* 3125, *F. verticillioides* 3120, *F. verticillioides* 3674 and *F. verticillioides* 5146. Of the four species, only *F. dimerum* and *F. chlamydospoum* had similar levels of 0.6016 nkat/ml and 0.5280 nkat/ml, respectively. *F. verticillioides* 6525 had a low level of activity in comparison to all other isolates.

TABLE 3.7 also illustrates β-D-xylosidase activities for the different *Fusarium* isolates. *F. verticillioides* 5550 produced a very low activity of 0.07424 nkat/ml, while the highest level was produced by *F. verticillioides* 1059, *F. verticillioides* 3120, *F. verticillioides* 6525 and *F. verticillioides* 3674. Most of the other isolates had levels ranging around 1.09 nkat/ml. *F. dimerum* and *F. semitectum* had very similar levels of β-D-xylosidase activity, while *F. solani* had a much lower level of 0.3912 nkat/ml. Most of the *F. verticillioides* strains had different levels with the exception of *F. verticillioides* 1059, *F. verticillioides* 3120, *F. verticillioides* 6525 and

F. verticillioides 3674 which had high activities and *F. verticillioides* 5085, *F. verticillioides* 8335, *F. verticillioides* E18, *F. verticillioides* 3125, *F. verticillioides* 1065, *F. verticillioides* 7390, *F. verticillioides* 5084, *F. verticillioides* 6019 and *F. verticillioides* 5146 which are medium producers of the enzyme.

TABLE 3.7 Enzyme activities (nkat/ml) of *Fusarium* species and strains

***Fusarium* SPECIES AND STRAINS**	**ENZYME ACTIVITY (nkat/ml)**			
	α-D-GALACTOSIDASE	**β-D-XYLOSIDASE**	**β-D-GLUCOSIDASE**	**α-D-MANNOSIDASE**
***F. dimerum* E21**	0.6016	1.2912	1.2914	1.2736
***F. solani* E1398**	2.2576	0.3912	1.3912	1.1069
***F. chlamydosporum* E1364**	0.5280	1.1752	2.0376	2.3488
***F. semitectum* E1822**	0.8300	1.0839	5.5158	1.4870
***F. verticillioides* 1059**	1.3002	3.3046	14.2270	1.6777
***F. verticillioides* E110**	0.5154	0.7435	1.3922	1.1453
***F. verticillioides* 5085**	0.5981	0.9827	0.1086	0.9843
***F. verticillioides* 8335**	0.5198	1.0942	4.3729	0.8455
***F. verticillioides* E18**	1.1642	1.2674	2.9339	1.9578
***F. verticillioides* 3125**	1.3480	1.4763	4.9717	2.2751
***F. verticillioides* 1065**	0.8102	1.9270	6.6560	1.2629
***F. verticillioides* 7390**	0.9783	1.2058	3.4114	0.9801
***F. verticillioides* 5084**	0.4784	1.6816	1.4523	1.1320
***F. verticillioides* 3120**	2.8075	3.1420	12.2345	2.0058
***F. verticillioides* 6525**	0.0346	3.2048	7.9016	1.8672
***F. verticillioides* 5550**	1.0238	0.0742	2.3138	1.0051
***F. verticillioides* 6019**	0.6484	1.1497	1.3950	0.8912
***F. verticillioides* 3674**	1.5220	2.9339	7.3734	2.0044
***F. verticillioides* 5146**	1.4900	1.2256	4.2369	1.4790

TABLE 3.7 depicts the activities of β-D-glucosidase for the *Fusarium* species and strains. *F. verticillioides* 3120 and *F. verticillioides* 1059 are very high producers of the enzyme, in comparison to all other isolates. The lowest activity (0.10856 nkat/ml) was achieved by *F. verticillioides* 5085. Of the four species, *F. semitectum* produced medium levels of activity of 5.5158 nkat/ml, while the other three species produced similar levels of 1.2914 nkat/ml, 1.3912 nkat/ml and 2.0376 nkat/ml. No similarities in activities were observed for the *F. verticillioides* strains, since all produced very different activities of β-D-glucosidase.

Seven isolates, viz., *F. chlamydosporum* and *F. verticillioides* 1059, *F. verticillioides* E18, *F. verticillioides* 3125, *F. verticillioides* 3120, *F. verticillioides* 6525 and *F. verticillioides* 3674 produced high activities of α-D-mannosidase ranging from 1.6777 nkat/ml to 2.3488 nkat/ml. The lowest activity of 0.8455 nkat/ml was produced by *F. verticillioides* 8335 which was comparable to *F. verticillioides* 5085, *F. verticillioides* 7390, *F. verticillioides* 5550 and *F. verticillioides* 6019. *F. solani*, *F. dimerum*, *F. verticillioides* E110, *F. verticillioides* 5084 and *F. verticillioides* 1065 produced medium activity of approximately 1.2 nkat/ml. All four species as well as the 15 *Fusarium* strains had very different α-D-mannosidase levels.

β-D-Glucosidase produced the highest activity for most of the isolates. *F. verticilliodes* 1059 and *F. verticillioides* 3120 produced the highest β-D-glucosidase activity when compared to the other enzymes, i.e. 1.778 nkat/ml and 1.529 nkat/ml, respectively. In general, *F. verticilliodes* 5085 exhibited the least activity for all the enzymes.

It is evident that amongst all strains of *F. verticillioides*, β-D-glucosidase was highest, with the exception of *F. verticillioides* 5085 which produced very low levels of the enzyme. Amongst most isolates, α-D-galactosidase had the lowest activity, except for *F. solani* and *F. verticillioides* 5146.

3.5.1 Correlation of Enzyme Profiles with Toxin Production

The production of α-D-galactosidase, β-D-xylosidase, β-D-glucosidase, and α-D-mannosidase were correlated to the production of four mycotoxins, namely, FB_1, ZON, FA and MON. TABLE 3.8 illustrates the activity of each enzyme individually with the concentrations of the four toxins.

The only high enzyme producers were *F. solani* and *F. verticillioides* 3120 with activities of 2.2576 nkat/ml and 2.8085 nkat/ml, respectively. *F. solani* produced only medium levels of FA and MON while *F. verticillioides* 3120 was a low producer of FB_1 and MON and produced an intermediate level of FA. *F. verticillioides* E18 and *F. verticillioides* 3674 were high producers of FB_1. *F. verticillioides* 1065 was a high producer of ZON and *F. verticillioides* 6525 and *F. verticillioides* 5146 were high producers of FA. Moniliformin was produced at high concentrations by *F. semitectum* and *F. verticillioides* E110, *F. verticillioides* 3125, *F. verticillioides* 6019 and *F. verticillioides* 3674. It is evident that neither of the high α-D-galactosidase-producing isolates was capable of producing high toxin concentrations.

The highest β-D-xylosidase-producing isolates were *F. verticillioides* 1059, *F. verticillioides* 3120, *F. verticillioides* 6525 and *F. verticillioides* 3674. Of these strains, *F. verticillioides* 3674 produced high levels of FB_1 and MON with concentrations up to 7.06 μg/ml and 19.51 μg/ml, respectively. In addition to producing high enzyme levels, *F. verticillioides* 6525 was also a high producer of FA (9.008 μg/ml). It can be seen that the high MON-producers did not produce high levels of this enzyme as well as with the production of FA. The only high producer of ZON was *F. verticillioides* 1065 which produced a medium level of β-D-xylosidase.

TABLE 3.8 illustrates the correlation between toxin production and β-D-glucosidase activity of each of the 19 *Fusarium* isolates. *F. verticillioides* 1059 and *F. verticillioides* 3120 produced levels of 14.227 nkat/ml and 12.435 nkat/ml of this enzyme, respectively. However, these strains were not high producers of FB_1, ZON, FA or MON. It is evident that the high FB_1 producers, viz., *F. verticillioides* E18 and *F. verticillioides* 3674, produced medium levels of β-D-glucosidase. There is no relationship between the levels of the other toxins.

The highest α-D-mannosidase producing strains of the *Fusarium* isolates classified, were found to be *F. chlamydosporum*, *F. verticillioides* E18, *F. verticillioides* 3125, *F. verticillioides* 3120, *F. verticillioides* 6525 and *F. verticillioides* 3674. Of these isolates, only *F. verticillioides* E18 and *F. verticillioides* 3674 were high producers of FB_1, while the remaining strains produced low concentrations of FB_1 (TABLE 3.8).

TABLE 3.8 demonstrates the efficiency of each of the three techniques used in this study. It is evident that both the EN and RAPD technique achieved a number of common clusters.

