MOLECULAR APPROACHES TO THE STUDY OF TRICHOTHECENE PRODUCTION IN *FUSARIUM* SPECIES

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Submitted to the

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in

Biotechnology

by

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July 2008

Dedicated
To My Dearest

Pappa and Amma

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DECLARATION

I hereby declare that the thesis entitled "**MOLECULAR APPROACHES TO THE STUDY OF TRICHOTHECENE PRODUCTION IN *FUSARIUM* SPECIES"** submitted to the **University of Mysore,** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY,** is the result of the research work carried out by me under the guidance of **Dr. ARUN CHANDRASHEKAR**, Scientist, Central Food Technological Research Institute, Mysore- 570 020, India, during the period 2003-2008.

I further declare that the results presented in this thesis have not been submitted for the award of any other degree.

Date: /07/08 Lincy Sara Varghese
Place: Mysore (Candidate)

Dr. ARUN CHANDRASHEKAR
Scientist
Department of Plant Cell Biotechnology

July, 2008

CERTIFICATE

This is to certify that the thesis entitled "**MOLECULAR APPROACHES TO THE STUDY OF TRICHOTHECENE PRODUCTION IN *FUSARIUM* SPECIES"** submitted by **Ms. Lincy Sara Varghese** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY** to the **UNIVERSITY OF MYSORE** is the result of research work carried out by her under my guidance in the Department of Plant Cell Biotechnology, CFTRI during the period 2003-2008.

ARUN CHANDRASHEKAR
(Research Guide)

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Lincy Sara Varghese

List of Abbreviations

%	- Percent
°C	- Degree celsius
µg	- Micro gram
AFLP	- Amplified fragment length polymorphism
ALP	- Alkaline phosphatase
APS	- Ammonium persulfate
ATA	- Alimentary toxic aleukia
BCIP/NBT	- 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium
BLA	- Banana leaf agar
BLAST	- Basic Local Alignment Search Tool
bp	- Base pairs
BSA	- Bovine serum albumin
cfu	- Colony forming units
cm	- Centi metre
DAS	- Diacetoxyscirpenol
DMAP	- 4-Dimethylaminopyridine
DMF	- N, N'-dimethylformamide
DMSO	- Dimethyl sulphoxide
dNTP	- Deoxy-nucleotide tri phosphate
DON	- Deoxynivalenol
DTT	- Dithiothreitol
EDTA	- Ethylene diamine tetra acetic acid
EF-1α	- Translation elongation factor-1α
ELISA	- Enzyme linked immuno sorbent assay
eV	- Electron volts
FHB	- Fusarium head blight
FPP	- Farnesyl pyrophosphate
g	- Gram
GC	- Gas chromatography
MS	- Mass spectrometry
h	- Hours
HPLC	- High performance liquid chromatography
HT-2	- Hydroxy T-2 toxin
ICRISAT	- International Crop Research Institute for Semi Arid Tropics
Ig	- Immunoglobulin
IGS	- Inter genic spacers
IPTG	- Isopropyl-β-D- thiogalactopyranoside
ISSR	- Inter simple sequence repeats
ITS	- Internal transcribed spacer
kb	- Kilo base
kDa	- Kilo Dalton
kg	- Kilogram
l	- Litre
M	- Molar
mA	- Milli ampere

*MEGA*3.1	- Molecular Evolutionary Genetics Analyis 3.1
mg	- Milli gram
min	- Minute
ml	- Milli litre
mm	- Milli metre
mM	- Milli molar
mRNA	- Messenger RNA
N	- Normal
NC	- Nitrocellulose
NCBI	- National Centre for Biotechnology Information
NCIM	- National Collection of Industrial Micro-organisms
NIV	- Nivalenol
NJ	- Neighbour Joining
nm	- Nano metre
OD	- Optimal density
PAGE	- Polyacrylamide gel electrophoresis
PBS	- Phosphate buffered saline
PCA	- Plate count agar
PCR	- Polymerase chain reaction
PDA	- Potato dextrose agar
PEG	- Polyethylene glycol
PFA	- Prevention of Food and Adulteration Act
PMSF	- Phenyl Methyl Sulfonyl Fluoride
p-NPP	- *para*-Nitro Phenyl Phosphate
ppm	- Parts per million
RAPD	- Random amplified polymorphic DNA
RFLP	- Restriction fragment length polymorphism
RIA	- Radioimmuno assay
rpm	- Rotations per minute
RT-PCR	- Reverse transcriptase Polymerase Chain Reaction
SDS	- Sodium dodecyl sulphate
sec	- Seconds
T-2	- T-2 toxin
TBS-T	- Tris Buffer Saline-Tween
TDI	- Tolerable daily intake
TEMED	- N,N,N',N'-Tetramethyl ethylene diamine
TLC	- Thin layer Chromatography
TS	- Trichodiene synthase
U	- Units
UV	- Ultra violet
V	- Volts
v/v	- volume/volume
w/v	- weight/volume
X-Gal	- 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZEN	- Zearalenone

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Synopsis

SYNOPSIS

Fusarium is a wide spread group of toxigenic and plant pathogenic filamentous fungi, with a record of devastating infections on many kinds of economically important plants. *Fusarium* is the causative agent of Fusarium Head Blight (FHB) or scab, ear, stem or stalk rot in economically important cereals, especially wheat and barley, dry rot in potatoes and crown rot in banana. FHB infection adversely affect the quality and marketability of grain by reducing yield, discoloring and shriveling of seeds and contamination of the grain with mycotoxins that eventually result in heavy losses of several billion dollars to wheat and barley farmers worldwide. The importance of these fungi and their toxins in human health cannot be underestimated.

Fusarium has been established as the causative of alimentary toxic aleukia (ATA). *Fusarium* secretes a vast array of toxins that include trichothecenes, fumonisins, zearalenone, beuvericin etc. Trichothecenes are sequiterpenoids in nature and are classified into 4 groups. Type A trichothecenes such as T-2, Hydroxy T-2 (HT-2) and Diacetoxyscirpenol (DAS) are produced by *F. sporotrichioides*, *F. poae, F. equiseti* and the type B trichothecenes such as deoxynivalenol (DON) synthesized by *F. culmorum*, *F. graminearum* and Nivalenol (NIV) by *F. graminearum*, *F. poae, F. equiseti*. Trichothecenes are significantly phytotoxic and paves the way for *Fusarial* colonization of the plant and is probably important in controlling the spread of other fungal strains. Trichothecenes are commonly reported as contaminants in breakfast cereals or cereal products and vegetables, as they are stable under normal cooking conditions. DON is the most prevalent among the trichothecenes in wheat and wheat based products, followed by the toxins such as NIV, DAS and T-2.

Chapter 1

Introduction and Literature Review

An introduction to the genus *Fusarium* is provided at the beginning of this chapter. Morphological characters, their differences between the species in relation to traditional classification system and recently developed molecular tools that are helpful in taxonomy of *Fusarium* are discussed. An account of the various toxins produced by different species of *Fusarium* is presented, detailing their chemical nature, pathology and toxicology. A detailed account of the classification, physico-

chemical properties, toxicology and mode of action of the trichothecenes has been provided. Gene organization involved in the multi-step biosynthetic pathway of trichothecenes has been focused upon. Regulatory elements involved in the synthesis of trichothecenes are described. Analytical techniques that are widely used in the detection of trichothecenes have been tabulated. Reports on the worldwide occurrence of trichothecenes in food and feed materials have been collated. Control strategies employed for prevention of *Fusarium* infection and trichothecene production is detailed.

The present study was formulated after survey of the literature and concerned itself with identification of trichothecene producers in Indian foods, their detection using PCR and novel antibody based methods. Finally some work was vested towards understanding of environmental and molecular factors that control trichothecene synthesis. The study concluded with an investigation of the trichodiene synthase promoter.

Chapter 2
Materials and Methods

A detailed account of the common chemicals used and the general protocols employed during the execution of the present investigation is provided in this chapter. Protocols used specifically are described in relevant chapters.

Chapter 3
Isolation, Screening and Characterization of Trichothecene Production in *Fusarium*

This chapter deals with the isolation of *Fusarium* from various food materials and the screening for their ability to produce trichothecene. A total of 167 isolates of *Fusarium* were isolated and purified from various food materials (69 % from sorghum, and the remaining from various other food commodities). The isolates were initially screened by thin layer chromatography (TLC) for their ability to produce trichothecenes like T-2 toxin, deoxynivalenol (DON), diacetoxyscirpenol (DAS) and nivalenol (NIV); 45 isolates were found to secrete at least one of the trichothecene toxins such as T-2, DON, DAS or NIV. Positive amplification of a *tri 5* gene fragment by PCR occurred only from DNA extracted from the producers of toxin. The toxin

positive isolates were divided into different morphology groups from which 13 isolates were selected for further analyses. Trichothecene analysis of the 13 isolates carried out using GC-MS resulted in the identification of six deoxynivalenol (DON) producers, three DON and fusarenon-X (Fus-X) producers and one DON and nivalenol (NIV) producer. One isolate each was identified as producers of NIV alone, or NIV along with Fus-X or diacetoxyscirpenol (DAS) toxins.

Variations were noted among the 13 isolates selected from different morphology groups in inter simple sequence repeats-PCR (ISSR-PCR). Identification of these isolates to the species level was carried out after detailed observation of spore morphology and comparison of sequence of the elongation factor-1α-PCR from these isolates with those reported in the FUSARIUM-ID v. 1.0 database. The isolates were identified as *F. proliferatum* (5), *F. nelsonii* (2), *F. equiseti* (1), *F. thapsinum* (1), *F. sacchari* (1), *F. brevicatenulatum* (1), *F.* oxysporum (1) and *F. globosum* (1).

Chapter 4
Detection of Trichothecenes in Market samples of Food and Feed

Fusarium produces fumonisins, trichothecenes, zearalenone, fusarenon X etc as major types of toxins which are encountered as contaminants in a variety of food and feed materials worldwide. Screening of food and feed samples collected from the local market for presence of trichothecenes is presented in this chapter. Different food and feed materials (n=40) collected from Mysore and Hyderabad markets (India) were subjected to total yeast and mold count. The food samples were then directly subjected to PCR for the detection of presence of toxigenic *Fusarium*. Amplification of *tri 5* gene was obtained in seven samples (six sorghum samples and one poultry feed) and the *Fusarium* strains isolated from them. These food samples were contaminated with trichothecenes as shown by TLC and HPLC. Of the different types of *Fusarium* toxins studied, T-2 and DAS were encountered in the positive samples. Contamination with T-2 toxin and DAS was observed in the six sorghum samples whereas only T-2 toxin was noticed in poultry feed. Trichothecene levels of these samples were estimated and the levels of T-2 and DAS varied from 0.012 to 0.13 mg/kg and 0.014 to 0.084 mg/kg, respectively.

Chapter 5
Antibody to the Tri 5 Protein

Work on the development of antibodies against the Tri 5 protein and the use of these antibodies for studies on trichothecegenic *Fusaria* are presented in this chapter. Part of the *tri 5* gene outside the intron region was cloned in pRSETA to obtain pRA-T5 and over-expressed in *E. coli* BL21. The protein was purified and used as immunogen to raise antibodies in poultry. The antibody reacted specifically in western blot with the protein expressed in recombinant *E. coli* bearing pRA-T5 and with a protein from trichothecene producing *Fusarium* species. ELISA using the antibody against the Tri 5 protein revealed a positive correlation between presence of the protein and the ability of the fungus to produce trichothecene. The use of antibody against Tri 5 for the detection of trichothecenes or trichothecegenic *Fusarium* is a novel approach and is being reported for the first time.

The effect of different carbohydrates as carbon source and varying concentration of glucose and peptone on production of T-2 and DON in relation to Tri 5 protein levels in *Fusarium* were investigated. Among the different carbohydrates tested, glucose (5 % w/v) supported higher production of DON as well as the Tri 5 protein. Higher concentration of glucose (10 % w/v) in the growth media induced greater secretion of DON while decreasing synthesis of the Tri 5 protein. Fungi grown in different concentrations of peptone secreted similar amounts of DON while the level of the Tri 5 was unaltered. T-2 was secreted to the largest extent from cells grown in the presence of sucrose and mannose. Peptone in the media did not influence secretion of T-2 whereas high glucose concentration supported the production of more amount of T-2.

Chapter 6
Analysis of Promoters of Genes Involved in Trichothecene Biosynthesis

The promoter sequence of the *tri 5* gene from our isolate ICR-PQ-12 (*F. nelsonii*) was cloned and sequenced. Sequences 1200 and 800 bp upstream from the start site of this promoter was ligated in fusion with GUS in pCAMBIA1304 and the constructs were introduced into the isolate ICR-PQ-12 (*F. nelsonii*) using *Agrobacterium* mediated transformation. The presence of GUS was observed in the transformants bearing both the promoter constructs while such activity was not detected in untransformed *Fusarium*. *In silico* analysis of promoter for the three key

genes *tri 5*, *tri 6* and *tri 10* involved in trichothecene biosynthesis from different *Fusarium* species revealed both inter and intra specific variation. Binding sites for transcriptional factors such as those involved in carbohydrate metabolism (MIG1 and MSN4) were predicted on the promoters of *tri 5*, *tri 6* and *tri 10*. The possibility of such genes existing in *Fusarium* was investigated by searching the *Fusarium graminearum* genome database (FDGB). Similarly, motifs specific for binding of NIT2, a protein specific for activation of nitrogen mediated genes was predicted and the presence of sequences similar to that of NIT2 in the *Fusarium graminearum* genome database (FGDB) identified.

Chapter 7.

Future Perspectives

Future prospects of the topic are briefly highlighted in this chapter.

Chapter 1.

Introduction and Literature Review

1.1 *Fusarium*

Fusarium is a group of anamorphic fungi, commonly found in the soil and sub-terranean plant parts, especially in the tropics, but also found in contrasting climates such as deserts and arctic regions. The genus *Fusarium* consists of both saprophytes and plant pathogens. Many a species have also been known to incite diseases directly or indirectly in animals and humans. *Fusarium* species produce a wide array of secondary metabolites that are associated with most of the plant diseases and many growth defects in animals and humans as well. Most of them have acute carcinogenic and mutagenic properties and there are allegations against the use of *Fusarium* metabolites as biological weapons during World War II.

1.2. Growth and Maintenance

A carbohydrate rich medium like potato dextrose agar (PDA) is used and is principally suitable for observation of gross morphological appearances and the colouration of the colonies. *Fusarium* species show their full diversity and colour on this medium because of its highly available carbohydrate content, with profuse aerial mycelial growth and sparse sporulation. Cultures growing on sugar rich media such as minimal, complete and PDA sporulate poorly; usually take more than one month and the conidia produced are often atypical (Nelson et al, 1983). Minimal media such as corn leaf agar or carnation leaf agar (CLA) and banana leaf agar (BLA) have been reported as better sporulation media (Matsushima, 1971; Fisher et al, 1982; Seifert, 1996; Leslie and Summerell, 2006). Macroconidia (spore produced by *Fusarium*) from cultures grown on these media often are highly variable and quite different from those observed on CLA or other nutrient-poor media (Leslie and Summerell, 2006). Macroconidia produced on the leaf pieces are significantly different in morphology when compared to those obtained from the agar surface. Those spores present in the sporodochia tend to be more representative and specific than those produced elsewhere in the culture. Microconidia (spore produced by *Fusarium*) with characteristic features are produced when grown on CLA. Formation of chains is an important species character; some species form chains on KCl agar, but not on CLA. Chlamydospores on the other hand form more readily on Spezieller Nährstoffarmer agar (SNA) than on CLA (Leslie and Summerell, 2006).

1.3. Morphological Characters

Morphological characters are by far and away the most traditionally used criteria for the identification of any fungal species. *Fusarium* produces extensive mycelia that are cottony in nature with tinges of pink, yellow and purple. Some species produce either macroconidia or microconidia as asexual reproductive structures whereas in some other species we can see both (Jay, 1987). Members of the genus *Fusarium* are variable in cultural characteristics because changes in the environment in which they grow can bring about morphological changes in both culture and conidia.

1.3.1. Conidial Characters

The anamorphic genus *Fusarium* is characterized by the production of septate, hyaline, delicately curved, elongate macroconidia, distinct microconidia and chlamydospores along with other secondary characters like mycelial growth and pigmentation (Moss and Thrane, 2004).

1.3.1.1. Macroconidia

Macroconidia is the single most important cultural character for the identification of a culture to *Fusarium* species. The most typical character of macroconidia is its shape, followed by their size and number of septa and finally the nature of apical and basal or foot cell (Leslie and Summerell, 2006).

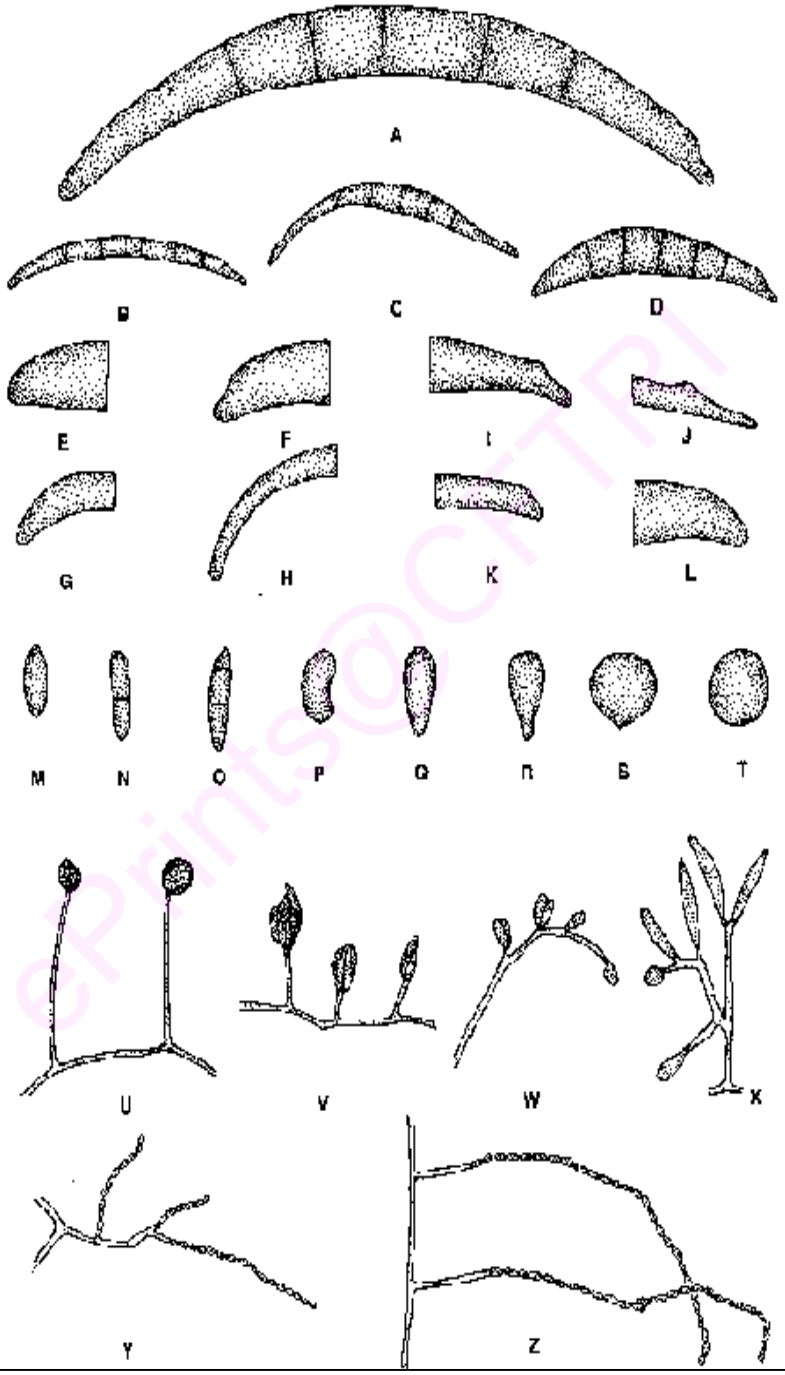
With respect to shape, most of the *Fusarium* species produces sickle shaped macroconidia that can be characterized into three types, (i) straight macroconidia which can appear almost needle like if they are thin *e.g. F. avenaceum*, (ii) macroconidia with dorsiventral curvature along all or a portion of the spore (these spores are almost of the same width along their entire length) *e.g. F. equiseti* and (iii) macroconidia in which the dorsal side is more curved than the ventral side (the cells in the middle of the spore are usually wider then those at the ends) *e.g. F. crookwellense* [(Fig. 1.1) (Leslie and Summerell, 2006)].

Macroconidia can be long (*F. armeniacum*) or short (*F. culmorum*), but in most cases spore size is a relatively constant character and major variations indicate improper culture conditions. Usually *Fusarium* macroconidia are 3-5 septate. The number of septa should be determined depending on the range and the average number of septa per spore.

Another important macroconidial character is the apical and basal cell forms. There are four general forms of apical cells: blunt *e.g. F. culmorum*, papillate *e.g. F. sambucinum*, hooked *e.g. F. lateritium* and tapering *e.g. F. equiseti* [(Fig. 1.1) (Leslie and Summerell, 2006)]. The apical cell length also can vary between species, but is usually constant within a species. The most important diagnostic features of apical cell are the degree of curvature, relative length and general form. The basal cell also has four general forms (Fig. 1.1): foot-shaped *e.g. F. crookwellense*, elongated foot-shaped *e.g. F. longipes*, distinctly notched e.g. *F. avenaceum* and barely notched e.g. *F. solani*. The basal cell is the least curved of all the cells in the macroconidia. The most important diagnostic characters are the relative length of the cell and its general form.

Fig. 1.1. Spore Morphology Characters Used in Making Species Diagnosis

(Adapted from Leslie and Summerell, 2006)



A-D: Macroconidial shapes, A: Typical *Fusarium* macroconidium, apical cell on left, basal cell on right; B: Slender, straight, almost needle like macroconidium, C: Macroconidium with dorsiventral curvature, D: Macroconidium with the dorsal side more curved than the ventral; E-H: Macroconidial apical cell shapes, E: Blunt, F: Papillate, G: Hooked, H: Tapering; I-L: Macroconidial basal cell shapes, I: Foot-shaped, J: Elongated foot shape, K: Distinctly notched, L: Barely notched; M-T: Microconidial spore shapes, M: Oval, N: Two-celled oval, O: Three-celled oval, P: Reniform, Q: Obovoid with a truncate base, R: Pyriform; S: Napiform, T: Globose; U-X: Phialide morphology, U and V: Monophialides; W and X: Polyphialides; Y-Z: Microconidial chains, Y: Short chains, Z: Long chains

Microconidia are not produced by all *Fusarium* species and so their presence itself is a potential diagnostic character in the *Fusarium* identification process. The important characters regarding microconidia are the microconidia themselves, the conidiogenous cells on which they are borne and their arrangement on or around the conidiogenous cell.

The first important character of microconidia is its size, which is not predetermined within a species. The general average size and the range for length of microconidia for a particular species should be determined. Microconidia can be aseptate or single septate and sometimes two septate.

Microconidia exist in seven basic shapes: (i) oval *e.g. F. sacchari*, (ii) reniform or kidney shaped *e.g. F. acuminatum*, (iii) obovoid with a truncate base e*.g. F. lactis*, (iv) pyriform or pear shaped *e.g. F. sporotrichioides*, (v) napiform or turnip like *e.g. F. beomiforme*, (vi) globose or spherical *e.g. F. globosum* and (vii) fusiform or spindle shaped *e.g. F. babinda* [(Fig. 1.1) (Leslie and Summerell, 2006)]. Some species of *Fusarium* can produce differently shaped microconidia *e.g. F. anthophilum* forms globose, pyriform and ovoid microconidia.

Another important diagnostic feature is the nature of conidiogenous cells which are of two basic types depending upon the number of openings per cell through which conidia are produced: monophialids with a single opening per cell *e.g. F. oxysporum* and polyphialids with more than one opening per cell *e.g. F. semitectum* [(Fig. 1.1) (Leslie and Summerell, 2006)]. The length of conidiogenous cells is also important. Two basic types, long (*e.g. F. solani*) and short (*e.g. F. oxysporum*) have been identified. Microconidia may be arranged singly (*e.g. F. semitectum*) or in chains, the length of the chain varyfrom few spores to very long "roller coaster"- like patterns on the phialides (*e.g. F. verticillioides*) [(Fig. 1.1) (Leslie and Summerell, 2006)]. Another important pattern is the arrangement of microconidia in false heads which are clumps of spores produced at the end of the phialides that superficially resemble spore heads (as seen in other fungi like *Aspergillus*) *e.g. F. solani, F. oxysporum.*

1.3.1.3. Chlamydospores

Chlamydospores are verrucose (rough) or smooth-walled structures, produced in single *e.g. F. solani*, double or paired *e.g. F. compactum*, clumps *e.g. F. scirpi* or chains *e.g. F. compactum*. Chlamydospores are produced rarely and take longer time (more than 6 weeks) when compared to macro- or microconidia. They are usually found in the aerial mycelia or embedded on the agar surface and is another important criterion used in the identification of species of *Fusarium*.

1.3.2. Other Important Characters

The characters discussed above are universally found in almost all *Fusarium* species. There are some other characters which are restricted to only few species of *Fusarium* and which serve as important delimitating factor in their identification.

Mesoconidia are the type of conidia having fusoid shape with up to 3-4 septa. They are typically produced by some species of *Fusarium*, particularly from polyphialids, in their aerial mycelium and they are not produced in sporodochia. Mesoconidia resemble macroconidia in their shape, but are smaller and lack the notched or foot shaped basal cell of macroconidia. Producers of mesoconidia include *F. camptoceras*, *F. chlamydosporum*, *F. semitectum*, *F. sporotrichioides*, *F. polyphialidicum*, and some species of *F. avenaceum* and *F. subglutinans* (Leslie and Summerell, 2006).

Teleomorphs are the unusual sexual stages seen in *Fusarium*, characterized by the development of sexual structures like ascus and ascospore maturation. These sexual stages are not commonly found in field conditions, except for *F. graminearum* (Broadhurst and Johnston, 1994; Summerell et al, 2001); though in other species they are made to generate under controlled laboratory conditions. Their morphology is difficult to make out but their presence may be a definitive species character (Leslie and Summerell, 2006).

Other characters like circinate (coiled) hyphae in the case of *F. circinatum*, formation of sclerotia (a compact mass of hardened mycelium stored with reserve food material) like structures etc are some other relevant characters which help in the primary identification process.

1.3.3. Secondary Characters

The most important and diagnostically potential secondary character is pigmentation (Leslie and Summerell, 2006). The different *Fusarium* species produce colours ranging from yellow to orange to carmine red (Joffe, 1974). The pattern of pigmentation can be easily assessed on PDA and a 12:12 h light:dark cycle is usually preferred. Pigments produced by these fungi may be sensitive to light or pH, may be diffusible or non diffusible into the growth media and most of the evaluations are carried out one week after incubation. The yellow pigments produced by cultures of *F. thapsinum* are highly characteristic and is an important diagnostic feature in its identification (Leslie and Summerell, 2006).

Another important character is the growth rate of the species, usually measured as colony diameter from PDA plates incubated with single spore culture and incubated at 25 or 30 ºC for 3 days. There are slow growing species like *F. lateritium*, *F. merismoides* and fast growing species like *F. culmorum*, *F. graminearum* etc (Leslie and Summerell, 2006). These characters, if most properly analyzed also, may not be clear and so are not usually preferred for identification of species.

Secondary metabolites and mycotoxins are also characteristic features which may impart a particular odor to the culture and serve as specific secondary character. The chemical background of the metabolites or mycotoxins can be used to primarily group the fungi which can further be analyzed to finally assign the fungi to a particular species. *Fusarium* is known to produce many toxins which can be effectively used for their specific identification.

1.4. Taxonomy of *Fusarium*

1.4.1. Conventional Taxonomic Identification Based on Morphology

Conventional taxonomic identification of filamentous fungi is based on morphological characteristics including colony colour, shape and size of sexual and asexual spores and spore-bearing structures, ability to produce various compounds like pigments, toxins etc or utilization of specific compounds like carbon, nitrogen etc (Leslie and Summerell, 2006). Taxonomy of *Fusarium* has been a matter of debate since 1930s (Nelson et al, 1994) due to the lack of clear morphological characters separating species

The *Fusarium* taxonomists have been categorized into three groups, "splitters", "lumpers" and "moderates" (Nelson et al, 1994). Splitters included Wollenweber and Reinking (based on (i) the presence or absence of microconidia, (ii) the shape of the microconidia, (iii) the presence or absence of chlamydospores, (iv) the location of the chlamydospores (intercalary or terminal), (v) the shape of the macroconidia, and (vi) the shape of the basal or foot cells on the macroconidia), Raillo and Bilai (form of apical cell) and Joffe (followed Wollenweber and Reinking's system). Lumpers included Snyder and Hansen (morphology of macroconidia), Messiaen and Cassini and Matuo [(followed Snyder and Hansen's system) (Nelson et al, 1994]. Moderates included Gordon (compromise between Wollenweber and Reinking's and Snyder and Hansen's systems) and Booth [(modified Gordon's system, based on nature of conidiophores and conidiogenous cells) (Nelson et al, 1994)].

According to Joffe (1974) the different species of *Fusarium* can be classified under 13 different sections depending on their morphological characters.

1. **Sporotrichiella Wr. em. Joffe**: Basis of classification in this section are (i) shape of microconidia, whether lemon or pear shaped, globose, ellipsoid, or elongate, dispersed in aerial mycelium, or formed in false heads; (ii) relative frequency of micro- and macroconidia (iii) sparse, small, oblong, narrowly fusoid to falcate, and pedicellate macroconidia formed in aerial mycelium or in sporodochia. (iv) intercalary or terminal chlamydospores, in chains or knots, and occasionally having plectenchymatous sclerotia. Perithecial states absent. Cultures white, yellow, carmine, red to purple. *e.g*. *F*. *poae*, *F*. *sporotrichioides*.
2. **Discolor Wr.**: Cultures white rose, peach, greyish, rose, red to brown. Microconidia absent. Macroconidia thick walled, present in aerial mycelium, sporodochia and pionnotes, either broad, falcate with short apical cell and well-developed foot-cell or spindle- or sickle-shaped, with elongated gradually narrowing apical cell, well marked basal cell, typically 5-septate. Chlamydospores intercalary or sometimes terminal, often in knots and chains. Sclerotia purple blue, brown to dark. Stroma yellow. *e.g*. *F*. *culmorum*, *F*. *graminearum, F*. *heterosporum, F*. *sambucinum, F*. *tumidum*.

3. **Martiella Wr. em. Joffe and Palti**: Cultures white, cream, orange-blue to brown. Microconidia abundant, oval or oblong, hyaline. Macroconidia in aerial mycelium, sporodochia or pionnotes, fusoid, cylindrical, curved or elongate, thickwalled with short round apical and foot cells. Chlamydospores globose, oval smooth or rough-walled, terminal and intercalary, single or in pairs, short chains or knots. *e.g. F. solani, F. javanicum*.
4. **Elegans Wr.**: Cultures white, light rose, orange, violet to purple. Mycelium felted, striate or floccose. Microconidia present abundantly in mycelium or false heads, variable, oval or elliptical, straight to curved, powdery. Macroconidia in aerial mycelia and sporodochia, sometimes in pionnotes, falcate, elongated subulate, spindle- or sickle-shaped, narrowing at both ends. Chlamydospores abundant, terminal and intercalary, smooth to rough walled. Perithecial states unknown. *e.g. F. oxysporum.*
5. **Liseola Wr.**: Cultures white-cream-brown, orange, violet. Microconidia in aerial mycelium, usually in long chains or small false heads, oval, fusiform oblong, rarely pyriform. Thin walled macroconidia, present in sporodochia, pionnotes and aerial mycelium, subulate, spindle shaped to cylindrical, straight or curved, with narrow apex and base cells, typically 3-septate. Chlamydospores absent. *e.g. F. moniliforme, F. proliferatum etc.*
6. **Gibbosum Wr. em. Joffe**: Culture white-pale pink, pale ochre, olive, carmine red. Microconidia absent or sparse, in aerial mycelium. Macroconidia in pionnotes and sporodochia, falcate, narrowing at both ends with elongated apical cell, and well developed pedicellate foot cell, dorsiventral, parabolic or hyperbolic, typically with 5, rarely 3-septate. Chlamydospores intercalary, abundant, smooth or rough-walled, single, or in chains and knots, yellow-brown. *e.g. F. equiseti.*
7. **Arachnittes Wr.**: Cultures white, rose-pale, yellow to brown. Macroconidia in aerial mycelium, rarely in sporodochia, curved apedicellate. Chlamydospores and sclerotia absent. *e.g. F. nivale, F. larvarum.*

8. **Eupionnotes Wr.**: Cultures pale, yellow, orange, yellow-brown, rose to green, growing slowly on PDA. Aerial mycelium usually sparse, slimy. Macroconidia in pionnotes, rarely in sporodochia, sublunate, cylindrical or spindle shaped, falcate, elongate or curved, apedicellate, sometimes pedicellate. Chlamydospores and sclerotia present or absent. Perithecial states present in some of the species. *e.g. F. dimerum*, *F. epispharia, F. tabacinum, F. merismoides, F. buxicola.*
9. **Lateritium Wr.**: Cultures white, rose, yellow, orange, carmine-red, violet to dark blue. Microconidia oval, elliptical, sparse or absent. Macroconidia elongated, cylindrical, straight, or slightly curved, with beaked apical cell and pedicellate base, formed in aerial mycelium or sporodochia, rarely in pionnotes, Chlamydospores intercalary, sparse in mycelium or macroconidia. *e.g. F. lateritium, F. stilboides, F. xylarioides*.
10. **Arthrosporiella Wr.**: Cultures white, peach, ochre-yellow to carmine red. Microconidia absent or sparse in aerial mycelium, elliptical, oval or pyriform. Macroconidia formed in aerial mycelium, in sporodochia, rarely in pionnotes, falcate, lanceolate, with wedge-shaped pedicellate basal cell and with narrow apex, typically 3-5 septate. Chlamydospores intercalary, single, sometimes in chains or knots, terminal chlamydospores are present or absent. *e.g. F. semitectum, F. camptoceras, F. concolor*.
11. **Roseum Wr.**: Cultures yellow, ochre, carmine, purple or red. Microconidia absent or sparse. Chlamydospores absent. Macroconidia in sporodochia, pionnotes or aerial mycelium, subulate, slender, almost filiform, falcate with thin walls, narrowing at both ends, pedicellate. White, yellow, purple to brown sclerotia. Stroma yellow, red. *e.g. F. avenaceum, F. arthrosporioides*.
12. **Macroconia Wr.**: Cultures white-rose, yellow-orange, slow-growing, slimy. Microconidia rare or absent. Macroconidia multi-septate, large, thick-walled, cylindrical, slightly curved, pedicellate, in sporodochia. Chlamydospores absent or occasionally in the macroconidia. *e.g. F. coccidicola, F. coccophilum, F. gigas*.
13. **Spicarioides Wr.**: Cultures rose, rose-red or purple. Microconidia in aerial mycelium in long chains or false heads, globose, oval. Macroconidia in

sporodochia or pionnotes, thick walled, slightly curved or straight, pedicellate, beaked at the tip with well marked foot cell. Chlamydospores absent. *e.g*. *F. decemcellulare*.