TABLE 3.8 Intra-specie differentiation of *Fusarium* by hydrolytic enzymes, volatile compounds and RAPD's

Fusarium ISOLATE	LEVEL OF TOXIN				LEVEL OF ENZYME				VOLATILE COMPOUND CLUSTERS	RAPD CLUSTERS (%)
	FB_1	ZON	FA	MON	GAL	XYL	GLU	MAN		
F. dimerum E21	ND	ND	M	M	L	M	L	M		
F. solani E1398	ND	ND	M	M	H	L	L	L		
F. chlamydosporum E1364	ND	ND	M	M	L	M	L	H		
F. semitectum E1822	ND	ND	M	H	M	M	M	M		
F. verticillioides 1059	L	ND	L	L	M	H	H	M	5084 5085	E110-93 6019-92 5085-93; 100 5550-100 5084-100
F. verticillioides E110	L	ND	M	H	L	L	L	L		5085-92; 100 1059-93 1065-92
F. verticillioides 5085	H	ND	M	M	L	M	L	L	1059 8335 7390	E110-100 1059-93 1065-95 8335-82.5 5084-100
F. verticillioides 8335	L	ND	M	M	L	M	M	L	5085	5085-82.5
F. verticillioides E18	H	ND	M	M	M	M	M	H		
F. verticillioides 3125	L	L	M	H	M	M	M	H	3674 5084 5550	5084-93.9 3674-92
F. verticillioides 1065	L	H	M	M	M	M	M	M		6525-92.5; 100 5085-95

		HIGH (H)	MEDIUM (M)	LOW (L)
GAL	α-D-galactosidase	>1.9 nkat/ml	0.75-1.9 nkat/ml	<0.75 nkat/ml
XYL	β-D-xylosidase	>1.9 nkat/ml	0.74-1.9 nkat/ml	<0.74 nkat/ml
GLU	β-D-glucosidase	>8.0 nkat/ml	2.35-8.0 nkat/ml	<2.35 nkat/ml
MAN	α-D-mannosidase	>1.75 nkat/ml	1.2-1.75 nkat/ml	<1.20 nkat/ml

TABLE 3.8 Intra-specie differentiation of *Fusarium* by hydrolytic enzymes, volatile compounds and RAPD's (cont.)

Fusarium ISOLATE	LEVEL OF TOXIN				LEVEL OF ENZYME				VOLATILE COMPOUND CLUSTERS	RAPD CLUSTERS (%)
	FB_1	ZON	FA	MON	GAL	XYL	GLU	MAN		
F. verticillioides 7390	L	L	M	M	M	M	M	L	5085	6019-94.1 5084-100
F. verticillioides 5084	L	L	M	L	L	M	L	L	1059 3125	6019-92.5 3125-93.9 7390-100 5550-100 1059-100
F. verticillioides 3120	L	ND	M	L	H	H	H	H		
F. verticillioides 6525	L	ND	H	M	L	H	M	H		1065-92.5; 100 5550-85
F. verticillioides 5550	L	ND	L	L	M	L	L	L	3674 6019 3125	6019-92 6525-85 1059-100 3674-100 5084-100 5085-100
F. verticillioides 6019	L	ND	L	H	L	M	L	L	5550	5084-92.5 7390-94.1 1059-92 5550-82.5; 92 5084-92 5085-92
F. verticillioides 3674	H	ND	ND	H	M	H	M	H	5550 3125	5550-100 3125-92 5146-87.2
F. verticillioides 5146	L	ND	H	M	M	M	M	M		3674-87.2

			HIGH (H)	MEDIUM (M)	LOW (L)
GAL	-	α-D-galactosidase	>1.9 nkat/ml	0.75-1.9 nkat/ml	<0.75 nkat/ml
XYL	-	β-D-xylosidase	>1.9 nkat/ml	0.74-1.9 nkat/ml	<0.74 nkat/ml
GLU	-	β-D-glucosidase	>8.0 nkat/ml	2.35-8.0 nkat/ml	<2.35 nkat/ml
MAN	-	α-D-mannosidase	>1.75 nkat/ml	1.2-1.75 nkat/ml	<1.20 nkat/ml

CHAPTER FOUR : DISCUSSION

Fusarium is a heterogenous genus with a world-wide distribution that includes many species. The differentiation of *Fusarium* species and strains is essential in establishing the correct assessment strategy for preventing plant and human diseases. Correct early identification of this genus is becoming increasingly important due to the toxin-producing capabilities of some of these species, especially the fumonisin-producers (Marasas *et al.*, 1986; Ross *et al.*, 1991). In the introduction to the illustrated manual for identification of *Fusarium* species, Nelson *et al.* (1983) highlight that workers interested in *Fusarium* species often encountered difficulties with the correct identification of *Fusarium* strains. This still remains a problem and thus, with the advent of molecular and genomic advances, alternative strategies need to be investigated.

The aim of this study was to determine if toxin-producing *Fusarium* isolates could be differentiated based on either their genetic profile or through specific biochemical markers. This research was therefore embarked on, to establish suitable detection methods based on RAPD's and their production of volatile compounds and hydrolytic enzymes. A total of 19 species and strains were selected for this study based on their toxin production. These included known isolates from two culture collections, viz., ppri and MRC which had known high and low FB_1-producers, as well as isolates from a previous study on fusarial isolates from keratitis infections.

Prior to the commencement of this investigation, each of the 19 *Fusarium* isolates was verified by the observation of colony morphology and hyphal pigmentation. Based on their cultural and microscopic features (Nelson *et al.*, 1983), four belonged to different species, viz., *F. semitectum*, *F. dimerum*, *F. solani* and *F. chlamydosporum* and the remaining 15 isolates were verified as *F. verticillioides*.

Colony colour was an important criterion for *Fusarium* species verification. Sabouraud dextrose agar which is a fungal growth medium, was utilized as the culture medium since it is rich in carbohydrates and was able to enhance the colony colours that were produced by the *Fusarium* isolates. The common *Fusarium* growth medium is Potato dextrose agar, however, previous researchers observed a level of mutation using this medium (Seifert, 1996) and it was therefore not used in this study.

The toxin levels for FB_1, ZON, FA and MON of each isolate was determined. The extraction and HPLC methods used were optimized and our results indicated a recovery of 72.4% from broth spiked with 10 μg/ml FB_1 standard when using SAX cartridges. Stack and Eppley (1992) recovered 67% FB_1 from maize using SAX cartridges while Thiel *et al.* (1993) reported an 84% recovery of FB_1 from maize spiked at 5μg/ml. Eighty-three percent of ZON was recovered from broth spiked with 20 μg/ml of the commercial standard when using Sep-pak cartridges. Percentage recoveries of ZON are high and have ranged from 70-110% (Leatherhead Food International, 2003b). In our study, a percentage recovery of 84.5% was obtained when 20 μg/ml of FA standard was spiked into broth. Venter and Steyn in 1998 obtained a recovery of 96.0% when FA was

extracted from maize. The extraction efficiency for MON was proven to be 84.8% from broth. Filek and Lindner (1996) recovered 70% MON from spiked wheat. The variation in recovery rates was probably due to the difference in sample matrices since broth samples were used in this study as opposed to maize and wheat samples used in other studies.

High performance liquid chromatography is a very sensitive method which is able to quantify toxin production. The detection limits for FB_1, ZON, FA and MON were 0.01 µg/ml, 0.1 µg/ml, 0.05 µg/ml and 0.1 µg/ml, respectively. High performance liquid chromatography analyses revealed similar retention times for FB_1, ZON, FA, and MON samples to those of the commercial standards. Variations in the retention times that occurred between the extracted toxins and the standards were investigated by spiking the extracted compounds with the respective standards. An increase in the resulting peak confirmed the retention time of the extracted toxin. This shift is generally due to certain changes such as pressure and flow rate within the HPLC system.

The 19 *Fusarium* isolates produced different toxins and different levels of the four toxins. (TABLE 3.2). Since this study was undertaken to differentiate *Fusarium* strains with varying toxin-producing capabilities, they were grown under the same conditions in a broth culture. Toxin levels were categorized subjectively as high, medium and low.

Fumonisin B_1 was produced by all *F. verticillioides* but not by any of the other four *Fusarium* species. Our results are similar to those of Nelson *et al.* (1991) who showed

that *F. verticillioides* and *F. proliferatum* are the only species of *Fusarium* capable of FB_1 production. In addition, *F. proliferatum*, *F. napiforme*, *F. nygamai* and *G. fujikuroi*, were reported by Keller and Sullivan (1996), Nelson *et al.* (1992), Smith *et al.* (1994) and Desjardins *et al.* (1997), respectively to produce fumonisins. The levels ranged from 0.25 μg/g to 8.14 μg/g which is comparable to levels found in contaminated maize by Kedera *et al.* (1999), viz., 0.20 μg/g. Two strains were classified as high FB_1-producers, i.e. *F. verticillioides* 3674 which produced 7.06 μg/ml and *F. verticillioides* E18 which produced 8.14 μg/ml of FB_1 and 12 as low FB_1-producers (concentrations ranged from 0.25 μg/ml – 1.63 μg/ml).

Of the 19 *Fusarium* isolates only four *F. verticillioides* strains produced ZON with levels ranging from 0.14 μg/ml to 0.81 μg/ml. None of the other species produced this toxin. Although ZON does not seem to be widely-produced, two of the four strains produced levels above that tolerated in foods (60 μg/kg - 200 μg/kg) [Leatherhead Food International, 2003b]. All *Fusarium* isolates, except *F. verticillioides* 3674, produced FA. Two strains produced relatively high levels (9.01 μg/ml and 11.27 μg/ml) of FA compared to the other strains although all were low producers compared to that reported in the literature in solid (20 μg/kg - 1 000 μg/kg) and liquid (320 – 150 mg/ml) culture [Bacon *et al.*, 1996]. In keeping with investigations by Betina (1980), Bacon *et al.* (1996) and Porter *et al.* (1996) who established *F. solani*, *F. verticillioides*, *F. oxysporum*, and *F. lateritium* as FA-producers, we found it to be commonly produced.