1.4.2. Molecular Taxonomy of *Fusarium*

Different molecular tools such as randomly amplified polymorphic DNA [(RAPD) (Yli-Mattila et al, 1996; Tran-Dinh et al, 1999; Fungaro et al, 2004)], amplified fragment length polymorphism [(AFLP) (Kroon et al, 2000; Majer et al, 1996; Schmidt et al, 2004)], restriction fragment length polymorphism [(RFLP) (Fernandez et al, 1994; Appel and Gordon, 1995; Talbot et al, 1996)], DNA sequences of inter-genic spacers [(IGS) (Appel and Gordon, 1995; Chilliali et al, 1998; Konstantinova and Yli-Mattila, 2004)], internal transcribed spacers [(ITS) (Chilliali et al, 1998; Klemsdal et al, 2000), β-tubulin (Mach et al, 2004; Yli-Mattila et al, 2004), translation elongation factor-1α [(EF-1 α) (O'Donnel et al, 1998a; O'Donnel et al, 1998b; Knusten et al, 2004; Geiser et al, 2004)] have been used for the differentiation and diagnosis of fungal strains.

1.4.2.1. Randomly Amplified Polymorphic DNA (RAPD)

RAPD is a PCR methodology where the primers (usually 10-12 bp in length) randomly bind to complementary sequences of the genomic DNA of a given organism and leads to the generation of consensus sequence patterns which serve as fingerprints for the organism (Dassanayake and Samaranayake, 2003). This technique works in such a way that nucleotide sequence variations due to insertions, additions or base substitutions, inversion of priming site, conformational changes in the template DNA etc in the PCR priming regions, especially at the 3' ends, prevent primer annealing and results in different sized PCR fragments that are highly specific for a particular species. RAPD assays have been effectively used for genome analysis of different bacteria and fungi (Sakallah et al, 1995; Tran-Dinh et al, 1999; Fungaro et al, 2004). Altomare et al (1996) have used RAPD assay along with isozyme analysis for studying the taxonomic relationships between *F. sporotrichioides*, *F. acuminatum* and *F. tricinctum*. RAPD-PCR has also been used for the analysis or identification of other *Fusarium* species such as *F. oxysporum*, *F. avenaceum*, *F. poae*, *F. solani* and *F. moniliforme* (Yli-Mattila et al, 1996; Kerńnyi et al, 1997; Hue et al, 1999; Paavanen et al, 1999). In spite of the advantages, the

need for fastidious PCR conditions and poor reproducibility of the results prevent the use of RAPD in fungal taxonomy.

1.4.2.2. Amplified Fragment Length Polymorphism (AFLP)

AFLP is a modified version of RAPD, strictly a PCR method, which is based on the amplification of adapter ligated restriction fragments obtained after double digestion of genomic DNA with two restriction enzymes, one a rare cutter and the second a frequent cutter. In the PCR, adapter specific primers carrying bases complementary to the restriction site sequences with specific 3' terminal bases are used. These primers will amplify limited set of restriction fragments from the genomic DNA digest, those having the specific complimentary base at the 3' end and extends into the fragment, producing large array of amplicons which constitute the AFLP fingerprint. This method has been widely used in the evaluation of genetic diversity of a variety of organisms (Janssen et al, 1996; Majer et al, 1996; Travis et al, 1996; Masiga et al, 2000). Leissner et al (1997) has used AFLP to discriminate between different isolates of *F. graminearum*. AFLP fingerprinting was used to distinguish *F. langsethiae* from *F. poae* and *F. sporotrichioides* by Schmidt et al (2004). AFLP has also been used for phylogenetic and taxonomic analyses of *Fusarium* species (Baayen et al, 2000; Abdel-Satar et al, 2003; Schmidt et al, 2004; Bogale et al, 2006). AFLP patterns may be used for discrimination of populations, mating type and even more closely related species, down to the clonal level (Chulze et al, 2000), but not suited for distinguishing taxa above 'species' level (Schmidt et al, 2004).

1.4.2.3. Inter-Genic Spacers (IGS)

Inter-genic spacers are regions separating nuclear ribosomal DNA (rDNA) repeat units which consist of highly conserved genes and more variable spacer regions (Taylor et al, 2000). The number of rDNA repeats varies among different species which results in variations in the length and restriction sites of IGS, leading to different RFLP patterns that act like fingerprints for the identification of a particular species (Hills and Dixon, 1991). IGS-RFLP has been used for the analysis of genetic variation within and between closely related species or populations (Mishra et al, 2006; Singh et al, 2006; Mbofung et al, 2007). RFLP analysis of IGS region have been effectively used for phylogenetic

analysis of closely related species of *Fusarium* such as *F. langsethiae*/*F. sporotrichioides* and *F. poae*/*F. kyushuense* which allowed clear differentiation between the two species of the former and the latter group (Konstantinova and Yli-Mattila, 2004; Yli-Mattila et al, 2004). Polymorphisms in the IGS rDNA region have been used for studying genetic diversity in *F. oxysporum* (Alves-Santos et al, 1999). The studies on aggressiveness versus molecular characteristics of *F. culmorum*, among isolates from Europe and America, using IGS-RFLP recorded little correlation of mycotoxin profile and aggressiveness of the fungus (Toth et al, 2004).

1.4.2.4. Internal Transcribed Spacers (ITS)

Internal transcribed spacers ITS1 and ITS2 are the spacer regions between the 5.8S-18S and 28S like rDNA repeat units. The ITS1 and ITS2 spacers undergo more variations even within closely related species and hence are widely used for identification process and also for studying the evolutionary events (O'Donnel, 1992). ITS-RFLPs have been widely used for phylogenetic analysis and identification of *Fusarium* (Young-Mi et al, 2000). Variations occurring in ITS1 and ITS2 sequences have been used to study the genetic relationship between different *Fusarium* species (Young-Mi et al, 2000). Sequence of the ITS2 region was useful in the identification of *F. solani* while that for *Fusarium* species in the Sporotrichiella section and its closely related species failed to separate *F. langsethiae* from its close relative *F. sporotrichioides* (Yli-Mattila et al, 2004). Turner et al (1998) have reported similar results wherein the use of ITS-RFLP failed to differentiate between *F. avenaceum* and *F. tricinctum*.

1.4.2.5. β-tubulin

β-tubulin gene sequences have been widely used for phylogenetic investigations in various fungi (Samson et al, 2004; Amrani and Corio-Costet, 2006). The phylogenetic investigations of Yli-Mattila et al (2004) based on β-tubulin have clearly distinguished between the species *F. sporotrichioides*, *F. langsethiae*, *F. poae* and *F. kyushuense*, but it failed to resolve phylogenetic relationships between *F. langsethiae* and *F. sporotrichioides.* Schmidt et al (2004) have also used DNA sequences of β-tubulin along with other marker genes for the taxonomic study of *F. langsethiae*, *F. poae* and *F.*

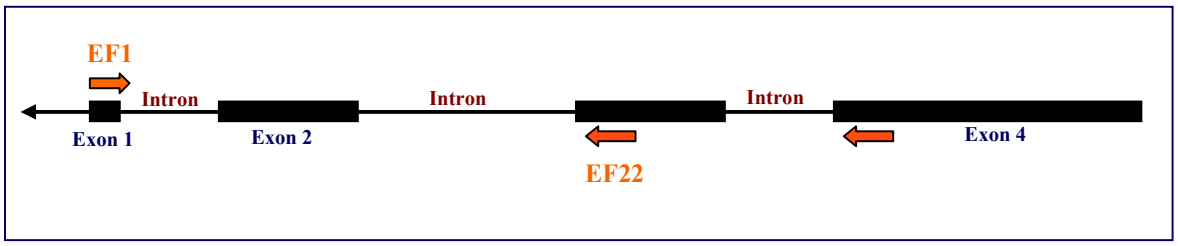
sporotrichioides. β-tubulin has been used in the molecular phylogenetic analyses of *F. xylarioides* (Geiser et al, 2005).

1.4.2.6. Translation Elongation Factor-1α (EF-1α)

EF-1α coding for an essential part of the protein translation machinery has been widely used as a phylogenetic marker especially for *Fusarium* identification due to the following reasons, (i) they are highly informative at the species level of *Fusarium*, (ii) non-orthologous copies of the gene have not been detected in the genus, (iii) universal primers have been designed that work across the phylogenetic breadth of the genus (Geiser et al, 2004). The gene has been primarily utilized for studying the species and generic level differences in *Heliothus* moths by Cho et al (1995).

EF-1α gene consists of three introns spanned by four exons around which three primers EF1, EF2 and the nested primer EF22 have been designed by O'Donnel et al (1998b). The priming sites of the EF1 primers are depicted in Fig.1.2. The EF1-EF2 primer pair amplifies PCR fragments spanning the three introns, the sequences of which are proved to be highly informative at the sequence level across the different species of various filamentous fungi, including *Fusarium*. Knusten et al (2004) have used EF-1α gene sequences for the phylogenetic analysis of *F. poae*, *F. langsethiae* and *F. sporotrichioides*. The method has been successfully used in separating the two subgroups of *F. langsethiae* and the strains of *F. sporotrichioides*.

Fig. 1.2. Translation Elongation Factor-1α Gene showing the Priming Sites of EF1, EF2 and EF22 Oligonucleotides



Geiser et al (2004) have assembled the EF-1α sequences of 463 isolates of *Fusarium* and created FUSARIUM-ID v. 1.0 database which is accessible at http://fusarium.cbio.psu.edu. The database contains a BLAST search tool where the unknown sequence can be searched against the database for identification purposes (Geiser et al, 2004).

Other genes such as calmodulin, cellbiohydrolase-C and topoisomerase II has also been used for the identification of *Fusarium* (Hatsch et al, 2004; Mule et al, 2004).

1.5. Mycotoxins

Mycotoxins are the secondary metabolites produced by a large number of molds and consist of relatively low molecular weight, non-volatile compounds with diverse chemical structures ranging from simple moniliformin to complex polypeptides. Most of them have carcinogenic and mutagenic properties and are toxic to animals, crops and humans (Jay, 1987). Few of the commonly occurring mycotoxins are aflatoxins (*Aspergillus flavus*), tenuazoic acid (*Alternaria alternata*), citrinin (*Penicillium citrinum*), ochratoxins (*Aspergillus* sp., *Penicillium* sp.), patulin (*Penicillium expansum*), penicillic acid (*Penicillium* species), ergot alkaloids (*Claviceps purpuria*), sterigmatocystin [(*Aspergillus* sp., *Penicillium* sp.) (D'Mello and MacDonald, 1997)].

The genus *Fusarium* produce a number of toxins of varied chemical background; the important and commonly encountered ones are fumonisins, moniliformin, zearalenone, fusaric acid and the trichothecenes.

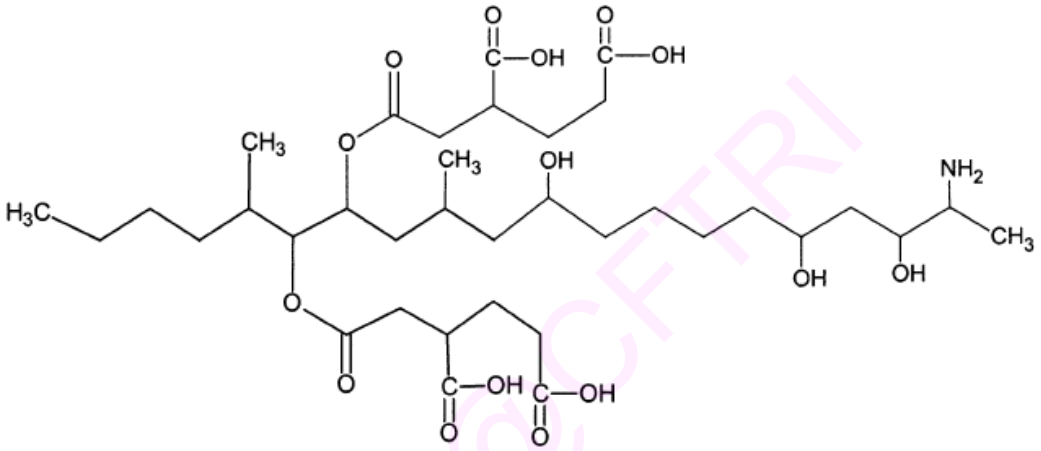
1.5.1. Fumonisins

Fumonisins, one of the relevant secondary metabolites produced by *Fusarium*, have a long-chain hydrocarbon unit (similar to that of sphingosine and sphinganine) which plays a significant role in their toxicity (Wang et al, 1992). The major producers of this toxin are *F. proliferatum* and *F. verticillioides*. Fumonisins are classified into three types, FB_1, FB_2 and FB_3 (Fig. 1.3). FB_1 is the most toxic of all fumonisins and the most predominant contaminant of small grain cereals especially maize, and other grains such as wheat, oats, rye etc (Dutton and Kinsey, 1995; Wang et al, 1995b; Fadl-Allah et al, 1997; Ritieni et al, 1997; Srobárová and Pavlová, 1997; Pancaldi and Alberti, 2001). FB_1 have been reported to promote tumor in rats (Gelderblom et al, 1988). Co-contamination

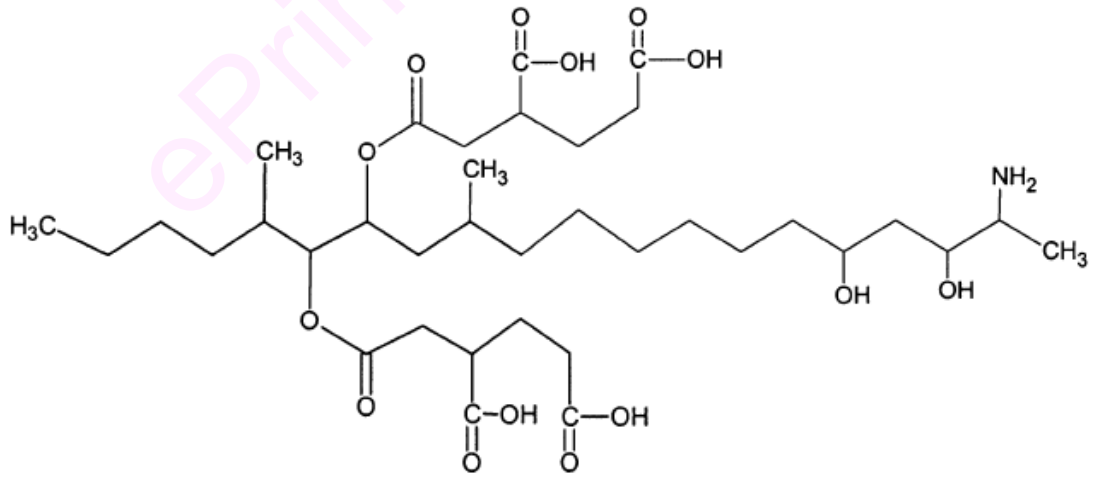
of maize with fumonisins and other *Fusarium* toxins such as trichothecenes, fusaproliferin and beauvericin has been reported (Wang et al, 1995a; Yamashita et al, 1995; Ritieni et al, 1997).

Fig. 1.3. Structure of Fumonisins (Adapted from Hussein and Brasel, 2001)

A. Fumonisin B_1



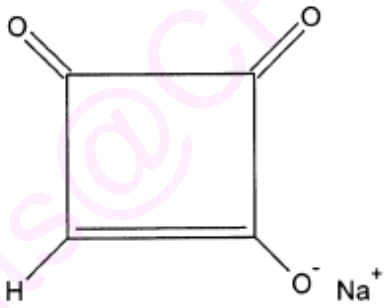
B. Fumonisin B_2



1.5.2. Moniliformin

The major producers of moniliformins are *F. avenaceum*, *F. tricinctum* and to a lesser extend by *F. subglutinans* (Bottalico and Perrone, 2002). The chemical structure of moniliformin is provided in Fig. 1.4. Moniliformins have cytotoxic activity, mainly attributed to the inhibition of pyruvate dehydrogenase, glutathione peroxidase and glutathione reductase (Gathercole et al, 1986; Chen et al, 1990). Moniliformins increase cardiac permeability in young rats and ducklings and may induce Keshan disease (a fatal cardiomyopathy endemic to certain rural areas of China) in humans (Zhang and Li, 1989).

Fig. 1.4. Structure of Moniliformin (Adapted from Hussein and Brasel, 2001)

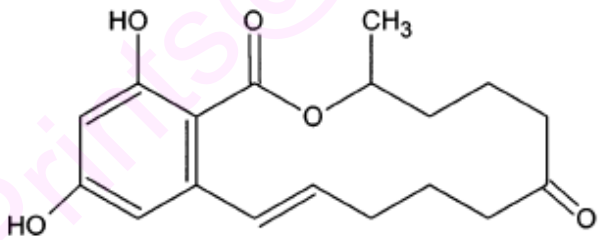


1.5.3. Zearalenone

Zearalenone (ZEN), previously known as F-2 toxins, is the group of toxins produced by all toxigenic and pathogenic species of *Fusarium*, mainly *F. graminearum*, *F. crookwellemse*, *F. culmorum*, and *F. semitectum* (El-Nezami et al, 2002). *F. graminearum* is mainly responsible for most of the estrogenic effects found in farm animals (Hussein and Brasel, 2001). These are the group of metabolites that have properties similar to the hormone estrogen. Chemically they are known as 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid μ-lactone [(Fig. 1.5) (Hussein and Brasel, 2001)]. Different derivatives of ZEN, including α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), monohydroxylated, dihydroxylated and formylated ZEN, have been isolated from cultures of *Fusarium* (El-Nezami et al, 2002). ZEN is less toxic than the

trichothecenes. It has an LD 50 value of 1-10 g/kg body weight of mice (Placinta et al, 1999). The estimated safe intake of ZEN for humans has been reported to be 0.05 µg/kg of body weight/day (Kuiper-Godman et al, 1987). ZEN and its metabolites act as growth stimulants (El-Nezami et al, 2002). Early onset of puberty in children associated with consumption of ZEN contaminated corn has been reported from Puerto Rico (Schoental, 1983). In mammals, levels as low as 1.5-3 mg of ZEN /kg of diet cause infertility, reduced milk production and hyper-estrogenism (Placinta et al, 1999). Incidence of contamination of food commodities with ZEN has been reported worldwide (Müller and Schwadorf, 1993; Adler et al, 1995; De Nijis et al, 1996; Vrabcheva et al, 1996; Gao and Yoshizawa, 1997; Srobárová and Pavlová, 1997). A common feature of ZEN is their co-occurrence with certain trichothecenes like DON and their derivatives, thus accounting for majority of the FHB infections in small grain cereals in Europe (Bottalico and Perrone, 2002).

Fig. 1.5. Structure of Zearalenone (Adapted from Hussein and Brasel, 2001)

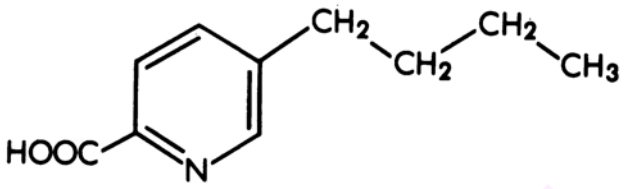


1.5.4. Fusaric Acid

Fusaric acid, chemically 5-butylpicolinic acid (Fig. 1.6), is a well known phytotoxin, produced by several species of *Fusarium*, particularly *F. oxysporum* (Bacon et al, 1996). The toxin was first reported from *F. heterosporum* by Yabuta et al (1934). Fusaric acid alone is mildly toxic to animals, but it elicits synergistic interactions with other mycotoxins and enhances their toxicity (Smith and Sousadias, 1993). In addition to its toxic effects on animals, fusaric acid has antibiotic, insecticidal, pharmacological and

phytotoxic activities (Burmeister et al, 1985). Fusaric acid has been reported from corn and sorghum samples and other food and feed products (Burmeister et al, 1985).

Fig. 1.6. Structure of Fusaric Acid (Adapted from Burmeister et al., 1985)



1.5.5. Trichothecenes

Trichothecenes are mycotoxins, named after the discovery of the antifungal compound 'trichothecin' from the fungus *Trichothecium roseum* in the year 1948 (Desjardins et al, 1993). They are the family of compounds belonging to the class of sequiterpenoids. Sesquiterpenoids are the class of terpenes that consists of three isoprene units and have the molecular formula $C_{15}H_{34}$ (courtesy to http://en.wikipedia.org/wiki/Diterpenes). *Fusarium*, the commonly occurring phytopathogenic fungi in monocoteledonous plants like wheat and barley and the etiological agent of FHB, are the major producers of the simple alcohols and short chain esters in this class, the type A (T-2, HT-2, DAS) and type B trichothecenes [(DON, NIV and their derivatives) (Rocha et al, 2005)].

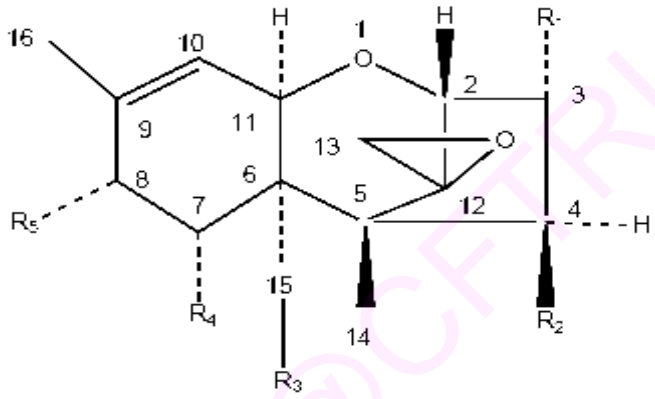
Trichothecenes are produced by several species of molds: *Fusarium*, *Myrothecium*, *Trichoderma*, *Trichothecium, Cladosporium, Verticimonosporium, Cephalosporium*, *Verticimonosporium*, *Stachybotrys* etc. (Ueno, 1989; Sweeney and Dobson, 1999). Certain plant species such as *Baccharis coridiofolia* and *Baccharis artemisioides* also produce macrocyclic trichothecenes (Rizzo et al, 1997).

1.5.5.1. Structure and Properties

Trichothecenes are produced as secondary metabolites by wide genera of fungi, though *Fusarium* appears to be the sole source of these toxins in agricultural products. More than 170 types of trichothecenes have been identified so far (Langseth and Rundberget, 1998).

Trichothecenes have molecular weight in the range of 200-500. They are distinguished by the presence of a trichothecene ring, having an olefinic bond at C-9, 10; and an epoxide group at C-12, 13 (Fig. 1.7).

Fig. 1.7. Basic Structural Unit of Trichothecenes (Adapted from Wannemacher and Weiner, 1997)



1.5.5.2. Classification of Trichothecenes (Joffe, 1986)

Trichothecenes are classified into 4 types based on their chemical nature (Table 1.1.) and fungal origin.

Type A: Include T-2 toxin, diacetoxyscirpenol (DAS), neosolaniol (NEO), HT-2 toxin. Produced by different species of *Fusarium*. They lack the ketone function at C-8 and contain an H, OH, or ester group at C-8.

Type B: Include nivalenol (NIV), diacetylnivalenol (DAN), deoxynivalenol (DON), fusarenone X (Fus-X). Produced by different species of *Fusarium.* It is characterized by a ketone function at position C-8.

Type C: Include crotocin. Produced by *Cephalosporium crotocinigenum*. It contains an oxirane ring at C-7/C-8, characterized by an epoxide function at C-7-8 position.

Type D: Include macrocyclic derivatives of verrucarins and verrucarol, roridins and satratoxin H. Produced by several fungi such as *Myrothecium, Stachybotrys,*

Cylindrocarpon, Verticimonosporium etc. They have an ester or ether bridge between C-5 and C-15.

Table 1.1. Specific Side Groups of Most Abundant Trichothecenes

(Adapted from Wannemacher and Weiner, 1997)

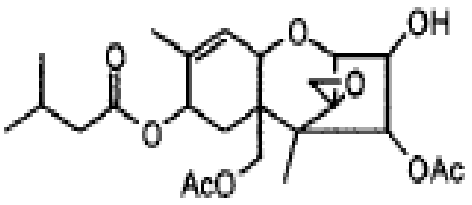
Trichothecene	R1	R2	R3	R4	R5
T-2	-OH	$-OCOCH_3$	$-OCOCH_3$	-	$-OCOC_3$
HT-2	-OH	-OH	$-OCOCH_3$	-H	$-OCOC_3CH(CH_3)_2$
DAS	-OH	$-OCOCH_3$	$-OCOCH_3$	-H	-H
Neosolaneol					
NIV	-OH	-OH	-OH	-OH	=O
DON	-OH	-H	-OH	-OH	=O
Fusarenon-X	-OH	$-OCOCH_3$	-OH	-OH	=O
Macrocyclic	-H	-	-O-R'-O-	-H	-H

Among the four groups of trichothecene toxins, *Fusarium* strains are known to produce only group A and B trichothecenes. Few of the commonly occurring trichothecenes of *Fusarium* origin are

1.5.5.2.1. T-2 toxin

Chemically it is 12,13-epoxytrichothec-9-ene-3,4,8,15-tetraol 4,15-diacetate 8-(3-methylbutanoate) [(molecular formula - $C_{24}H_{34}O_9$) (Fig. 1.8)] with a molecular weight of 466.58 (SCF 2001). T-2 toxin is rapidly metabolized to HT-2 toxin *in vivo*. There is limited or no evidence of the toxin being carcinogenic to humans or animals. The plasma half-life for T-2 toxin is less than 20 minutes (Wannemacher and Weiner, 1997).

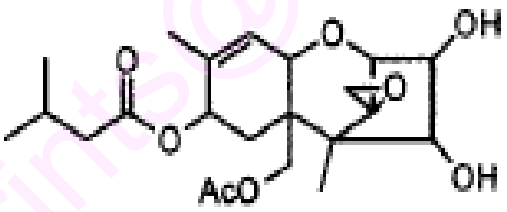
Fig. 1.8. Structure of T-2 Toxin (Adapted from SCF, 2001)



1.5.5.2.2. HT-2

Chemically it is 12,13-epoxytrichothec-9-α,4-β,8-α,15-tetraol-5-acetate-8-isovalerate [(molecular formula - $C_{22}H_{32}O_8$) (Fig. 1.9)] with a molecular weight of 424.54 (SCF 2001). HT-2 toxin is the major metabolite of T-2 toxin and is produced by the action of carboxyesterase on T-2 toxin (Johnsen et al, 1988). Clinical effects resemble that of T-2 toxin.

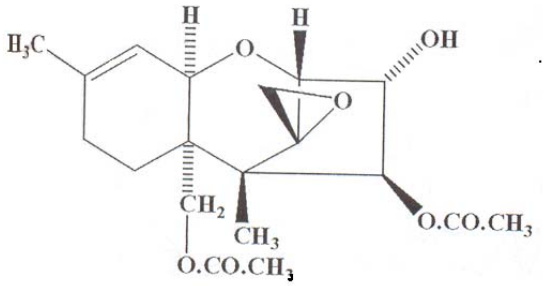
Fig. 1.9. Structure of HT-2 Toxin (Adapted from SCF, 2001)



1.5.5.2.3. DAS

Chemically it is 12,13-epoxytrichothec-9-ene-3,4,25-triol-4,15-diacetate [(molecular formula - $C_{19}H_{26}O_7$) (Fig. 1.10)] with a molecular weight of 366.41 (SCF, 2001). The toxin, also known as 'anguidine', had been used in clinical evaluations as an antitumor drug during the late 1970s and 80s. Patients developed signs and symptoms of severe mycotoxicosis due to which its administration as a chemotherapeutic agent was discontinued (Emanuel et al, 1975; SCF, 2001).

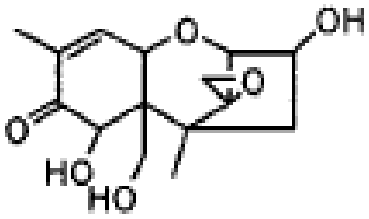
Fig. 1.10. Structure of DAS (Adapted from Moss and Thrane, 2004)



1.5.5.2.4. DON

Chemically it is 4-Deoxynivalenol (DON, vomitoxin, dehydronivalenol, RD-toxin): 12,13-epoxy-3,4,15-trihydroxytrichotec-9-en-8-one [(molecular formula- $C_{15}H_{20}O_6$) (Fig. 1.11)] with a molecular weight of 296.32 (SCF,1999). DON has been first characterized as a toxic metabolite of *F. graminearum* in 1979 during which the consumption of damaged corn induced vomiting in swine. DON is also known as 'vomitoxin' due to the characterized induction of vomiting feed refusal in animals after the ingestion of contaminated feed (Rocha et al, 2005). No signs of carcinogenicity has been associated with DON toxicosis (Iverson et al, 1995).

Fig. 1.11. Structure of DON (Adapted from SCF, 1999)

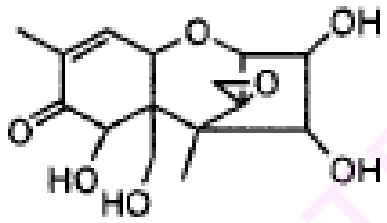


1.5.5.2.5. NIV

Chemically it is trichothec-9-en-8-one,12,13-epoxy-3,4,7,15-tetahydroxy-,(3-alpha,4-beta,7-alpha) [(molecular formula - $C_{15}H_{20}O_7$) (Fig. 1.12)] with a molecular weight of 312.35 (SCF 2000). Nivalenol was first isolated from *F. nivale* Fn2B, an

atypical strain of *F. sporotrichioides*. There is no evidence for carcinogenic effect of NIV in experimental animals. No human data is available for any of the toxicity effects. This could be because the intake levels of NIV are always far below the t-TDI of 0.7 µg/kg body weight (discussed in section 4.1.2. Tolerable Daily Intake Level).

Fig. 1.12. Structure of NIV (Adapted from SCF, 2000)



1.5.5.3. Natural Production of Trichothecenes

Trichothecene production among the different isolates of *Fusarium* varies from species to species. The toxins produced by some of the commonly occurring *Fusarium* species are collated in Table 1.2.

According to Moss and Thrane (2004), the common toxin producers of *Fusarium* species could be grouped under three sections of the *Fusarium* classification pattern (section1.4.1). Their method mainly make use of the metabolite profile in addition to other features like physiological and ecological factors.

1. **Liseola group** (see section 1.4.1.5)**:** No known trichothecene producers in this group. Mainly fumonisin producers. *e.g. F. verticillioides, F. proliferatum.*
2. **Sporotrichiella group** (see section 1.4.1.1)**:** Major producers of type A trichothecenes, although few strains are known to produce type B toxins along with T-2 and DAS. Most of the members are saprophytes, commonly associated with cereal debris in damp cool environments. *e.g. F. sporotrichioides, F. poae.*

3. **Discolor group** (see section 1.4.1.2)**:** Common producers of type B trichothecenes. Based on the type of metabolites produced they are further divided into three chemotypes.
    - Chemotype Ia: DON and 3-acetyl DON (3-ADON); ***e.g. F. culmorum***.
    - Chemotype Ib: DON and 15-acetyl DON (15-ADON); ***e.g. F. graminearum.***
    - Chemotype II: NIV and 4-acetyl NIV (ANIV) or Fusarenon X; ***e.g. F. cerealis*** (=*F. crookwellense*).

In the literature survey we have noted the following discrepancies regarding *Fusarium* classification in relation to trichothecene production.

1. The members of the *Giberella fujikuroi* species complex are mainly comprised of *Fusarium* species that are classified under Liseola section of the *Fusarium* classification system of Joffe (1974), and described as trichothecene non producers by Moss and Thrane (2004). On the contrary, the species of Liseola group ie, *F. moniliforme* and *F. sacchari* have been reported to produce trichothecene toxins such as DON and T-2, respectively (Table 1.2). Cantalejo et al (1999) have reported the occurrence of trichothecene producing isolates of *F. moniliforme* from cereals and feed stuffs.
2. The species of other sections of *Fusarium* classification such as Martiella (*F. solani*) and Elegans (*F. oxysporum*) have also been reported to produce trichothecenes (Table 1.2).

1.5.5.4. Chemical and Physical Properties

Trichothecenes are highly stable under different environmental conditions including normal cooking conditions (Lauren and Smith, 2001), irradiation (O'Neill et al, 1993), physiological saline (Duffy and Reid, 1993) and temperature (Widerstrand and Pettersson, 2001). They are non-volatile and insoluble in water, but soluble in a variety of organic solvents [(acetone, ethyl acetate, dimethyl sulphoxide-DMSO, ethanol, methanol, propylene glycol) (Cole and Cox, 1981)]. They have low vapour pressure, but vaporize when heated in organic solvents. They form yellow greasy liquid on extraction with suitable solvents and white crystals when purified. They are resistant to autoclaving but are inactivated at 900 °F for 10 minutes/500 °F for 30 minutes, or in 3-5 % solution of sodium hypochlorite (Wannemacher and Weiner, 1997).

1.5.5.5. Toxicology

The trichothecene mycotoxins are generally toxic to humans, other mammals, birds, fish, a variety of invertebrates, plants, in short to all eukaryotic cells.

1.5.5.5.1. Effects of Trichothecenes on Humans

The major toxicological effects of trichothecenes in animals and humans include gastric and intestinal lesions, hematopoeitic and immunosuppressive effects, central nervous system toxicity (nausea, anorexia, lassitude, emesis, loss of coordination), suppression of reproductive function (abortion, infertility), vascular effects leading to hypotension and increased cell death (Larsen et al, 2004). The rate of toxicity varies somewhat with the particular toxin and animal species studied, but mostly depends on the nutritional status of the host, stress, liver damage, intestinal infections (all of these affecting trichothecene metabolism) and route of administration [(affecting mitochondrial electron transport system and cellular energetics) (Wannemacher and Wiener, 1997)]. These toxins are more toxic via the lungs when compared to other routes of administration (Table 1.3). They are more toxic via intranasal, intrathecal, and inhalational exposures (Larsen et al, 2004). Once inside the systemic circulation, regardless of the route of exposure, they can affect rapidly proliferating tissues (Ueno, 1989; Wannemacher et al, 1991).

High doses of trichothecenes severely injures actively dividing tissues including bone marrow, lymph nodes, thymus, spleen, intestinal mucosa, finally resulting in

immuno-suppression (decreased serum IgG and IgM levels, decreased resistance to pathogens, impaired delayed hypersensitivity response etc). Hematotoxicity is a common major symptom characterized by thrombocytopenia, leukopenia, coagulation disorders and compromised resistance to infections that lead to septicemia and massive hemorrhage. *In vitro* studies showed that WBCs are more susceptible to trichothecenes than platelets and RBCs. Ocular and upper respiratory effects are also found to be associated with this mycotoxicoses. Myelotoxicity is highest for T-2 and HT-2 toxins and lowest for DON and NIV. Chronic exposure to trichothecenes causes Alimentary Toxic Aleukia in humans-ATA (Yagen and Joffe, 1976; Larsen et al, 2004; Čonkova′ et al, 2003).