Moniliformin was produced by every *Fusarium* specie and strain used in this study. All strains produced MON at a high concentration than the other three toxins, except for *F. verticillioides* 5146 which produced a higher level of FA. Various research works (Betina, 1980; Cole and Cox, 1981; Marasas *et al.*, 1984; Rabie *et al.*, 1982; Joffe, 1986) also reported on the production of MON by *F. verticillioides* and *F. chlamydosporum.*

The next area of research entailed generating RAPD fingerprints, which were used to investigate genetic variability between *Fusarium* species and *F. verticillioides* strains isolated from human and plant origin. In this evaluation, 17 short primers (10-mer) of arbitrary nucleotide sequences were used to amplify segments of genomic DNA from *Fusarium* isolates. This procedure proved to be quite reproducible under the set PCR conditions and was therefore, doubled as a marker for all agarose gels owing to the lack of stock of commercial molecular weight marker VI. Other researchers, however, have contrasting views on the repeatability of this technique (Jones *et al.*, 1997; Penner *et al.*, 1993; Skroch and Nienhuis, 1995).

In this analysis, multiple bands were produced with the primers. The banding patterns observed with primers UBC 204, UBC 270 and UBC 284 were polymorphic and revealed a high degree of diversity for *Fusarium* species at the inter-species level. Similar findings were reported in a number of studies based on the same technique where researchers also detected specie differentiation amongst *Fusarium* (Donaldson *et al.*, 1995; Jimenez *et al.*, 2000; Khalil, *et al.*, 2003; Schilling *et al.*, 1994; Voigt *et al.*, 1995).

The construction of dendrograms (hierarchic representation of linkage levels between pairs of groups), was used for cluster analysis which allowed the comparison and grouping of individuals by calculating the similarity between all possible pairs in a gel. In this investigation, all *F. verticillioides* strains, except *F. verticillioides* 5550, *F. verticillioides* E18 and *F. verticillioides* 3125 produced different clusters with different primers. *F. verticillioides* 5550, formed four different banding patterns with four primers, and gave four clusters with six different strains. *F. verticillioides* 5550 with *F. verticillioides* 3674, and *F. verticillioides* 6525 with *F. verticillioides* 1065 were the only strains that clustered together by two different primers (TABLE 3.3). Therefore, an analysis of different strains using these 17 primers does not lead to any conclusive diagnostic tool towards intra-specie variation. In this study, we were able to distinguish between the different species but not strains using RAPD's. This is in keeping with the recent studies of Khalil *et al.* (2003) who were also able to distinguish among strains of *F. verticillioides*, *F. solani*, *F. oxysporum* and *F. avenaceum*. However, they are incongruent to the results of Ouellet and Seifert (1993) who were able to distinguish strains between *F. graminearum* strains. A PCR-RAPD typing system for *Agrobacterium* was also able to classify strains with four 10-mer primers (Llop *et al.*, 2003).

An important aspect of this study was to evaluate the RAPD technique as means of differentiating between toxin-producing and non-producing *Fusarium* isolates, as well as discriminating between high, medium and low FB_1-producers. This technique enabled the differentiation of Fusarial strains which produced FB_1. The two closely-situated

bands of 1 050 kb and 842 kb as well as the 360 bp band produced by primer UBC 204 were found to be associated with FB_1 production in *F. verticillioides* strains (Figure 3.7). Primer UBC 270 was equally successful in differentiating FB_1-producing *F. verticillioides* strains, except *F. verticillioides* E18 (Figure 3.8). Discrimination of *F. verticillioides* strains could also be achieved with primer UBC 284 (Figure 3.9). The five bands common to all *F. verticillioides* strains, excluding E18 seems to be related to FB_1 production. None of the primers could differentiate between the levels of FB_1 amongst these isolates. Jimenez *et al.* (2000), on the other hand, were able to characterize FB_1 and FB_2 isolates using six primers and also discriminate between high and low FB_1-producers among *Fusarium* isolates. *F. verticillioides* E18, which was a high FB_1-producer was consistently being missed as an FB_1-producer by all three of the primers. The exclusion of *F. verticillioides* E18 from the common banding profiles could be attributed to the possibility that it underwent mutation or is different from the other isolates because it is a human pathogen that was isolated from an eye infection.

In this investigation, RAPD's were capable of discriminating between *Fusarium* species and FB_1 production, but not between strains. In addition, no correlation could be made with respect to the levels of toxin produced. The technique of RAPD's is, as the acronym suggests, rapid. It is technically simple and often detects variation among isolates that are invariant with RFLP analysis. The utilization of this technique is dependent on producing reproducible specie or strain fingerprints, which is influenced by the annealing temperature, template concentration, primer length and primer sequence.

Fungi generally produce volatile compounds as they begin to colonize nutrient-rich substrates. Pasanen *et al.* (1996) suggested that volatile organic compounds may be used as markers for the activity of mycotoxigenic species. An AromaScanner comprising 32 polymer senses was used in this study to qualitatively differentiate between the *Fusarium* species and *F. verticillioides* strains. The first study by Keshri and Magan (2000), based on the differentiation of two *Fusarium* strains using EN technology limited the investigation of general volatile production patterns to the early stages of growth (up to 96 h). Since toxins are secondary metabolites and are produced during the latter stages of fungal growth (after 21 days), this analysis was designed to analyze the production of volatile compounds after the production of the four predominant fusariotoxins by way of prolonged incubation, i.e. 25 days.

The aroma profiles generated for the 19 isolates enabled differentiation of the five species of Fusaria as none of them clustered together (TABLE 3.5). This finding is in accordance with a number of studies (Korpi *et al.*, 1998; Larsen, 1997; Larsen and Frisvad, 1994; Wilkins *et al.*, 1997), including that of Filer *et al.* (2001) who screened 12 fungal species for over 150 volatile compounds and found unique profiles for each specie. In addition, Keshri and Magan (unpublished data) suggested that the general volatile patterns produced by an ochratoxigenic strain of *A. ochraceus* and *A. niger* were very different and unique.

The use of the EN to differentiate strains or toxin profiles was not clear. Our results showed the formation of nine clusters of similar *F. verticillioides* strains.

F. verticillioides 3674, *F. verticillioides* 5550 and *F. verticillioides* 3125 were found to be similar based on their volatile compound profiles. Data clustering also demonstrated *F. verticillioides* 5085, *F. verticillioides* 1059, *F. verticillioides* 8335 and *F. verticillioides* 7390 to be similar while *F. verticillioides* 6019 clustered with *F. verticillioides* 5550. Keshri and Magan, however, in 2000 proved that strains of *F. proliferatum* and *F. verticillioides* could be separated by CA. Other research workers (Larsen and Frisvad, 1995; Nilsson *et al.*, 1996) also found some volatile organic compounds to be strain-specific.

The results produced by the electronic nose for each *Fusarium* isolate, and the levels of FB_1, ZON, FA and MON (TABLE 3.6), showed no association. This is in contrast to the research of Keshri and Magan (2000), who found that some of the non-toxic and toxic strains of *F. proliferatum* and *F. verticillioides* could be identified by their volatile profiles. They did, however, report on some overlapping between a *F. verticillioides* fumonisin-producing strain and a ZON- and fumonisin-producing strain. There was also an overlap between a trichothecene and fumonisin producer and a fumonisin producer for *F. proliferatum*. Another study carried out by Jelen *et al.* in 1995, demonstrated that toxic and non-toxic *F. sambucinum* strains produced distinctive and characteristic volatiles.

Conclusions arrived at in this area are based entirely on qualitative findings. Further research is recommended in order to identify the volatile compounds produced by each of the isolates. This can be achieved by gas chromatography–mass spectrophotometry (GC-

MS) which will allow one to determine the exact difference between each isolate by the production of their specific volatile compounds.

Results of duplicate samples produced a variation among four strains. It is explicable that this inconsistency may occur due to conditions created within each individual sample bottle. It is impractical that replicate samples would be identical since factors such as temperature and spore counts, and consequently, amount of mycelium growth would affect the production of volatiles. This discrepancy, even though slight, still implies a drawback of this device.

The final phase of this research involved the determination of four hydrolytic enzyme production profiles, namely, α-D-galactosidase, β-D-xylosidase, β-D-glucosidase and α-D-mannosidase. Not much research has been carried out in this area and therefore, limited literature is available. It was evident that the production of hydrolytic enzymes allowed for the differentiation of *Fusarium* species. However, it was not possible to discriminate between *Fusarium* strains in relation to their toxin-producing abilities. Results indicated that of the four enzymes produced, β-D-glucosidase exhibited the highest activity with the exception of four isolates. In a vast variety of biological systems, β-glucosidase catalyzes the hydrolysis of terminal, non-reducing β-D-glucose residues in numerous glucosides with the subsequent release of β-D-glucose (Lin *et al.*, 1999). It is therefore logical to attain such a result due to the high glucose content in the growth medium (SDA).