Table 1.3. Comparative Toxicity of T-2 Toxin

(Adapted from Wannemacher and Weiner, 1997)

Route of Administration	**Mammals tested**						
	Mouse	**Rat**	**Guniea pig**	**Rabbit**	**Cat**	**Pig**	**Monkey**
	T-2 toxin LD_{50} values (mg/kg)						
Intravenous	4.2-7.3	0.7-1.2	1.0-2.0	-	-	1.2	-
Intraperitoneal	5.2-9.1	1.3-2.6	-	-	-	-	-
Subcutaneous	2.1-3.3	0.6-2.0	1.0-2.0	-	< 0.5	-	-
Intramuscular	-	0.5-0.9	1.0	1.1	-	-	0.8
Intragastric	9.6-10.5	2.3-5.2	3.1-5.3	-	-	-	-
Intranasal	-	0.6	-	-	-	-	-
Intratracheal	0.16	0.1	-	-	-	-	-
Inhalational	0.24	0.05	0.6-2.0	-	-	-	-
Intracerebra	-	l0.01	-	-	-	-	-
Dermal in DMSO	6.6	4.3	2.2	10	-	-	> 8.0
Dermal in Methanol	-	> 380	> 80	-	-	-	-

'-' : Not determined

1.5.5.5.1.1. Acute Toxicological Effects

Single dose exposures to low levels of trichothecenes result in acute toxicological effects. The major effects caused by trichothecene intoxication via oral, parenteral, dermal or aerosol exposures are gastric and intestinal lesions, hematopoeitic and immunosuppressive effects, central nervous system toxicity (characterized by nausea, anorexia and lassitude), suppression of reproductive organ function and acute vascular effects leading to hypotension and shock (Wannemacher and Wiener, 1997). Ocular exposures are characterized by tearing and burning sensation of eyes and conjunctivitis (Wannemacher and Wiener, 1997). Infection of the upper respiratory tract results in itching of nose and pain rhinorrhea when nose is affected, sore thoat, aphona and voice changes when throat is affected and cough hemoptysis, dyspnea, deep chest pain and chest pressure when tracheobronchial tree is affected (Wannemacher and Wiener, 1997).

Most of the reports on acute toxicological effects of trichothecenes have been from Southeast Asia, particularly in Afghanistan, Laos and Campodia during World War II after their use as a biological weapon where rocket, aerial bomb or cylinder explosion was used to spread a yellow oily droplet within 100 m of the explosion site (Mirocha et al, 1983; Ember, 1984; Watson et al, 1984; Wannemacher and Wiener, 1997). The incident called “yellow rain” was followed by the development of immediate symptoms such as severe nausea, vomiting, burning superficial skin discomfort, lethargy, dizziness and loss of co-ordination in the victims (Mirocha et al, 1983; Watson et al, 1984; Wannemacher and Wiener, 1997). 3-12 hours after exposure, the vctims had developed dyspnea, coughing, sore mouth, bleeding gums, epistaxis, hematemesis, abdominal pain and central chest pain. Exposed skin turned red, swollen, painful and pruritic. Development of vesicles, small or large bullae or petechial rashes and skin necrosis were also observed. Severe poisoning was associated with bloody ooze from mouth and nares followed by hematochezia. Death occurred from minutes to days after exposure and was often preceded by tremors, seizures and coma; most of the dying individuals were hypothermic and hypotensive finally resulting in tachycardia. DAS isolated from an autopsy case when injected into rabbit eyes had produced characteristic symptoms such as reddening of eyes, edema and cornea opacity (Wannemacher and Wiener, 1997). The common symptoms in both Southeast Asia and Afghanistan included vomiting (71%);

diarrhea (53%); skin irritation, burning, and itching (44%); rash or blisters (33%); bleeding (52%); and dyspnea [(48%) (Ember, 1984; Stahl et al, 1985)]. Similar dermal irritations after trichothecene toxicosis have been reported in laboratory personnel working with trichothecegenic organisms (Bamburg et al, 1968; Bamburg and Strong, 1971) or those handling fodder using infected straw for fuel, or after sleeping on mattresses made of infected straw contaminated with trichothecene producing molds (Forgacs, 1972).

Outbreaks of illness with symptoms similar to that of vomitoxin (DON) consumption have also been reported from Japan, India, USA and China. Scabby grain toxicosis has been reported from Japan where the victims developed nausea, vomiting and drowsiness after consuming *Fusarium* infected rice (Ueno, 1971). In China multiple outbreaks of vomiting illnesses have been attributed to the consumption of moldy grains (corn and wheat) contaminated with DON and ZEN during 1961-1985 that had affected more than 7000 people (Luo, 1988a, Luo, 1988b, Luo, 1988c). Acute trichothecene mycotoxicoses characterized by gastrointestinal illnesses have been reported from the Kashmir valley of India where nearly 100 people were sick, after consumption of mold contaminated wheat (Bhat et al, 1989). Varying levels of trichothecene mycotoxins such as T-2, DON, ADON (acetyl DON) and NIV were recovered from the samples tested. Outbreak of human mycotoxicoses due to the consumption of DON producing *Fusarium* contaminated wheat and sorghum has been reported from India during 1982-1984 (Ramakrishna et al, 1989). T-2 toxin contamination in rice leading to trichothecene toxicosis has been reported from China by Wang et al (1993). Reports from USA during 1997-1998 have stated that approximately 1700 US children became ill after eating contaminated burritos and suffered from vomiting, nausea, headache, and abdominal cramps. The gastrointestinal illnesses and other symptoms associated with the consumption of burritos were attributed to some preformed toxin or any such short acting agent. The vomitoxin (DON) levels in the burritos were less than 1 ppm [the advisory level proposed by Food and Drug Administration (FDA) in food for adults]. This advisory level that has been set for adults may not be applicable to children (Centers for Disease Control and Prevention, 1999) and hence the chances of vomitoxin being the etiological agent of the illness was not be eliminated. Acute toxicosis has been reported

from USA in 2002 from urine samples of patients suffering from serous health effects like headache, vomiting, diarrhea, asthma, loss of concentration and balance (Croft et al, 2002).

1.5.5.5.1.2. Chronic Toxicological Effects

Chronic exposure to sub-acute doses of trichothecenes is responsible for Alimentary Toxic Aleukia toxicosis (ATA) in humans and mycotoxicoses in animals. Chronic mycotoxicosis occurs when the toxins are repeatedly introduced iatrogenically as subacute doses (Joffe, 1971).

1.5.5.5.1.2.1. Alimentary Toxic Aleukia (ATA)

The clinical course of ATA is divided into four stages (Wannemacher and Wiener, 1997).

1. **First Stage:** Characterized by vomiting, diarrhea and abdominal pain. The victims also develop excessive salivation, headache, dizziness, weakness, fatigue and tachycardia, sometimes fever and sweating. Most of these symptoms develop immediately or few hours to several days after the consumption of trichothecene contaminated food materials.
2. **Second Stage:** Also called leukopenic stage or latent stage. Characterized by leukopenia, granulopenia, and progressive lymphocytosis. The victims may develop the next stage of infection if the ingestion of the toxin-contaminated food is not interrupted or if large doses are consumed.
3. **Third stage:** Characterized by the appearance of bright red or dark red petechial rashes, first localized in small areas, on the skin of the chest, but later becoming numerous and spreading to other areas of the body. In most cases intensive ulceration and gangrenous leisures develop in the larynx which may lead to aphonia and death by strangulation. The affected individuals also develop severe hemorrhagic diathesis of nasal, oral, gastric and intestinal mucosa.
4. **Fourth stage:** Also called recovery stage. Characterized by the fall of body temperature and healing of the necrotic lesions. Development of secondary infections like pneumonia is common. Convalescence is

prolonged and generally requires several weeks to few months, particularly for bone marrow to return to normal.

The first report on chronic trichothecene mycotoxicoses and ATA has been from Russia during the Second World War when peasants consumed gains contaminated with trichothecene mycotoxins (GajdusÏek, 1953; Wannemacher and Wiener, 1997). Chronic inhalation mycotoxicoses have been reported from Chicago where infestation with *Stachybotrys atra* had occurred. The symptoms included chronic recurring cold and flu-like symptoms, sore throat, diarrhea, headache, fatigue, dermatitis, intermittent focal alopecia and generalized malaise. Trichothecene mycotoxins have been isolated from the contaminated household materials in USA which also have been proved to be toxic to experimental animals (Croft et al, 1986). The incident called 'Sick- building syndrome' claiming 4 lives, occurred due to the contamination of building walls and ceiling fibre boards with *Stachybotrys atra* which produced many toxins including satratoxin, the macrocyclic trichothecene toxin (Croft et al, 1986). 'Sick- building syndrome' has also been reported from Canada due to the inhalation or dermal exposure to dust from ventilation systems where the culprits had been T-2 toxin, T-2 tetraol, DAS and roridin (Smoragiewicz et al, 1993). Similarly, isolation of *Stachybotrys* from the bronchoalveolar lavage fluid of a child with chronic cough, recurrent pneumonias and chronic fatigue also suggested that the symptoms could have been due to the inhalation of satratoxin produced by the fungus (Elidemir, 1999). Satratoxin H have been identified in a water-damaged office building in New York City where the workers were exposed to the *Stachybotrys chartarum* that resulted in the development of non-specified disorders of the lower airways, eyes and skin, fevers and flu-like symptoms, and chronic fatigue (Johanning, 1996).

1.5.5.5.1.2.2. Urov or Kashin-Beck Disease

The disease occurs endemically among the Cossacks in the valley of Urov River in Eastern Siberia and also in North Korea and Northern China (Nelson et al, 1994). It brings about chronic disabling, deforming, dystrophic osteoarthosis involving the peripheral joints and spine. Pre-school children or children of school age have been the most susceptible to the disease. The disease begins without any visible symptoms (Nelson et al, 1994). Early stages of the disease are characterized by pain in some of their

joints and the joints become thickened. The disease progresses slowly to a chronic stage that is characterized by shortening of the long bones, thickening and subsequent deformity of the joints, flexor contractures and muscular atrophy (Nelson et al, 1994). This disease has been reported to be caused by some strains of *F. poae* but the mycotoxin associated with it has not been identified yet (Nelson et al, 1994).

1.5.5.5.2. Effects of Trichothecenes on Animals

Consumption of contaminated feed materials is responsible for mycotoxicosis in animals.

1.5.5.5.2.1. Haemorrhagic Syndrome

This syndrome is characterized by bloody diarrhea, necrotic oral lesions, hemorrhagic gastroenteritis, and extensive hemorrhages in many organs (Nelson et al, 1994) and has been reported from North Central United States in animals such as cattle, pigs and poultry after ingestion of moldy cereals, particularly corn. *F. sporotrichioides* and *F. poae* were isolated from contaminated feeds. Hemorrhagic syndrome in animals is closely related to ATA in humans. Both of these diseases are caused by T-2 and DAS toxins that are produced primarily by *F. sporotrichioides* (Marasas and Nelson, 1987).

1.5.5.5.2.2. Estrogenic Syndrome

Consumption of cereals, particularly corn and barley, contaminated with zearalenone after infection by *F. graminearum* results in this disease which is characterized by swollen mammary glands and vulva and in severe cases vaginal and rectal prolapse (Nelson et al, 1994). The disease mainly affects genitals and reproductive organs. Pigs are the most sensitive animals where true estrus is not observed in females whereas the young males undergo feminizing effect, characterized by the enlargement of the mammary glands, atrophy of the testes and swelling of the prepuce. The syndrome finally results in infertility, reduced litter size and weak piglets (Nelson et al, 1994).

1.5.5.5.2.3. Feed Refusal and Emetic Syndromes

Majority of the field outbreaks of emetic syndromes in pigs are caused after the consumption of cereals contaminated with DON, produced by *F. graminearum*. The clinical symptoms include feed refusal (associated with nausea and vomiting) resulting in decrease in weight gain and slower growth rates (Nelson et al, 1994).

1.5.5.5.2.4. Fescue Foot

This disease in cattle has been reported from winter pastures of tall fescue (*Festuca arundinacea* Schreb.) in the United States, Australia and New Zealand and is characterized by lameness, loss of weight, arched back, elevated body temperature and dry gangrene, involving the hind feet, tail tip and ears, with sloughing of the most distal parts of these extremities (Nelson et al, 1994). *F. sporotrichioides* have been identified from among the various *Fusarium* isolates from toxic hay (Marasas and Nelson, 1987).

1.5.5.5.2.5. Degnala Disease

The disease has been reported in buffaloes and cattle from India and Pakistan following consumption of rice straw from rice grown in low-lying, waterlogged areas. Degnala disease occurs during winter and is characterized by edematous swelling of the legs and necrosis, gangrene, and sloughing of the extremities (Nelson et al, 1994). Isolates of *F. equiseti* and *F. semitectum* have been procured from the toxic rice straw, but their toxins were not identified from the contaminated material and so these species cannot be considered as the sole etiological agents of the disease (Marasas and Nelson, 1987).

1.5.5.5.2.6. Moldy Sweet Potato Toxicosis (Atypical Interstitial Pneumonia)

This is a fatal respiratory disease of cattle reported from United States and Japan after ingestion of moldy sweet potatoes contaminated with furanoterpenoides, produced by *F. solani*. The clinical symptoms are rapid respiratory rate, typical extension of the head and neck associated with dyspnea and frothy exudate around the mouth and finally death of the animal (Nelson et al, 1994).

1.5.5.5.3. Effects of Trichothecenes on Plants

Trichothecenes are responsible for phytotoxic and cytotoxic activity of some of the *Fusarium* species. The major toxic effects include wilting, chlorosis, necrosis and other symptoms (Rocha et al, 2005). In infected plants, *Fusarium* invasion can reduce kernel set and kernel weight resulting in reduction of yield. Unlike in animals, the type A trichothecenes such as T-2, HT-2 and DAS are less toxic to plants when compared to the type B toxins. DON and 3-ADON are the most toxic to plants which brings about growth retardation, inhibition of seed germination, inhibition of green plant regeneration etc (McLean, 1996).

1.5.5.5.3.1. Fusarium Head Blight (FHB)

Fusarium head blight, also known as scab of wheat is the most destructive of the diseases caused by *Fusarium* (Kang and Buchenauer, 2002). FHB was first reported from England by W. G. Smith in 1884 (Arthur, 1891). The disease gained world recognition during the early 19th century. Outbreaks of FHB have been reported from different parts of the world since then (Mesterhazy, 1984; McMullen et al, 1997; Ellner 1999). The disease has been a serious problem to the cereal breeders, producers and to the entire food industry. Economic losses caused by FHB since 1990 have been estimated to be $3 billion US for wheat and $0.4 billion US for barley (Windels, 2000). Causative agents of the disease are *F. graminearum*, *F. culmorum* and *F. avenaceum* (Bai and Shaner, 1994; Parry et al, 1995). The disease mainly affects wheat and barley that are grown in humid and semi-humid climates. Symptoms associated with FHB include premature necrosis and brown or gray discolouration of the spike tissue resulting in cereals with lower grain yield, lower test weight, reduced grain quality and reduced milling yield (Stack, 1999). Infection with *Fusarium* also cause destruction of starch granules and their cell walls and affects endosperm storage proteins resulting in poor quality product (Larsen et al, 2004). Wheat heads are highly susceptible to *Fusarium* infection at the early flowering stage (Bushnell et al, 2003).

1.5.5.5.3.2. Akakabi-byo (Red Mold Disease or Scab)

Akakabi-byo or scab of cereal grains is another disease caused by *Fusarium* species, particularly *F. graminearum* (Nelson et al, 1994). The disease has been reported mainly from Japan where it affected wheat, barley, rye, oats and rice. Consumption of infected cereals finally resulted in outbreaks of human mycotoxicosis, characterized by anorexia, nausea, vomiting, headache, abdominal pain, diarrhea, chills, giddiness, and convulsions in Korea, Japan and former USSR (Nelson et al, 1994).

1.5.5.6. Mode of Action

1.5.5.6.1. In Animals and Humans

Trichothecenes exerts their action primarily by inhibiting the protein synthesis machinery. Studies with radiolabelled trichothecenes have demonstrated two types of interactions with the cell (i) free, bidirectional movement of these toxins across the plasma membrane and (ii) specific, high affinity to ribosomes (Middlebrook and

Leatherman, 1989). Binding of trichothecenes to 60 S ribosomal subunits during translation results in blocking of the initiation or elongation of peptide chains and thereby inhibits protein synthesis (Thompson and Wannemacher, 1984; Wannemacher and Weiner, 1997). Protein inhibition in Vero cells occurred after 5 minutes of exposure to T-2 toxin (Thompson and Wannemacher 1984). Secondary effects of inhibition of protein synthesis include inhibition of DNA and RNA synthesis. Substantial inhibition of RNA synthesis has been observed in HeLa cells [(86% inhibition) (McLaughlin et al, 1977)] compared to that of Vero cells [(15% inhibition) (Thompson and Wannemacher, 1984)]. *In vivo* studies in mice have shown that trichothecenes suppressed DNA synthesis in almost all tissues, but the rate of inhibition was not so substantial when compared to that of protein synthesis. Other than the inhibitory effects on DNA, RNA or protein synthetic machinery, none of the cell model studies could demonstrate either mutagenic effects or DNA damage (Busby and Wogan, 1981).

Being highly lipophilic, trichothecenes are easily absorbed through skin, gut and pulmonary mucosa. Exposure to high concentrations of aerosolized T-2 toxin resulted in the death of rodents and guinea pigs within 1-12 hours (Marrs et al, 1986; Creasia et al, 1990). In contrast oral exposure to T-2 toxin resulted in immediate damage to intestinal mucosa (Matsumoto et al, 1978). Their entry is more rapid through the pulmonary and intestinal mucosa than through skin (Kemppainen and Riley, 1984). Once inside the cell, these toxins bind to subcellular structures and exert multiple effects on the membrane structures which consequently stimulate lipid peroxidation especially in liver, kidney, spleen, thymus and bone marrow (Suneja et al, 1989). Trichothecenes disrupts or alters the morphology of mitochondria, rough endoplasmic reticulum, myofibres and membranes, thereby seriously affecting cellular energetics and cytotoxicity (Yarom et al, 1983; Trusal and O'Brien, 1986; Wannemacher and Wiener, 1997). Trichothecenes exert multiple effects within mitochondria which includes inhibition of succinate dehydrogenase activity, resulting in decreased levels of succinate, pyruvate and malate oxidation with effects on electron transport activity and inhibition of mitochondrial protein synthesis (Pace, 1983; Pace et al, 1988). The cascade of cytotoxic effects terminates with apoptosis or increased cell death in a variety of cell types by means of mitochondrial and non mitochondrial mechanisms (Shifrin and Anderson, 1999; Yang et

al, 2000; Ishigami et al, 2001; Poapolathep et al, 2002). Rapidly proliferating tissues such as intestine and bone marrow are adversely affected. Furthermore, they readily cross the placenta also and bring about increased cell death in mouse fetuses (Ishigami et al, 2001).

1.5.5.6.2. In Plants

During infection, spores of the fungus are deposited on the florets where they germinate and initiate infection. Initially, the fungal hyphae establish it on the florets and glumes and allow the fungus to grow towards the stomata and other susceptible sites within the spike. This may lead to the direct penetration of the epidermal cells (Bushnell et al, 2003). The fungi may also enter the plant through stomata and underlying parenchyma, partially or fully exposed anthers, openings between the lemma and palea of the spikelet or through the base of the glumes where the epidermis and the parenchyma are thin walled (Lewandowski and Bushnell, 2001; Bushnell et al, 2003). Infections at the very early stage results in poorly developed kernels, which greatly affect the yield and quality of grain. The initial 48-72 hours post infection is crucial for *Fusarium* infection during which the fungus develops a biotrophic relationship with the host plant. After 72 hours post infection, the fungus switches on the necrotrophic stage which is associated with an increased fungal colonization of the host plant and eventually plant death (Goswami and Kistler, 2004). Bushnell et al (2003) have suggested that the trichothecene toxins play a role in the shift from biotrophic to necrotrophic stage. During infection of cereal grains the fungal mycelia progresses from the surface of the kernel to the endosperm. The phenolics present in kernel cell wall play a pivotal role in the inhibition of growth and reproduction of a wide variety of fungal genera (McKeehen et al, 1999). Kernel phenolics such as flavonoids and phenolic acids of maize and wheat are directly associated with resistance to *Fusarium* infection (Reid et al, 1992). Reid et al (1992) have reported of increasing concentration of phenolic compounds in FHB resistant varieties of wheat when compared to that of the susceptible ones. The decrease in the amount of phenolids has been attributed to degradation through the pathogenic metabolites or inhibition of their synthesis through trichothecenes. Inoculum containing a mixture of *Fusarium* species has been reported to be more aggressive than infection with a single species (Arseniuk et al, 1999).

The role of trichothecenes as phytotoxic determinants has been demonstrated by Proctor et al (1995a) and Desjardins et al (1996). Species of *Fusarium* devoid of the ability for trichothecene production have been identified as less pathogenic to susceptible wheat cultivars (Proctor et al, 1995a). However, the trichothecene production of a *Fusarium* species in relation to its ability to induce FHB needs to be established. Positive correlation between FHB symptoms and trichothecene levels (i.e, the low level of FHB symptoms in presence of lower trichothecene levels), is generally expected. On the contrary, wheat varieties developing low FHB symptoms in presence of higher concentration of DON and *vice versa* have been reported by Mesterházy et al. (1999). Ittu et al (1995) proposed that the plant defence mechanism may slow down in presence of trichothecenes so as to enable the easy establishment of the producer *Fusarium* in the host plant. It may be expected that since trichothecenes inhibit protein synthesis and that synthesis of protein is essential to the defense by plants, trichothecegenic fungi would find it easier to overcome host barrier. Trichothecenes are different in their ability to induce disease condition in various hosts. Carter et al (2002) have reported that the NIV chemotypes of *F. graminearum* were more aggressive to corn than to wheat.

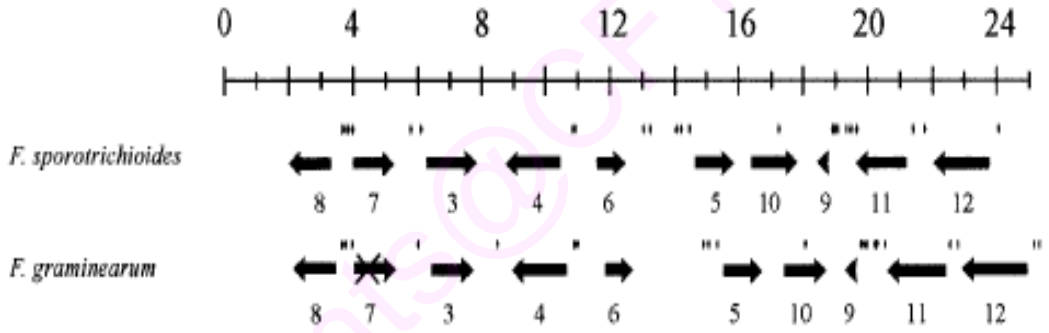
1.5.5.7. Biosynthesis and Gene Organization

Biosynthesis of trichothecenes starts from an isoprenoid synthesis intermediate namely farensyl pyrophosphate (FPP). The pathway begins with cyclisation of farensyl pyrophosphate to trichodiene by the enzyme trichodiene synthase, which is a dimer with a subunit molecular mass of 45 KDa, isolated from *F. sporotrichioides* (Hohn and Beremand, 1989a). 10 to 12 co-regulated orthologous genes within a 26 Kb distance, involved in different steps of the trichodiene biosynthetic pathway have been identified mainly in *F. graminearum* and *F. sporotrichioides* [(Fig.1.13.) (Brown et al, 2002; Brown et al, 2003)].

The genome has 3 co-regulated gene loci (Brown et al, 2003). The core trichothecene gene cluster consists of 12 genes (*tri 3* to *tri 14*) which include those encoding trichodiene synthase [(*tri 5*) (Hohn and Beremand, 1989a)], P-450 monooxygenases [(*tri 4* and *tri 11*) (Hohn et al, 1995; Alexander et al, 1998)], a C-15 acetyl transferase [(*tri 3*) (McCormick et al, 1996)], a C-14 acetyl transferase [(*tri 7*) (Brown et al, 2001)], two transcriptional regulatory factors [(*tri 6*) (Proctor et al, 1995b)]

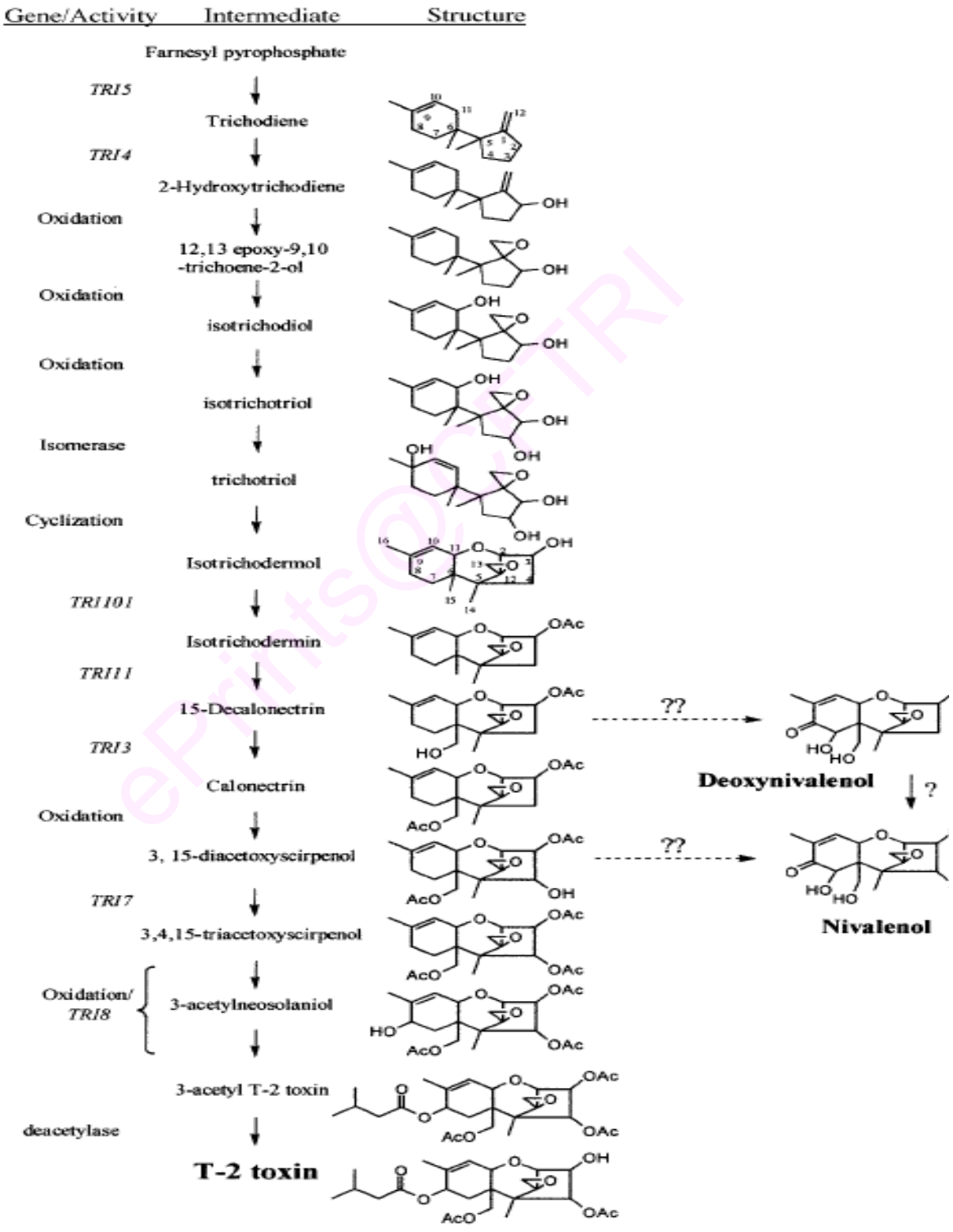
and [(*tri 10*) (Tag et al, 2001)], a toxin efflux pump [(*tri 12*) (Alexander et al, 1999)], a C-4 hydroxylase, [(*tri 13*) (Brown et al, 2002)], a C-3 esterase [(*tri 8*) (McCormick and Alexander, 2002)], and two unidentified hypothetical proteins, [(*tri 9* and *tri 14*) (Brown et al, 2004)]. Fig. 1.14 shows the cascade of reactions involved in the biosynthesis of various trichothecenes.

Fig. 1.13. Organization of Predicted ORFs for *F. sporotrichioides* and *F. graminearum*. The arrowheads indicate direction of transcription. Numbers underneath each large arrow refer to the specific genes, e.g., 5 indicate *tri 5*. Genes from different *Fusarium* species with the same number are homologues. (Adapted from Brown et al, 2001).



The second locus consists of a single *gene tri 101*, required for the 3-*O*-acetylation of trichothecene ring that confers resistance to the DON producer *F. graminearum* (Kimura et al, 1998a). The third locus consists of two genes *tri 1* and *tri 16*, outside the trichothecene gene cluster (Brown et al, 2003). The gene *tri 1* encodes the cytochrome P-450 monooxygenase required for C-8 hydroxylation of trichothecene ring (Meek et al, 2003; McCormick et al, 2004) and *tri 16* is required for the esterification of C-8 position (Peplow et al, 2003).

Fig. 1.14. The Proposed Biochemical and Genetic Pathway for Trichothecene Biosynthesis (Adapted from Brown et al, 2001)



1.5.6. Trichothecene Metabolism and Degradation

Unlike the other commonly occurring mycotoxins, trichothecenes do not require any metabolic activation to elicit its toxic effect (Busby and Wogan, 1981). They can directly cause skin or intestinal mucosa irritation leading to other clinical effects immediately to hours after any sort of dermal or oral exposure. Whether inside the body or in cell free systems such as culture media, they have the capability of direct reaction with cellular components leading to inhibition of protein, DNA and RNA synthesis, disaggregation of polyribosomes and rough endoplasmic reticulum, inhibition of mitochondrial functions and finally apoptosis [(1.5.5.6) (McLaughlin et al, 1977; Pace, 1983; Shifrin and Anderson, 1999)].

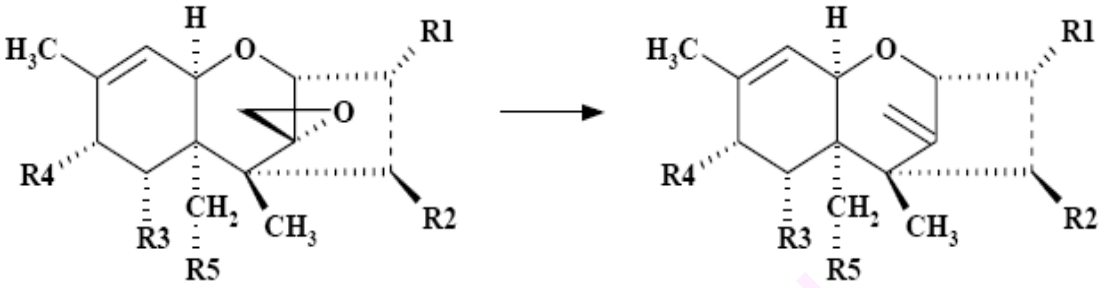
Liver is the major organ of trichothecene metabolism (Wannemacher and Wiener, 1997). Studies with radiolabelled mycotoxins have shown that the radioactivity appeared in bile, liver and gastrointestinal tract irrespective of the route of administration of the toxin [(oral, intramuscular, intravenal, dermal) (Matsumoto et al, 1978)]. In addition to liver, tissues in the intestine are also involved in the metabolism of the trichothecene mycotoxins. In the intestine trichothecenes are metabolically altered rather than degraded.

Of all the trichothecene toxins, the metabolic fate of T-2 has been well studied. The major metabolic actions on T-2 toxin are deepoxydation (i.e., the removal of oxygen from the epoxide ring at the C-12, 13 positions to yield a carbon-carbon double bond (Fig.1.15) and oxidation of the C-3 and C-4 positions on the isovaleryl side chains (Westlake et al, 1987; Swanson et al, 1988). The de-epoxy metabolites have been shown to be significantly less toxic than their epoxy parents in a variety of cytotoxicity tests (Swanson et al, 1987; Shima et al, 1997; Eriksen et al, 2004). Epoxide reduction is possibly the only single-step reaction for detoxification of trichothecenes. The studies of Swanson et al (1988) have shown that T-2 toxin was metabolized to its deepoxy derivatives such as deepoxy HT-2 and deepoxy triol by the intestinal microflora of a variety of animals. Similar has been the case of DAS where it was deacetylated and deepoxidated by the intestinal microflora of cattle, swine and rats. Investigations on the intestinal metabolism of T-2 toxin in rat intestine using tritium labeled T-2 toxin has also revealed HT-2 toxin as the main metabolite, though traces of 3'-OH-T-2 toxin, T-2 tetraol, and 4-deacetylneosolaniol were observed (Conrady et al, 1988). The deepoxide

metabolites of trichothecenes have not hitherto been reported in plants (Boutigny et al, 2007)

In liver a nonspecific carboxyesterase (EC 3.1.1.1) of microsomal origin participate in the selective hydrolysis of T-2 toxin at C-4, giving rise to HT-2 toxin as the only metabolite (Ohta et al, 1977). The C-4 acetyl residues of diacetoxyscirpenol, monoacetylnivalenol, fusarenon-X and diacetylnivalenol have been selectively hydrolyzed by microsomal esterase to yield the corresponding C-4-deacetylated metabolites such as monoacetoxyscirpenol, nivalenol and 15-acetylnivalenol, respectively (Ohta et al, 1978). In addition to liver, this esterase activity has been observed in the microsomes of brain, kidney, spleen, intestine, white blood cells and erythrocytes of laboratory animals (Ohta et al, 1978). Recent studies on the degradation of trichothecene mycotoxins by chicken intestinal microflora have shown that the non-acylated trichothecenes such as DON, NIV and verrucarol were completely converted to their deepoxy metabolites whereas the monoacetyl trichothecenes 3-ADON, 15-Al-DON and fusarenon X underwent deacetylation (Young et al, 2007). The type A trichothecenes such as T-2, DAS and neosolaniol also exhibited deacetylation. The 3-*O*-acetylation of trichothecenes is another detoxification process used by *Fusarium* to protect themselves from their own toxins (Kimura et al, 1998b). The gene *tri 101* codes for the enzyme trichothecene 3-*O*-acetyltransferase that catalyzes the transfer of acetyl group to C-3 hydroxyl group of trichothecenes. Increased resistances to trichothecenes have been reported in the yeast species *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* by McCormick et al (1999) after the expression of *tri 101*. Transgenic plants containing tri *101* have been shown to accumulate less amounts of DON (Okubara et al, 2002; Manoharan et al, 2006; Kimura et al, 2006; Ohsato et al, 2007). Detoxification of trichothecenes has hitherto not been reported to occur naturally in plants (Boutigny et al, 2007).