Enzyme activities achieved in this study were very low and could be attributed to the growth medium. Sabouraud dextrose agar was used as the growth medium for the analysis of enzyme production so as to maintain consistent conditions of growth throughout this study. However, an experiment was carried out in which an inducer (xylan) was incorporated into the SDA medium to validate the use of this medium by determining whether increased enzyme activities would be achieved. An increase was indeed found to occur, though only negligible. Analyses, therefore continued with the use of SDA as the growth medium. Comparable results were reported in 2000 by Keshri and Magan who used wheat meal agar as an inducer. Lipolytic as well as amylolytic activities were not improved even when Rucka *et al.* (1998) added sunflower oil to Czapek-Dox medium and starch, for the respective enzymes.

Another fundamental cause of low enzyme activities could be the time of incubation of each culture. *Aspergillus* species produced high titres of glucoamylase at 120 h (Ellaiah *et al.*, 2002). Olama and El-Sabaeny (1993) reported that the maximum lipase production was obtained after eight days of incubation using *Aspergillus niger*. It was, hence, probable that 25 days of incubation could have had a reducing effect on the enzyme activity. As a result, an attempt to verify the effect of incubation time on enzyme activity was investigated. At seven days and 15 days each of the four enzymes were extracted and quantified. Again, a negligible increase was arrived at. Results were on par with those described by Keshri and Magan (2000) when incubation times of 0 h, 48 h and 96 h were tested.

A third contributing factor could be the use of solid media. It has been established that liquid is a superior medium of choice for the induction of enzymes due to the provision of agitation and aeration which improve enzyme production. Similar low enzyme activity levels were achieved by Keshri and Magan in 2000 when they used a solid medium (wheat meal agar).

The activities of these enzymes were quantified after the onset of mycotoxin production (on the 25th day of incubation) in order to determine any relationship between the two aspects. Analyses, however, revealed that no specific correlation existed between mycotoxin and enzyme production for *Fusarium* species and strains. Results showed that no differentiation between species and enzyme production was possible. Additionally, it was not possible to correlate enzyme production with intra-specie variation.

The strains and species that formed the basis of this study, were acquired from various sources many of which lacked complete detail regarding their origin. A further limitation was that in this study there were no non-toxigenic *Fusarium* isolates. *F. verticillioides* 7390 was supposedly a non-FB_1 producer, however, in this study, it was found to be a low FB_1-producer. Improved extraction procedures as well as low detection limits and sensitivity of the HPLC system, may have contributed to the detection of FB_1. Another possible reason could be mutation of this strain. When this study was initiated, the polyketide synthase (PKS) gene was not characterized and hence arbitrary primers were selected for the RAPD analysis.

CHAPTER FIVE : CONCLUSIONS

Taxonomy of Fusaria is important since it underpins current research in this field. Molecular methods utilizing the gene sequence or secondary metabolites can contribute to early detection and further characterization of this group of fungi. To address this, this study was undertaken to determine specie and strain variation using RAPD's. The volatile compounds and hydrolytic enzyme profiles were also investigated to ascertain their use for differentiation of this genus.

Results of this study showed that RAPD's could be applied to identify a number of *Fusarium* species, namely, *F. verticillioides*, *F. dimerum*, *F. solani*, *F. chlamydosporum* and *F. semitectum*. This technique was unsuccessful for *F. verticillioides* strains which were indistinguishable from each other based on their fingerprints obtained. Further molecular characterization of *Fusarium* isolates using microsatellites, RFLP's and electrophoretic karyotyping may be explored to achieve intra-specie differentiation as well as to ascertain any correlation with the organism's toxin producing ability. This study was undertaken to characterize specie and strain variation of 19 isolates of *Fusarium* which comprised five species and 15 *F. verticillioides* strains.

Primers UBC 204, UBC 270 and UBC 284 can be employed in the assessment of diseases and mycotoxin contamination of crop products using RAPD's. It is also possible to use these primers in the detection and assay of *Fusarium* species from environmental samples. This technique has demonstrated good repeatability, reproducibility and is simple to use.

The EN proved to be successful in the differentiation of *Fusarium* species but not between *F. verticillioides* FB_1-producers and non-producers. The pitfalls of this technique for differentiation appears to be the fact that it is qualitative and can only resolve whether similar strains cluster together. In order for the AromaScanner to be used for strain differentiation, further analysis using GS or GC-MS would be required.

The hydrolytic enzyme profiles were also capable of differentiating species, however, results were inconclusive in discriminating *F. verticillioides* strains. This technique was also unable to indicate strain variation among different toxin-producing isolates. Since the genes involved in fumonisin biosynthesis have been elucidated, it would be invaluable to consider the PKS gene and the enzymes responsible for the production of fumonisins in order to significantly differentiate *Fusarium* species and strains with a correlation to FB_1 production.

Although analysis of volatile compounds and hydrolytic enzymes was employed as a detection tool for FB_1-producing *F. verticillioides* strains, this technique did not allow such distinction. While RAPD analysis was found to be useful in differentiating between the various *Fusarium* species, it did not allow clear intra-specific discrimination amongst *F. verticillioides*. Since it is imperative that microorganisms be identified correctly in order for control strategies to be designed, these techniques have to coincide with taxonomic systems. Based on the findings of this study, cultural taxonomy still remains the cornerstone of any research even though time-consuming and requiring extensive work with pure cultures.

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APPENDIX A

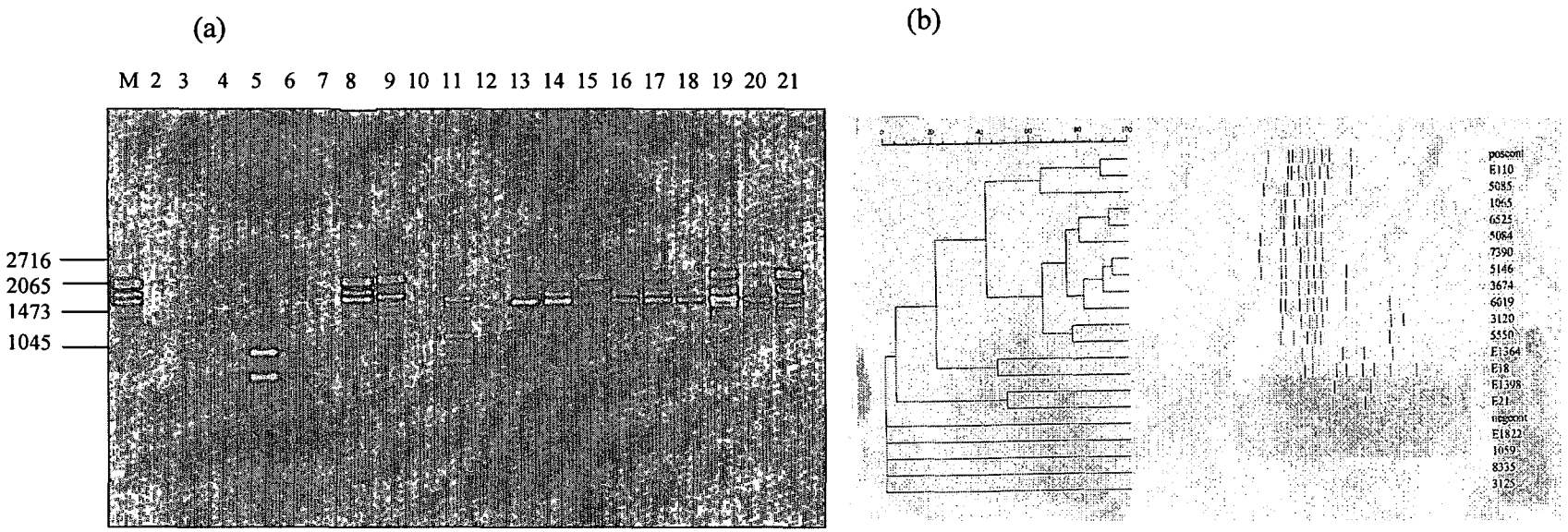

Figure A1 Agarose gel (a) and dendrogram (b) of 19 *Fusarium* isolates amplified by primer OPC 1. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

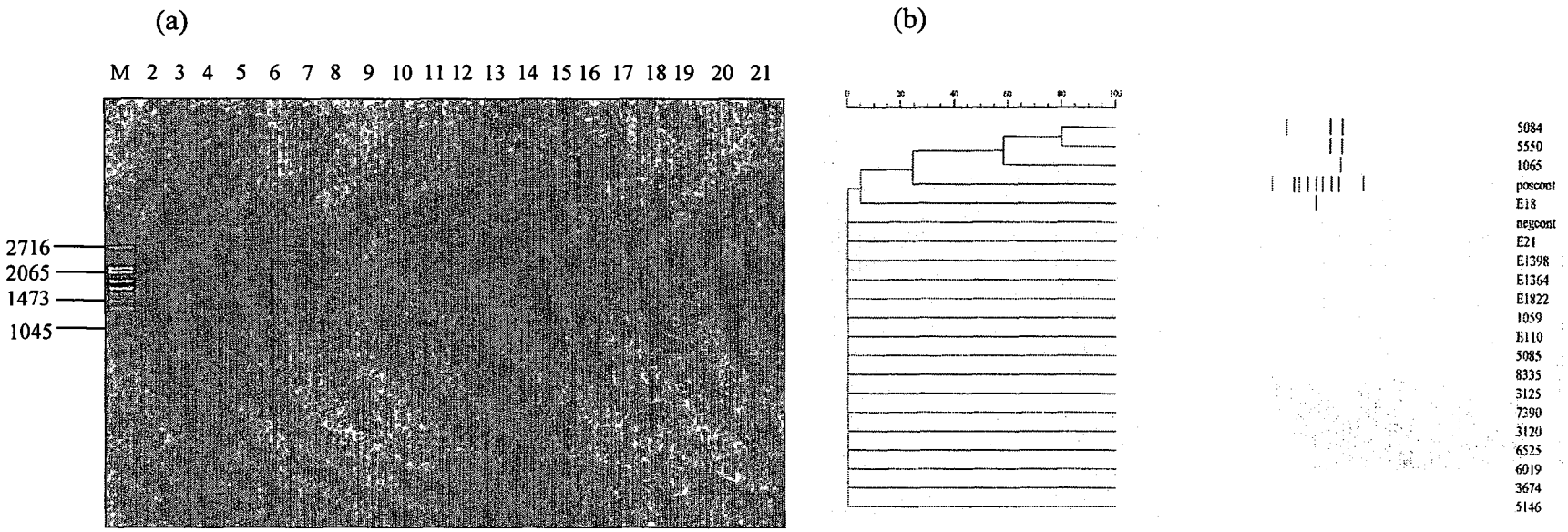

Figure A2 Agarose gel (a) and dendrogram (b) of 19 *Fusarium* isolates amplified by primer OPC 2. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