Fig. 1.15. Reduction of the C-12,13 Epoxide Group to C-C Double Bond (Adapted from EFSA, 2005)



Other than the intestinal microflora, few bacterial isolates are also capable of degrading and detoxifying trichothecenes. Effective detoxification have been exhibited by a rumen bacterial isolate *Eubacterium* DSM 11798. The bacteria metabolized trichothecene mycotoxins (both type A and B) to a less toxic form in one or more steps (Fuchs et al, 2002). This bacterium is incorporated as an active ingredient in the animal feed Biomin BBSH 797 with the intension of augmenting the existing detoxification capacity and thus helping in the protection of the animals. Trichothecenes at a safe dose range has also been recommended for use as a feed additive in trichothecene contaminated (or presumed contaminated) feed for piglets and feed for pigs and chickens for fattening (EFSA, 2005). A soil bacterium, *Curtobacterium* sp. strain 114, has been reported to effectively assimilate T-2 toxin to non toxic derivatives (Ueno et al, 1983). T-2 toxin was transformed to its less toxic derivative HT-2 toxin which was further transformed into T-2 triol. Shima et al (1997) have isolated a bacterial strain (E3-39) belonging to the *Agrobacterium-Rhizobium* group, capable of converting DON to a less toxic metabolite 3-keto-DON by means of oxidative biotransformation. In addition, the plant species *Baccharis sarothroides* (FS2) and *Baccharis megapotamica* (FS3) have been reported to metabolize T-2 into HT-2, T-2-tetraol, and 3'-hydroxy-HT-2 toxin (TC-3). These metabolites are appreciably less toxic than the parent T-2 toxin. However, DAS when deacetylated at the C-4 position forms a more toxic derivative monoacetoxyscirpenol [(MAS) (Mirocha et al, 1988)].

Very little to little amounts of the trichothecene toxins are excreted intact. Majority of the toxins entering the body are converted into nontoxic or less toxic metabolites as a result of extensive and rapid biotransformation. Trichothecenes are converted into their deacetylated or deepoxy metabolites or glucuronide conjugates of these metabolites, resulting in the elimination of toxicity. These metabolites are excreted in urine and feces (Corley et al, 1985).

1.5.7. Factors Controlling Trichothecene Production

The production of trichothecenes by *Fusarium* is controlled by both physico-chemical and genetic factors.

1.5.7.1. Physico-Chemical Factors Controlling Trichothecene Production

Fusarium toxins encountered in food are generated primarily in the field although some toxin synthesis may occur during storage. The two climatic parameters affecting fungal infection, toxin production and distribution are temperature and moisture conditions (Xu and Berrie 2005). Wet and cool weather during flowering followed by late season rainfall has been found conducive to infection with *Fusarium* (Larsen et al, 2004). On the contrary, incidence of spikelets with FHB symptoms and concentration of mycotoxins has increased with increasing length of wetness period and temperature. Mycotoxin production is greatly enhanced by high temperatures ($\geq$ 20°C) during the course of initial infection. Moisture levels during harvest, transport and storage are key factors in the amount of trichothecenes that is likely to be found in damaged food materials (Larsen et al, 2004). Moisture levels of 17-19 % are required for the profuse growth of many of the *Fusarium* species in grain. Toxin production is enhanced by 15-30 % moisture for wheat, oat, rye and barley whereas for maize, the range is 30-40 % (Larsen et al, 2004). The extent of *Fusarium* infection and trichothecene accumulation increases with wet weather at harvest and with storage under high moisture conditions. The grains should be harvested at the optimal conditions of 14-15 % moisture level at 15 °C to avoid fungal growth and toxin production.

Changes in the carbon-nitrogen ratio have significant effect on trichothecene production (Desjardins et al, 1993). Ueno et al (1975) observed increase in trichothecenes production in liquid flask culture with increase in concentration of carbon/nitrogen sources in the media. High concentration of glucose and low concentration of nitrogen in

the culture medium are the important prerequisites for initiation of trichothecene production by *F. sporotrichioides* (Hohn and Beremand, 1989b; Ueno et al, 1975). On the contrary, decrease in production of trichothecenes has been observed for *F. graminearum* in presence of high concentration of glucose (Miller et al, 1983). Metal ions such as Mg^{2+}, Mo^{2+}, Cu^{2+}, Fe^{2+} and Zn^{2+} have no effect on trichothecene production (Ueno et al, 1975). Among the different carbon sources, sucrose and glucose have been identified as good substrate for maximal production of DON (Fromtling, 1998).

Agronomic practices also play an important role in *Fusarium* infection and toxin production. The factors mainly affecting trichothecenes production include crop rotation, varietal resistance, fungicide applications and cultivation techniques (Larsen et al, 2004). Insect infestations resulting in tissue damage also have a positive correlation to trichothecene contamination in maize (Larsen et al, 2004).

1.5.7.2. Molecular Factors Regulating Trichothecene Biosynthesis

Transcription factors are specific proteins that help in regulation of gene expression by binding to specific sites namely DNA binding domains in the promoter region. There are different families of transcription factors based on their similarity in DNA binding domains; (i) zinc finger, (ii) Cys_6 binuclear cluster, (iii) steroid receptor, (iv) helix turn helix, (v) homeodomain and (vi) leucine zipper proteins (Proctor et al, 1995b). The most commonly occurring DNA binding domain in the transcription factors of yeast and filamentous fungi is the Cys_6 zinc binding motif. They have the consensus amino acid sequence Cys-X_2-Cys-X_6-Cys-X_{5-9}-Cys-X_2-Cys-X_{6-7}-Cys (Coleman, 1992). However other types are also present in fungal transcription factors. Cys_2 His_2 zinc finger motiff has been observed in *Aspergillus nidulans* brlA (Krizek et al, 1991) and *Saccharomyces cerevisiae* (Covitz et al, 1991; Estruch et al, 1991).

Two major factors regulating trichothecene biosynthesis have been well studied and characterized; Tri 6 and Tri 10. The regulatory genes coding for these proteins namely *tri 6* and *tri 10* flank the gene for the first biosynthetic step of the trichothecene pathway, the *tri 5*.

1.5.7.2.1. Tri 6

The gene *tri 6*, 650 bp in length and situated upstream of *tri 5* was identified to have a regulatory effect in trichothecene biosynthesis (Proctor et al, 1995b). The gene

tri 6 codes for a 217 amino acid protein with a molecular weight of 25.3 kDa (Tri 6), the carboxy terminal of which has amino acid sequences similar to Cys_2 His_2 zinc finger motif found in eukaryotic transcription factors. Proctor et al (1995b) identified the presence of a peptide with regions similar to Cys_2 His_2 zinc finger DNA binding proteins, involved in the regulation of trichothecene biosynthesis. Carboxy terminal end of the protein is characterized by relatively high proportion of positively charged amino acids whereas the amino terminal end has more of negatively charged amino acids (Proctor et al, 1995b). The amino acid sequence of Tri 6 deviates from the established sequence of Cys_2 His_2 zinc finger motifs. The Tri 6 protein has 3 regions, ZF1, ZF2 and ZF3 of which ZF3 has 9 amino acid sequences, rather than the normal 2-4 between the two Cys residues, and Leu is replaced by Met. This has also been reported in *Drosophila* and *Xenopus* respectively, by Vincent et al (1985) and Ginsberg et al (1984). The two other regions ZF1 and ZF2 lack Phe and Leu residues which have also been reported in other genes (Archambault et al, 1992; Vincent et al, 1985). Both ZF1 and ZF2 residues possess higher number of residues (14 for ZF1 and 23 for ZF2) between the second Cys and the first His. Studies of Hohn et al (1999) have revealed that the ZF3 motiff was capable of binding DNA and that a ZF3 mutant carrying alanine instead of cysteine failed to bind *to tri 5* promoter.

Gene disruption studies have shown that the *tri 6* disrupted mutants are unable to convert trichothecene intermediates to the final T-2 toxin (Proctor et al, 1995b). Tri 6 is capable of binding sequences in the promoter regions of the gene *tri 5*. The Tri 6 binding sites contain TNAGGCCT as the core binding sequence (Hohn et al, 1999). The *tri 5* promoter contains 3 copies of this motif. Tri 6 can bind to even 39 bp sequence carrying this motif. Even single nucleotide sequence change within the proposed binding abolishes Tri 6 binding. The TNAGGCCT motif has been observed in the *tri 5* promoters of *F. graminearum* and *F. sambucinum* and also in trichothecene producing fungi of other genus such as *M. roridum* (Hohn et al, 1999). Tri 6 binding has been observed in other trichothecene pathway genes like *tri 7*, *tri 9 tri 10* and *tri 12*. The promoter regions of *tri 3* and *tri 4* do not contain the TNAGGCCT motif as such. The promoter region for *tri 4* has two sequences T4A (TCAGGCCC) and T4B (CCAGGCCT), which closely resembles the Tri 6 binding motif. Similar differences has been observed in the Tri 6

binding motifs of *tri 3* (T3A-TAAGGCCC and T3B-CCAGGCCC) and *tri 11* (T11A-TNAGGCCT and T11B-CAAGGCCT) genes and thereby suggested a minimum required sequence of YNAGGCC for Tri 6 binding (Hohn et al, 1999).

1.5.7.2.2. Tri 10

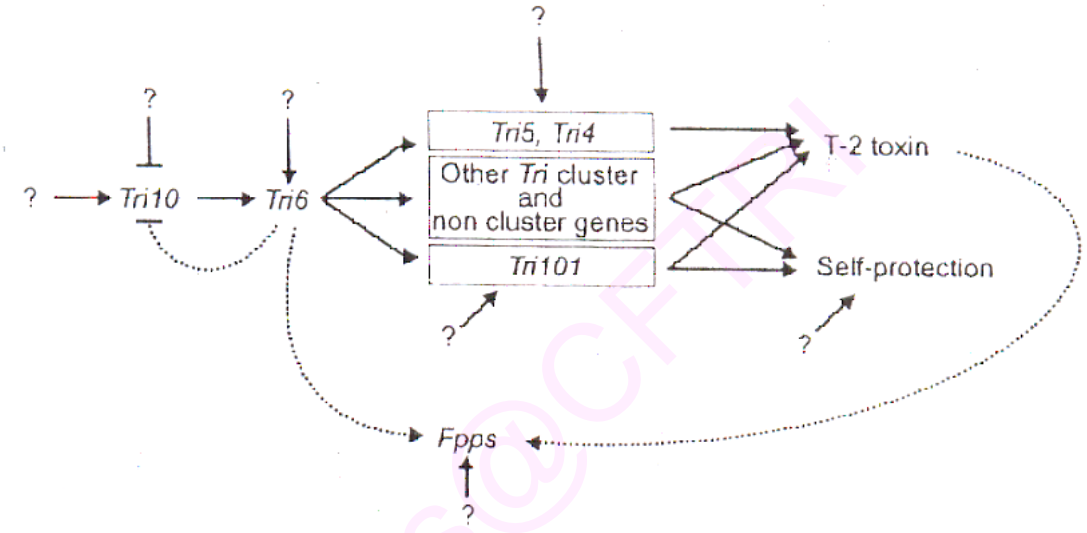
Tag et al (2001) have reported the characterization of a second regulatory gene, *tri 10* within the trichothecene gene cluster. The gene *tri 10* has 1260 bp open reading frame, placed 619 bp downstream of the *tri 5* stop codon. The gene codes for a putative protein of size 47.427 kDa (Tri 10), comprising of 420 amino acids. Tri 10 acts as a positive regulator of *tri 6* (Tag et al, 2001)

Gene disruption studies have shown that the *tri 10* disrupted mutants neither produced T-2 toxin nor accumulated trichothecene pathway intermediates (Tag et al, 2001). Expression of trichothecene biosynthetic genes *tri 5*, *tri 4* and *tri 6* was affected, showing that *tri 10* has a positive role in trichothecene biosynthesis (Tag et al, 2001). Self protection of *F. sporotrichioides* to T-2 toxin was reduced considerably in transformants with deletion of *tri 10*. Those mutants showed reduced growth to even low levels of trichothecenes when compared to the wild type fungi (Tag et al, 2001). The gene *tri 10* lacks *tri 6* binding site upstream of its open reading frame which again proves that *tri 10* transcription is independent on *tri 6*. Studies of Tag et al (2001) have also shown the over-expression of *tri 10* in the *tri 6* disruption mutants which suggests a negative regulatory effect of *tri 6* on *tri 10*, unlike in the case of other pathway genes whose expression levels were considerably reduced in response to disruption of *tri 6*. Disruption of *tri 10* affected the primary metabolic pathway as was demonstrated by the reduced transcript levels of farnesyl pyrophosphate (FPP) in *tri 10* disrupted strains (Tag et al, 2001). Tri 10 levels were not affected in *tri 6* disruption mutants but there has been decrease in the levels of FPP, thereby suggesting a direct action of *tri 10* on FPP synthesis either by controlling the expression of *tri 6* or other genes or by the regulating the synthesis of trichothecenes.

The studies of Tag et al (2001) have provided evidence for a regulatory circuit linking the primary and secondary pathways involved in trichothecene biosynthesis. In the regulatory loop, the activation of *tri 10* upregulates transcription of *tri 6* and the activation of *tri 6* in turn directly or indirectly downregulates *tri 10* transcription (Fig.

1.16.). They have suggested the involvement of an independent mechanism- the interruption of a cluster regulatory gene upstream of *tri 10*- that downregulated *tri 10* gene expression, which would otherwise have been constitutive in the *tri 6* deletion strain.

Fig. 1.16. Proposed Regulatory Loop for Trichothecene Biosynthesis



It has been established from the literature survey that the physico-chemical factors, do play a major role in trichothecene biosynthesis. The mode of action of these factors or the stage of trichothecene biosynthesis at which their effect is brought about needs to be identified. As shown in the regulatory loop (Fig. 1.16), some external factors other than the enzymes and the intermediate compounds in the trichothecene core cluster may be involved in the regulation of trichothecene synthesis. These factors may affect primary metabolism, thereby controlling the accumulation of FPP, or control the primary genes involved in trichothecene biosynthetic pathway.

1.5.8. Detection of Trichothecenes

The risk of exposure of the variety of food and feed stuffs to various mycotoxins and the mycotoxicoses followed are not pathognomic. Monitoring or determining the cause of the specific condition primarily requires confirmation of the toxin in the sample. Also, monitoring of these toxins in animal feeds not only provide a healthier diet for animals, but also prevent any mycotoxin residue carryover to humans. Therefore the

development of specific, sensitive and simple methods for detection of mycotoxins has been constantly in demand. Diversity in the chemistry and physiochemical properties of the specific mycotoxin, the varied complex matrices in which mycotoxins occur, uneven distribution of the toxin in food sample and the need to separate primary and secondary fungal metabolites produced simultaneously with mycotoxins are the deciding factors for selection of techniques to be applied for the detection, analysis and characterization of mycotoxins.

Among the different techniques implied, chromatography has so far been the widely accepted because it allows efficient separation of primary and secondary metabolites (Lin et al, 1998). Of the different chromatographic techniques employed, thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) etc have been widely used for the detection of trichothecenes (Lin et al, 1998). Biological assay systems such as flow cytometric and calorimetric methods have also been developed for the detection of trichothecenes and other mycotoxins (Visconti et al, 1991; Robb et al, 1990). Immunochemical assays have also gained wide acceptance as more simple and cost effective method for detection of mycotoxins. Many types of immunoassays such as radioimmuno assay (RIA), enzyme linked immuno sorbent assay (ELISA), immunoaffinity column chromatography, biosensors etc have been developed (Chu, 1992).

1.5.8.1. Chemical Methods

1.5.8.1.1. TLC

TLC was the first analytical method applied for the analysis of trichothecenes (Langseth and Rundberget, 1998; Larsen et al, 2004). It is a well developed, fast, cost effective and suitable method which can be used for the analysis of a wide range of mycotoxins, for a vast number or of samples. Other attractive features of TLC are the possibility of use of pre and post chromatographic visualization techniques, possibility of applying bigger volume of sample avoiding the need for sample concentration and the feasibility of performing semi-quantitative and quantitative analysis of even underivatized samples with good precision and accuracy (Lin et al, 1998). For the visualization of mycotoxins on thin layer plates, two methods have been most frequently

used; (i) direct examination under UV light of long or short wavelength [254 and 365 nm, respectively (Munez et al, 1990; Jaruis et al, 1992; di Menna et al, 1997)] and (ii) examination under UV before and after spraying the plates with a chemical reagent that reacts with mycotoxins to produce a coloured or fluorescent product. Exposing the plates to X-ray films has also been used (Munger et al, 1987). The commonly used spray reagents are methanolic aluminium chloride (Martin et al, 1986; Scott, 1987; Bennet and Shotwell, 1990) and *p*-anisaldehyde solution in methanol, acetic acid and sulphuric acid (Scott et al, 1970). Other reagents used include chromotropic acid (Martin et al, 1986; Panter et al, 1991), 4-(*p*-nitrobenzyl) pyridine (Hewetson et al, 1987), sulphuric acid-methanol (Wei and Chu, 1986) etc. Even though TLC is less sensitive as compared to other chromatographic methods, detection range upto 50 ppb has been reported for the analysis of DON (Chu, 1992).

1.5.8.1.2. HPLC and LC-MS

HPLC methods using UV detection at 218 nm has been well developed for the analysis of underivatized type B trichothecenes like DON, NIV or their derivatives (Martin et al, 1986; Lauren and Greenhalgh 1987; Martins and Martins 2001). Several methods for the derivatization of type A trichothecenes for HPLC analysis has been reported recently (Mateo et al; 2002; Jiménez et al, 2000). The lack of a conjugated carbonyl-double bond system and the lack of UV absorptivity as a consequence of the different functional groups limit the use of a simultaneous HPLC method with UV detection for the type A and type B trichothecene toxins (Razzazi-Fazeli et al, 2002). Dall'Asta et al (2004b) reported a LC method with fluorescence detection for the simultaneous determination of eight trichothecenes (T-2, HT-2 and DAS of type A and DON, 3-ADON, 15-ADON, NIV, and Fus-X of type B). Fluorescent derivatives of the toxins were produced after a pre-column derivatization with coumarin-3-carbonyl chloride. The major disadvantage of HPLC methods are the need for high purity samples and cost versatility (Lin et al, 1998).