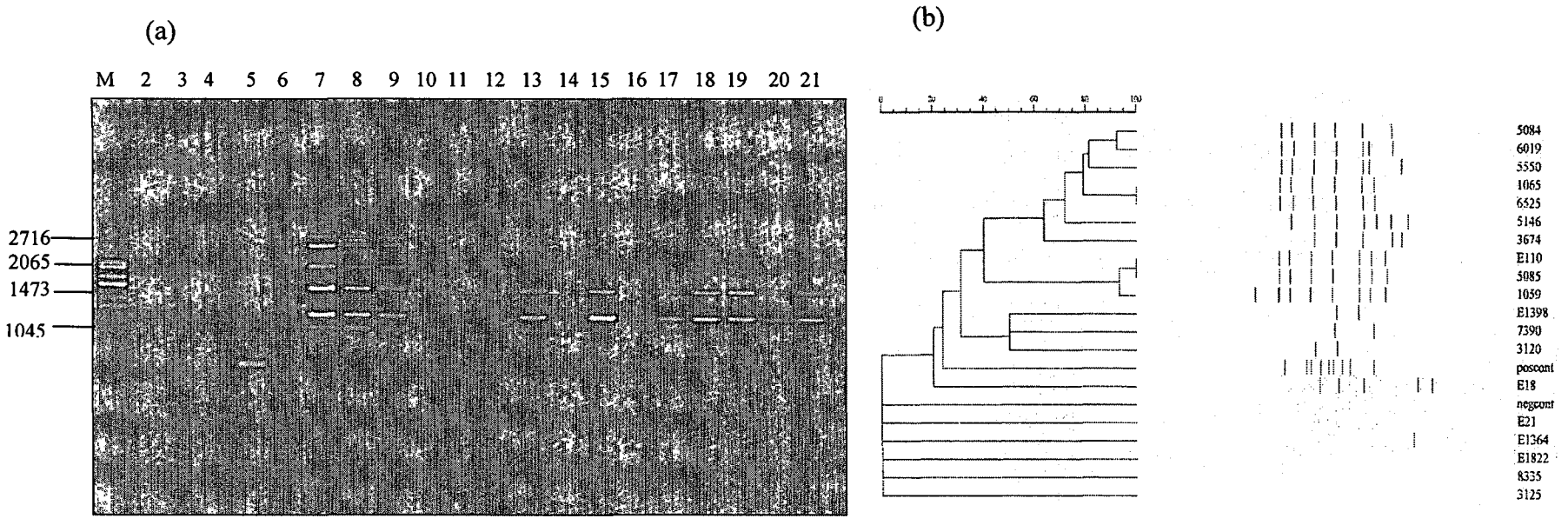

Figure A3 Agarose gel (a) and (b) dendrogram indicating banding patterns of the 19 *Fusarium* isolates amplified by primer OPC 3. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

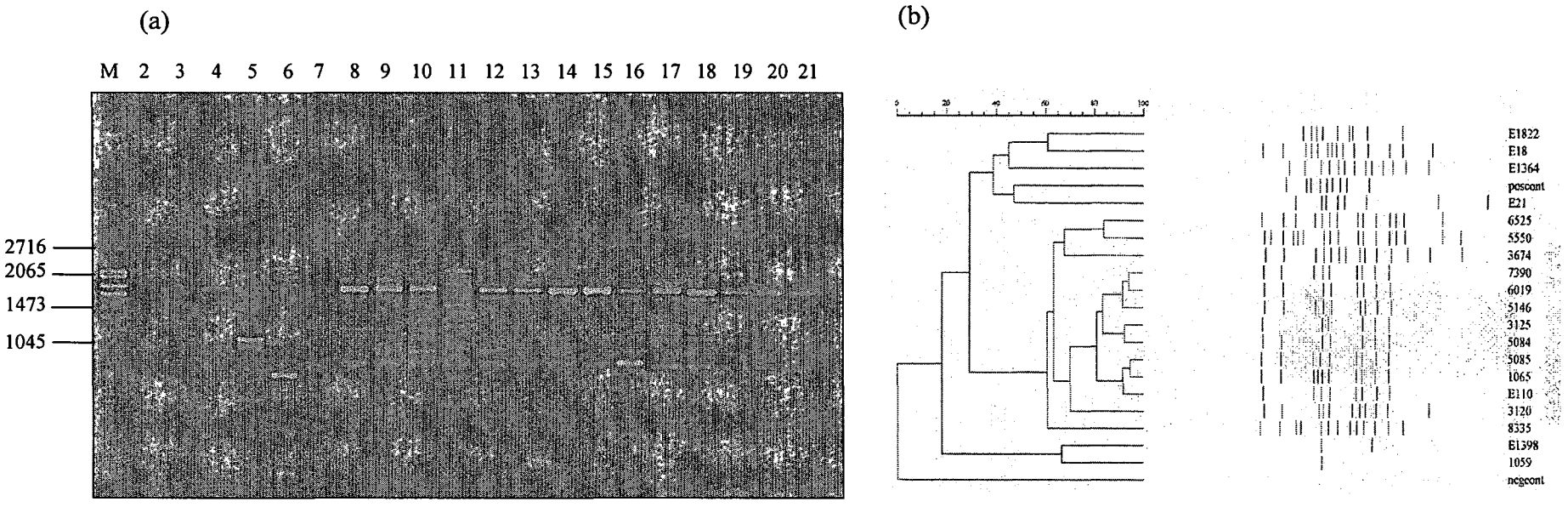

Figure A4 (a) Agarose gel and (b) dendrogram indicating banding patterns of the 19 *Fusarium* isolates amplified by primer OPC 4. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

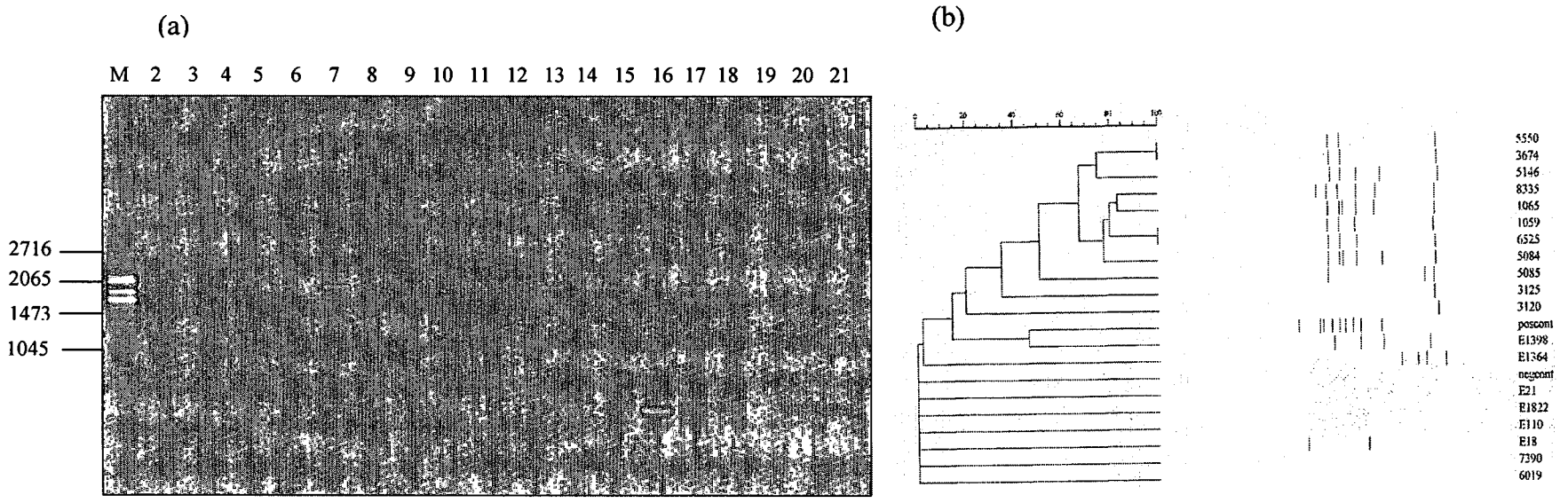

Figure A5 (a) Agarose gel and (b) dendrogram indicating banding patterns of the 19 *Fusarium* isolates amplified by primer OPC 6. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

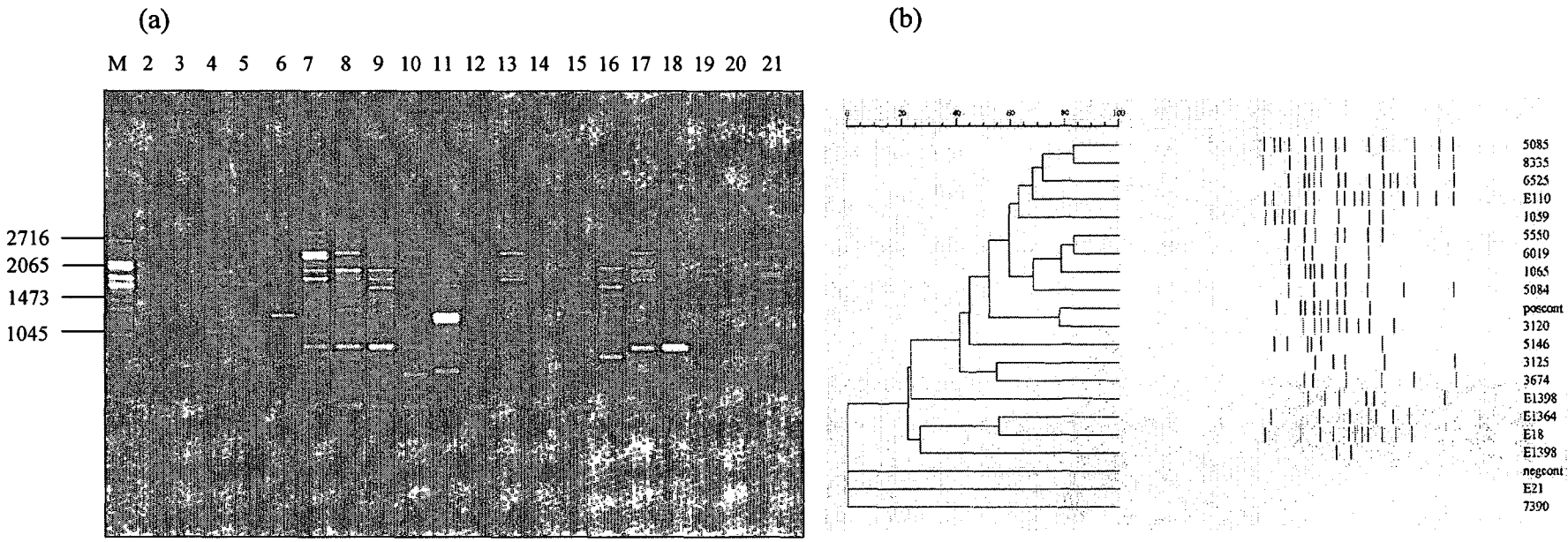

Figure A6 (a) Agarose gel and (b) dendrogram indicating banding patterns of the 19 *Fusarium* isolates amplified by primer OPC 7. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

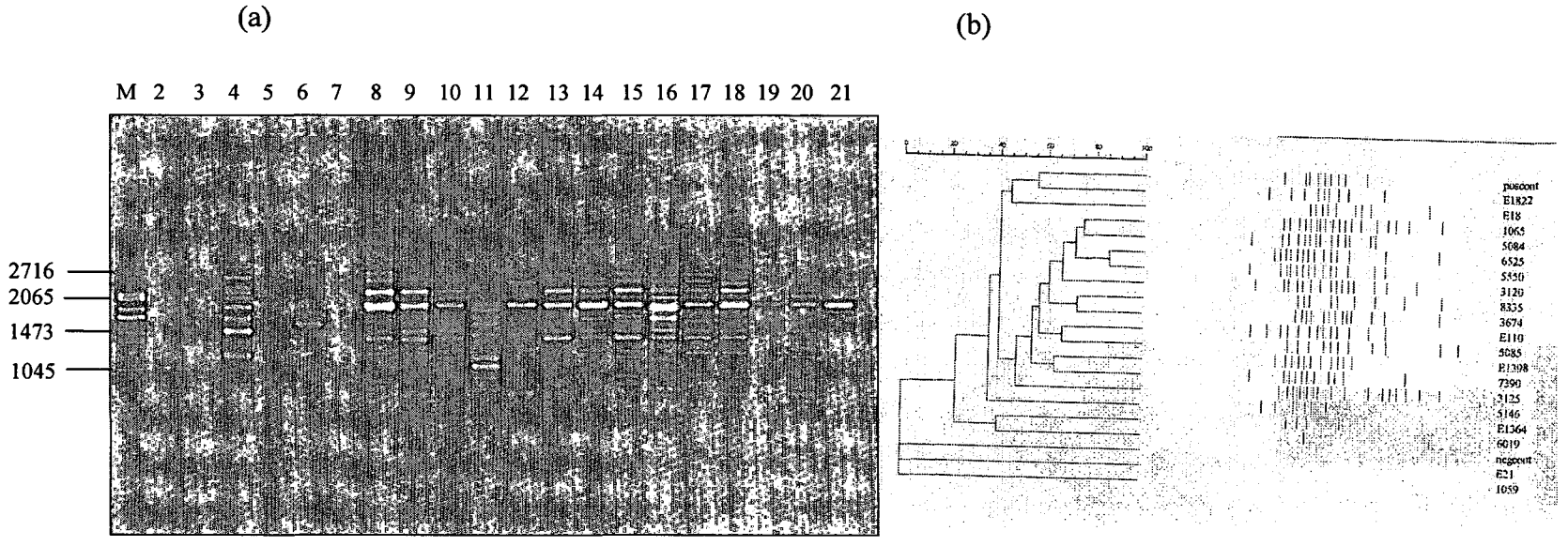

Figure A7 (a) Agarose gel and (b) dendrogram indicating banding patterns of the 19 *Fusarium* isolates amplified by primer OPC 8. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

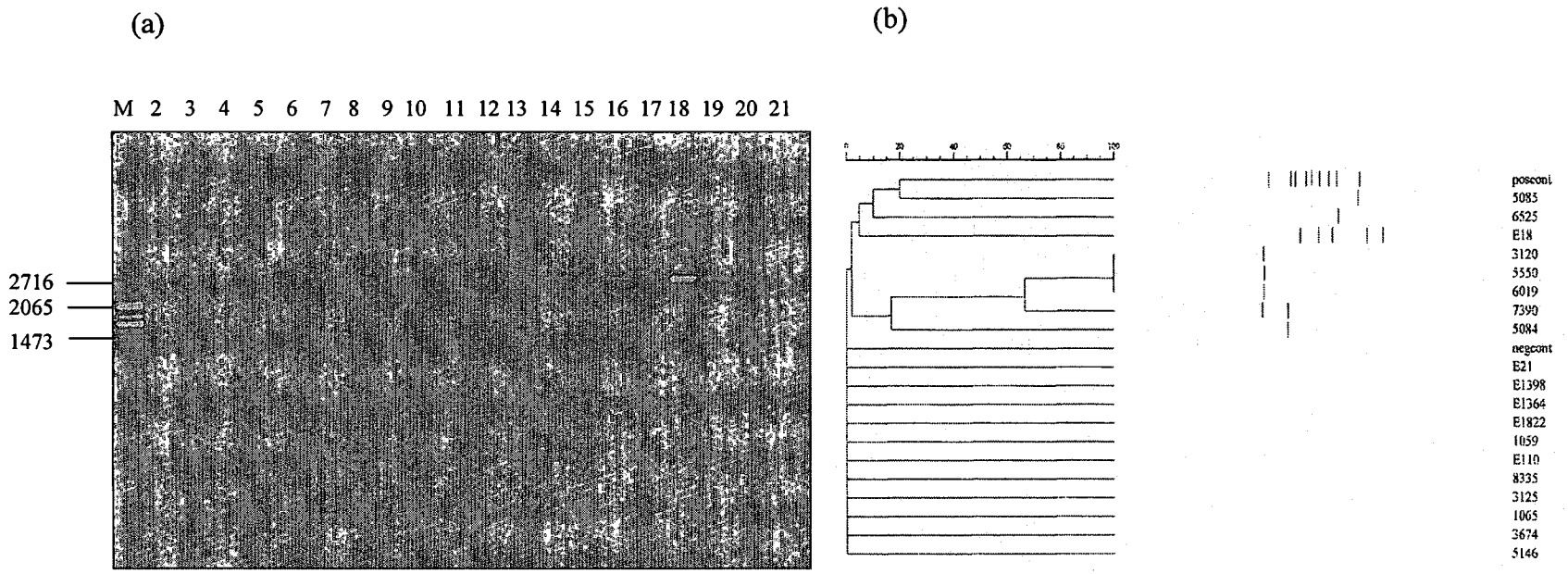

Figure A8 (a) Agarose gel and (b) dendrogram indicating banding patterns of the 19 *Fusarium* isolates amplified by primer UBC 229. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

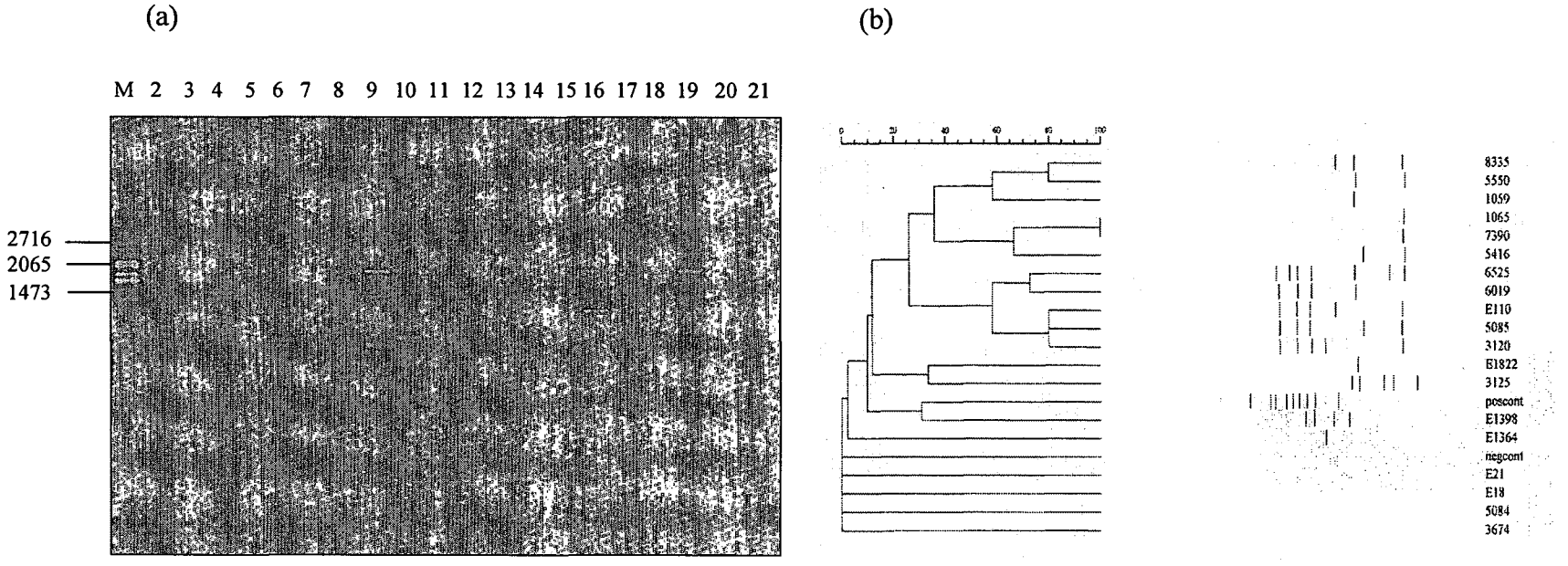

Figure A9 (a) Agarose gel and (b) dendrogram indicating banding patterns of the 19 *Fusarium* isolates amplified by primer UBC 230. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

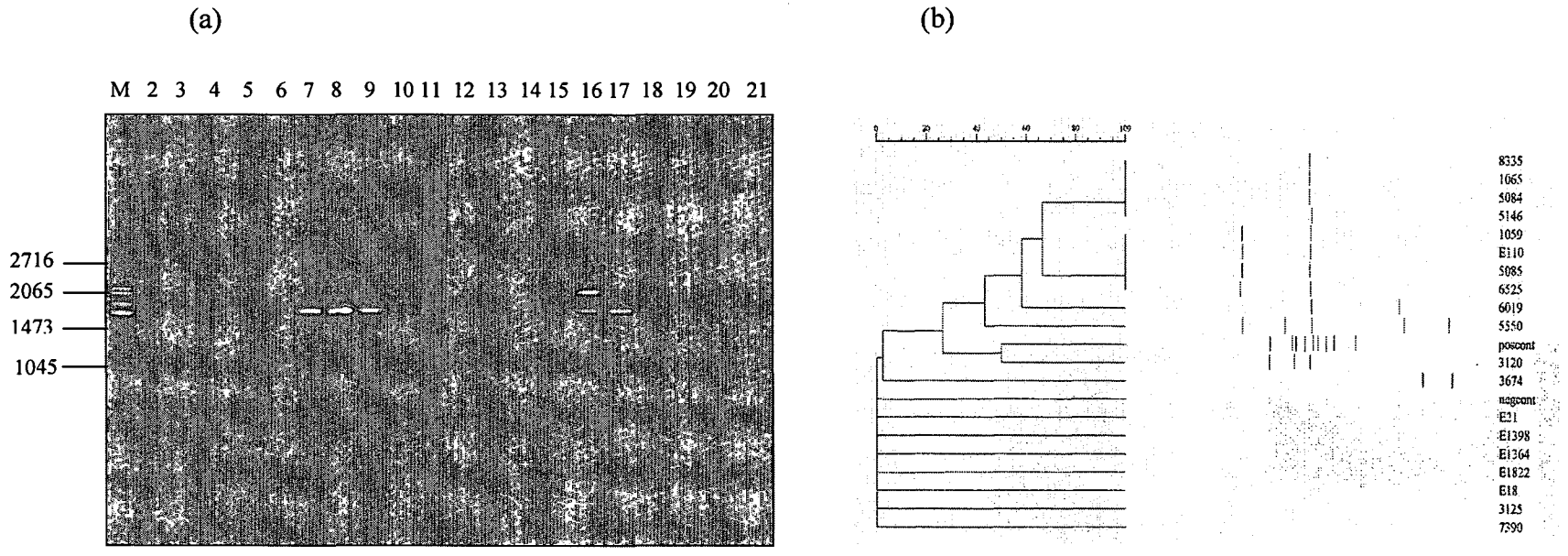

Figure A10 (a) Agarose gel and (b) dendrogram indicating banding patterns of the 19 *Fusarium* isolates amplified by primer UBC 246. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

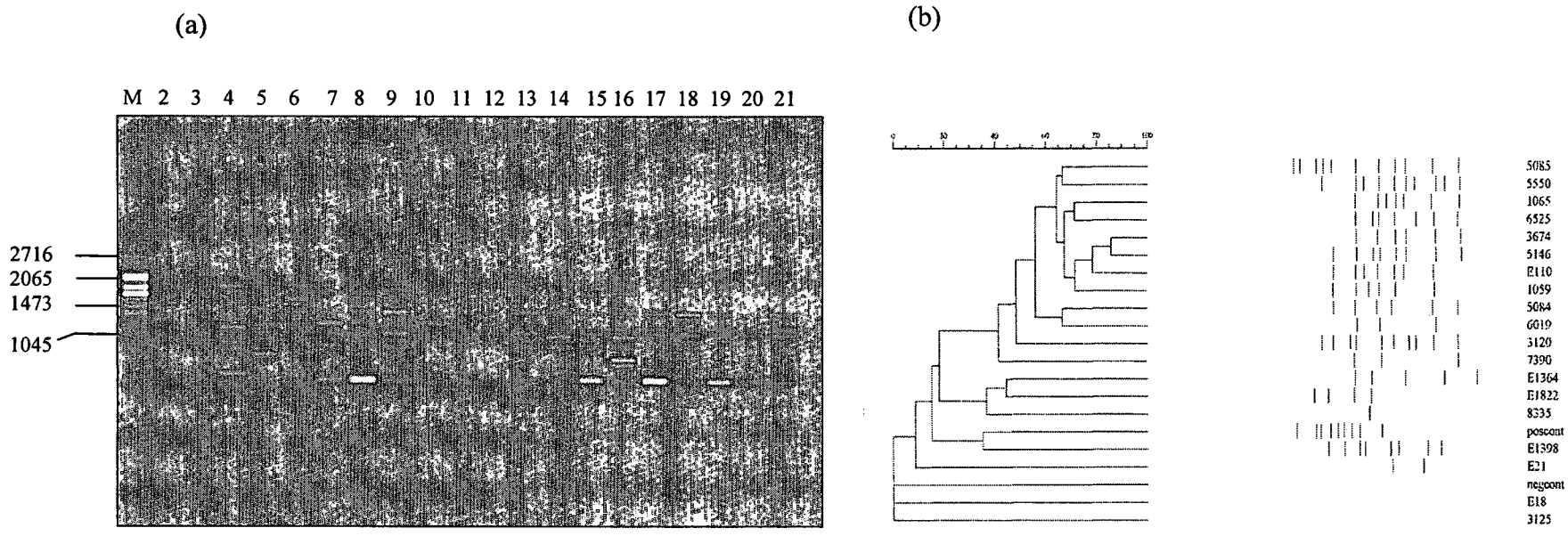

Figure A11 (a) Agarose gel and (b) dendrogram indicating banding patterns of the 19 *Fusarium* isolates amplified by primer UBC 285. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

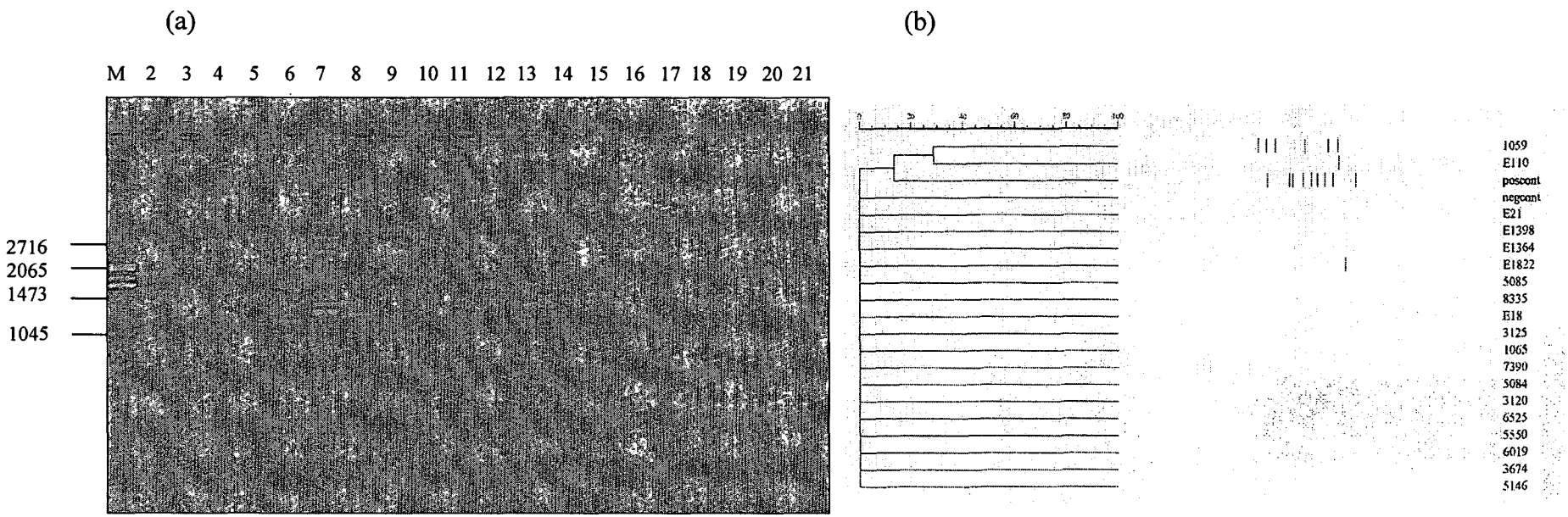

Figure A12 (a) Agarose gel and (b) dendrogram indicating banding patterns of the 19 *Fusarium* isolates amplified by primer UBC 286. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

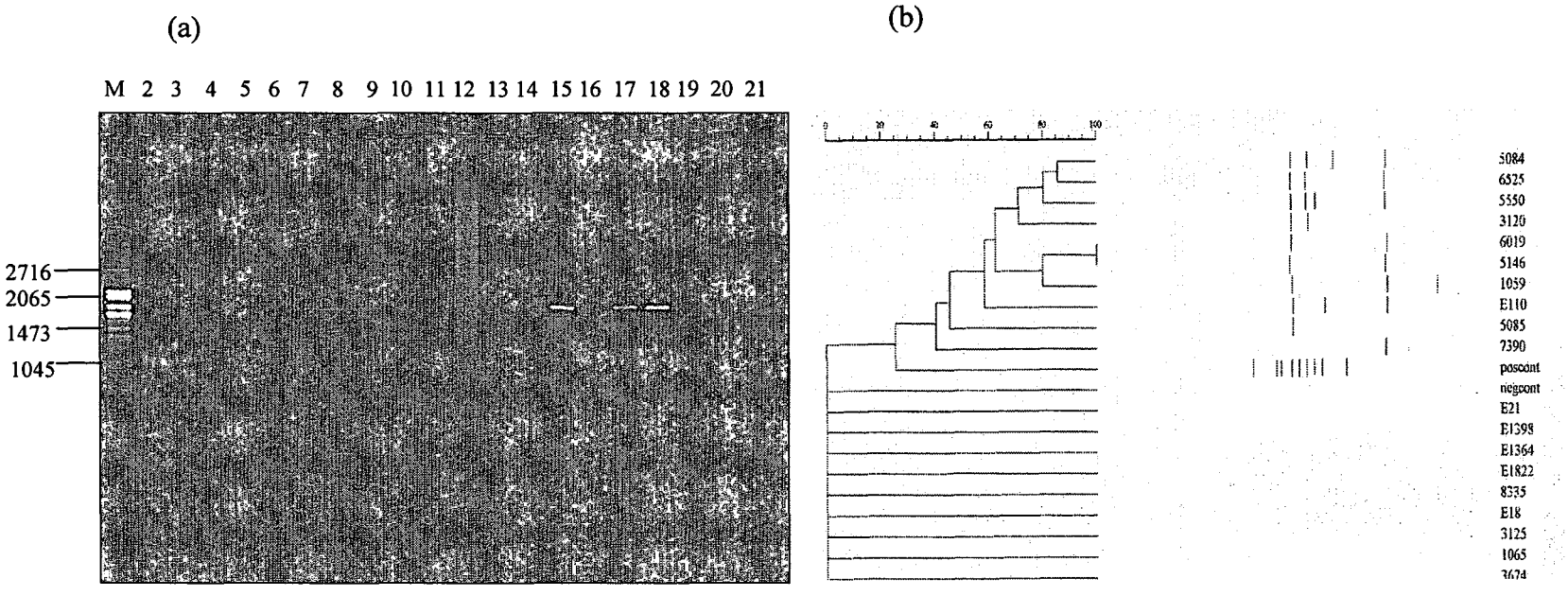

Figure A13 (a) Agarose gel and (b) dendrogram indicating banding patterns of the 19 *Fusarium* isolates amplified by primer UBC 295. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.

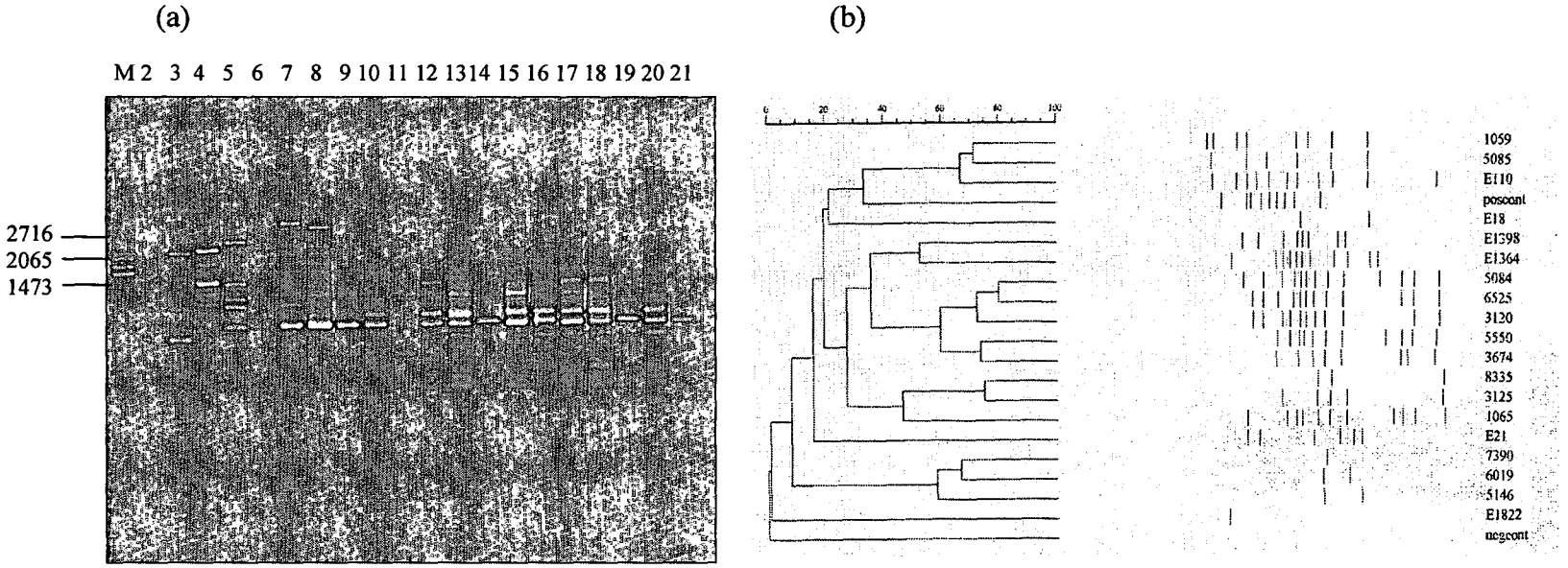

Figure A14 a) Agarose gel and (b) dendrogram indicating banding patterns of the 19 *Fusarium* isolates amplified by primer UBC 300. Lane 1 represents the marker and positive control, lane 2 is the negative control omitting DNA, lanes 3-6 represents DNA from different *Fusarium* species, i.e. *F. dimerum* E21, *F. solani* E1398, *F. chlamydosporum* E1364 and *F. semitectum* E1822. Lanes 7-21 represents *F. verticillioides* strains, i.e. ppri 1059, E110, ppri 5085, M8335, E18, M3125, ppri 1065, M7390, ppri 5084, M3120, ppri 6525, M5550, ppri 6019, M3674 and ppri 5146.