Recent developments in mycotoxin investigations have led to the universal application of high resolution MS detectors coupled with LC. This method is especially used for compounds that do not have a chromophore and so is well suited for trichothecene analysis (Mirocha et al; 1986; Hewetson and Mirocha 1987). Different MS

instrumentations such as fast atom bombardment-MS (FAB-MS), thermospray, plasmaspray, dynamic atom bombardment, chemical ionization etc have been used for trichothecene analysis in conjunction with LC (Mirocha et al, 1986; Ackerman et al, 1987; Kostiainen, 1991; Kostiainen and Kuronen, 1991; Park et al, 1985; Razzazi-Fazeli et al, 2002). LC-MS for simultaneous determination of type A and type B trichothecenes have been developed by Dall'Asta et al (2004a) and Berthiller et al (2005) using electrospray ionization interface in the positive mode and atmospheric pressure chemical ionization triple quadrupole mass spectrometry, respectively. Picogram levels of toxin are readily detected using the MS method (Chu 1992).

1.5.8.1.3. GC and GC-MS

GC has been widely used for the determination of *Fusarium* mycotoxins in cereals (Onji et al, 1998). GC methods require the compounds to be volatile and non polar. Those toxins which are not sufficiently volatile enough needs to be volatilized by suitable derivatization methods. About 90 % of the published data for trichothecene detection report the use of GC methodology for the analysis of type A toxins and 75 % for the analysis of type B toxins (Larsen et al, 2004). Techniques used more recently include GC with electron capture or flame-ionization detectors. Most of these analytical methods make use of various derivatization reactions such as silylation [(using trimethyl silane and its derivatives) (Gilbert et al, 1985; Möller and Gustavsson, 1992; Croteau et al, 1994)] or fluroacylation which includes polyfluroacylation, polyflurobutyration and polyfluropropionation [(using heptaflurioimidazole heptaflurobutyryl anhydride, pentafluropropionyl imidazole or anhydride etc) (Begley et al, 1986; Seidel et al, 1993; Onji et al, 1998)] for obtaining a volatile derivative which again adds on to the complexity of the procedure (Larsen et al, 2004). Lowest limits of detection of 2 and 10 µg/kg have been reported for type A and type B toxins, respectively. GC methods are often unreliable and produce conflicting results due to the interference from many of the endogenous components in the food matrix.

GC combined with Mass Spectrometry (GC-MS) is a recent advance which provides desired selectivity and sensitivity for analysis of most common trichothecenes (Jeleñ et al, 1997; Langseth and Rundberget, 1998; Onji et al, 1998; Mateo et al, 2001; Nielsen and Thrane, 2001). Resolving and improving the method by coupling MS

detectors with GC has lowered the detection limits to parts per billion levels of toxin in the sample (Chu, 1992). GC-MS have also been developed for simultaneous detection of type A and type B trichothecenes. Schollenberger et al (2005a) have employed a GC-MS method using Magnum Ion Trap system in the chemical ionization mode for the simultaneous detection of different trichothecenes after derivatization with trifluroaceticacidanhydride. GC-ECD for the simultaneous detection of trichothecenes has been reported by Seidel et al (1993) and Kotal et al (1999). A major problem associated with the use of GC-MS is the lack of clear structural information, even after derivatization of the sample. Another disadvantage is the simultaneous presence of interfering substances and mycotoxins having the same retention time at the selected mass (Onji et al, 1998; Chu, 1992).

1.5.8.2. Immunochemical Methods

Immunoassays for detection of mycotoxins have gained wide acceptance as a sensitive, specific and simple probe in the field of mycotoxin analysis. Immunoassays have been developed for the estimation of mycotoxins in foods, feed and biological fluids (Chu, 1991; Chu, 1992; Pestka et al, 1995). Antibodies against almost all commonly occurring toxins have been developed (Pestka et al, 1995). Primarily developed immunoassays for trichothecenes were based on the conjugation of the toxin to a protein or polypeptide carrier and injecting the conjugate to other animals yielding polyclonal antibodies (Fan et al, 1987b; Wang and Chu, 1991; Chu, 1992; Abouzied et al, 1993). With the advances in hybridoma technology, monoclonal antibodies have been developed and used widely for the detection of trichothecenes (Goodbrand et al, 1987; Sinha et al, 1995; Kohno et al, 2003). Gan et al (1997) have developed antibodies in chicken against the exoantigens and mycelia soluble antigens of *Fusarium*. Antibodies against exoantigens were specific to *Fusarium* genus whereas those to mycelia soluble antigens exhibited cross reactivity with antigens from other genera of fungi. The advantage of using immunoassays is the possibility of using samples directly for the assay, obviating the need for sample clean-up and is hence very useful in the analysis of food and feed commodities. The major problems associated with use of immunoassays are (1) need for a good labeled toxin as a marker and a specific antibody in the assay system, (2) good method for separation of free and bound toxin, (3) specificity of the antibody and (4)

possibility of structural analogues in the food matrifx that may react with the antibody (Chu, 1992).

The commonly used assay techniques include radio immunoassay (RIA) and enzyme linked immunosorbent assay (ELISA). Immunocolumns, holding the antibody in the purification column has also been widely used for clean-up or isolation of a specific toxin from the crude sample (Chu, 1992).

1.5.8.2.1. RIA

RIA involves incubation of a specific antibody simultaneously with a solution of unknown sample or a known standard and a constant amount of labeled toxin followed by quantifying radioactivity in the free and bound toxin. Concentration of toxin in the test sample can be calculated by comparing the results to a standard curve [(obtained by plotting ratio of radio activities in bound fraction and free fraction vs log concentration of the unlabelled standard toxin) (Chu, 1992)]. Use of RIA employing monoclonal and polyclonal antibodies has been widely reported for the detection of trichothecenes [(Wei and Chu, 1987- T-2, HT-2, DAS and their derivatives; Zhang et al, 1986- DON; Chu et al, 1984- Deoxyverrucarol (DOVE), DAS; Wang and Chu, 1991- Nivalenol)]. RIA allows detection of 0.25 to 0.5 ng of purified toxin in each analysis and 2 to 5 ng in food and feed samples (Chu, 1992). In spite of its many advantages, RIA faces major limitations like the need for use of a radioactive ligand, problems related to disposal and storage of the radioactive reagents used, necessity for specific instrumentation and the number of samples that can be conveniently analyzed (Pestka et al, 1981).

1.5.8.2.2. ELISA

Immunological assay system such as ELISA has been used widely as an ideal method for routine screening of large number of samples with minimal cleanup. The primary requirement for an effective ELISA is the antibodies that can display high degree of sensitivity and specificity against each toxin.

Two types of ELISA have been generally used for the analysis of mycotoxins

(i) Direct ELISA involving the use of a mycotoxin-enzyme conjugate and mono or polyclonal antibody specific to the mycotoxin or vice-versa.

(ii) Indirect ELISA involving the use of a protein-mycotoxin conjugate, a primary antibody specific to the mycotoxin and a secondary antibody to which an enzyme is conjugated (Chu, 1992).

Enzymes such as horse radish peroxidase (Pestka et al, 1981; Goodbrand et al, 1987: Abouzied et al, 1993; Sinha et al, 1995; Chung et al, 2003; Kohno et al, 2003), alkaline phosphatase (Kemp et al, 1986; Nagayama et al, 1988) and β-galactosidase (Chu, 1992) have been commonly used for conjugation to the secondary antibody. Presence of mycotoxin is determined by incubating with a substrate solution which is oxidized by the conjugated enzyme to give a colored complex. Advantage of indirect ELISA over direct ELISA is that less antibody (100 times less) is required for the former. Also, there is no need for preparation of a toxin-enzyme conjugate. ELISA is 10 to 100 times more sensitive than RIA when pure mycotoxins are used. Mycotoxin levels up to 2.5 pg can be measured efficiently with ELISA (Chu, 1992).

Both direct and indirect ELISA has been widely used for the detection of trichothecenes in food commodities and body fluids. Studies making use of ELISA for trichothecene testing are listed in Table 1.4.

1.5.8.2.4. Immunoaffinity Columns (IAF) and Immuno Chromatography

Antibodies attached to affinity columns have been efficiently used for clean-up or purification of mycotoxins prior to chemical analysis. Immunoaffinity columns have been primarily used for the recovery of aflatoxins from biological fluids which was later developed as a clean-up method for a number of mycotoxins (Groopman and Donahue 1988; Fremy and Chu, 1989; Patey et al, 1990; 1991).

Immuno chromatography, a method involving the use of ELISA as a post-column monitoring system for HPLC, has been employed for the analysis of group A trichothecenes. This method making use of generic antibodies against group A trichothecenes has been reported to efficiently monitor quantities as low as 2 ng of T-2 toxin and its derivatives (Chu and Lee, 1989; Chu, 1992).

Table 1.4. Use of ELISA for Detection of Trichothecenes

Sl. No.	Type of ELISA	Toxin detected	Sample	Reference
1.	Direct competitive ELISA	T-2	Artificially contaminated wheat and corn	Pestka et al, 1981
2.	Indirect ELISA	T-2	Urine, serum and milk	Fan et al, 1984
3.	Competitive ELISA	T-2	Corn	Gendloff et al, 1984
4.	Indirect ELISA	3-ADON	Rice	Kemp et al, 1986
5.	Indirect ELISA	T-2 and HT-2	Urine samples	Fan et al, 1987a
6.	ELISA	DON and its analogues	Corn	Casale et al, 1988
7.	Indirect competitive ELISA	T-2	*Fusarium* culture filtrate (screening)	Nagayama et al, 1988
8.	ELISA	DAS	*Fusarium* culture	Hack et al, 1989
9.	ELISA	NIV	Barley	Ikebuchi et al, 1990
10.	ELISA	DON	Wheat	Mills et al, 1990
11.	Direct ELISA	DON and T-2	Corn and wheat	Chu, 1991
13.	Indirect competitive ELISA	DNIV	Mouse and rabbit sera	Abouzied et al, 1993
14.	Direct competitive ELISA	DON and 15-ADON	Spiked corn and wheat	Sinha et al, 1995
15.	Indirect competitive ELISA	NIV and DON	Agricultural crops	Kohno et al, 2003

Flurometric assays [(using affinity columns carrying fluorescent labeled antibody) (Warden et al, 1987; Warden et al, 1990; Trucksess et al, 1991)], biosensors (Ligler et al, 1987; Williamson et al, 1989), HPTLC-ELISAgram [(separation of mycotoxins in HPTLC followed by membrane blotting and colour development using a suitable substrate) (Pestka, 1991)] etc have also been used for detection of trichothecenes.

1.5.8.3. Molecular Methods

Another approach for trichothecene detection is the identification of trichothecegenic *Fusaria* in the sample. Conventional identification methods are primarily used which involves microbiological investigation of infected samples on suitable agar media and enumeration of fungal propagules. In this case only the viable mycelia may be identified (Schnerr et al, 2001). Morphological features such as presence or absence of macroconidia, microconidia, their size, shape, colony morphology etc (discussed in section 1.3) are generally used as the means for identification which require great expertise and training (Bluhm et al, 2002). In case where interpretation of visual

symptoms is used, detection becomes possible only at later stages of infection when it is too late for any counter measures (McCartney et al, 2003).

Molecular characterization of trichothecene biosynthetic pathways has led to the development of molecular assays utilizing different genes making up the pathway. The gene *tri 5*, placed first in the trichothecene biosynthetic pathway, is a common functioning unit in all trichothecene producers irrespective of the group to which they belong (Desjardins et al, 1993). PCR assay based on this gene can easily detect trichothecene producers from the fungal lot in a given sample. Alignment of *tri 5* sequences from different trichothecene producers have shown that the *tri 5* open reading frame is conserved among all the chemotypes of *Fusarium* (Niessen and Vogel, 1998; Bakan et al, 2002). Differences in sequence have been observed in the intron region and the region upstream to *tri 5* gene (Hohn et al, 1999; Bakan et al, 2002; Li et al, 2005).

Many of the PCR methods available for detection of trichothecene producers have been based on utilizing primers targeting the *tri 5* gene (Wilson et al, 2004). Niessen and Vogel (1998) developed a PCR method for detection of trichothecene producing *Fusarium*. The primers Tox 5/1-Tox 5/2 amplified the 658 bp fragment of *tri 5* from all potential trichothecene producing *Fusaria* in contaminated cereals and malts. Differences in the genetic make up of *tri 5 gene* have been utilized for the development of qualitative and quantitative PCR assays for the detection of trichothecene producers in general, or for species specific and chemotype specific detection. Edwards et al (2001) developed a PCR assay based on *tri 5* where they observed good correlation between the *tri 5* DNA and concentrations of DON in *F. culmorum.* Bakan et al (2002), using primers targeted against *tri 5* and *tri 5-tri 6* intergenic regions, have developed a method for the detection of high and low DON producing stains of *F. culmorum.* They identified two separate clusters within the *F. culmorum* strains as a result of the variations in *tri 5* and the intervening sequence between *tri 5* and the adjacent *tri 6* that correlated very well with the secretion of DON. Li et al (2005) has reported the use of the intergenic sequence of *tri5-tri6* genes for the specific detection of DON and NIV chemotypes of *Fusarium*. Both uniplex and multiplex PCR assays have been developed for the detection of trichothecene producing *Fusaria* (Demeke et al, 2005). In the assay they used the Tox 5 primer sets of Niessen and Vogel (1998) for group specific detection of trichothecene producing

Fusaria and specific primers for detection of the isolates at the species level. Multiplex PCR has been successfully used for the simultaneous detection of toxigenic *F. culmorum, F. graminearum and F. sporotrichioides* in wheat samples from Canada. A RAPD-PCR based on 5' region of *tri 5* was developed by Wilson et al (2004) for the detection and differentiation of *F. sporotrichioides* and *F. langsethiae* from other species of *Fusarium*. PCR with the specific primers have amplified 300 and 360 bp fragments from the DON and NIV chemotypes, respectively. Other genes in the trichothecene biosynthetic pathway such as *tri 7* and *tri 13* have been employed for the characterization of *F. graminearum, F. culmorum* and *F. cerealis* in terms of their NIV and DON production (Lee et al, 2001; Chandler et al, 2003). Studies reporting the use of different genes involved in trichothecene synthesis for PCR analysis are listed in Table 1.5.

Table 1.5. Studies Using PCR for Detection of Toxigenic *Fusaria*

Sl. No.	Targetted gene	Purpose	Type	Source	References
1.	*tri 5* gene	Trichothecene producing *Fusarium*	Uniplex	Wheat	Niessen and Vogel, 1997
2.	*tri 5* gene	Trichothecene producing *Fusarium*	Uniplex	Trichothecene producers	Niessen and Vogel, 1998
3.	*tri 5* gene	Trichothecene producing *Fusarium*	Uniplex	*In vitro* and in planta	Doohan et al, 1999
4.	*tri 5* gene	Trichothecene producing *Fusarium and* quantification of *tri 5*	Uniplex	Trichothecene producers in FHB infections	Edwards et al, 2001
5.	*tri 5* gene	Large and small amounts of DON producers	Uniplex	Wheat	Bakan et al, 2002
6.	*tri 5* gene	Correlation between DNA content and conc. of DON	Real-time PCR	Wheat	Schnerr et al, 2002
7.	*tri 5* gene	Detection and taxonomy of trichothecene-producing species	Uniplex	Trichothecene-producers in the *Fusarium* section Sporotrichiella	Niessen et al, 2004.
8.	*tri 5* gene	Species specific identification of different *Fusarium* species and simultaneous detection of *F. culmorum*, *F. graminearum* and *F. sporotrichioides*	Uniplex and multiplex	Wheat	Demeke et al, 2005
9.	*tri 5* gene	Detection of toxigenic *Fusarium*	Real-time PCR	Toxigenic *Fusarium* in airborne and settled grain dust	Halstensen et al, 2006a
10.	*tri 5* gene	Level of *Fusarium* species	Semi-quantitative PCR, uniplex	Trichothecene-producing *Fusarium* species in grain dust	Halstensen et al, 2006b
11.	*tri 5* gene	Trichothecene producing *Fusarium*	Uniplex	Food and feed	Lincy et al, 2008
12.	*tri 6* and	Trichothecene and Fumonisin producing	Multiplex, real-time	Corn meal	Bluhm et al, 2002

	fum 5 genes	*Fusarium*			
13.	5' region of *tri 5* gene	Species specific detection and differentiation	RAPD and Touch down PCR	Wheat	Wilson et al, 2004
14.	*tri 5-tri 6* intergenic sequence	NIV and DON chemotypes	Uniplex	Wheat	Li et al, 2005
15.	*tri 3, tri 5* and *tri 7* genes	Distinguish NIV, 3- and 15-ADON chemotypes	Multiplex	A given species/population of the genus *Fusarium*	Quarta et al, 2006
16.	*tri 7* gene	DON and NIV producing chemotypes	Uniplex	Wheat	Lee et al, 2001

1.5.8.4. Commercial Trichothecene Detection Kits

To facilitate easy and rapid analysis of mycotoxins both in the field and in laboratory, many commercial mycotoxin detection kits have been developed with the concept of flexible "on-site" testing, that has eliminated the need for expensive and heavy electronic equipments. Evaluation and certification of kits as safety screening tools for food and other materials are facilitated by the Association of Official Analytical Chemists (AOAC) Research Institute, created by the AOAC international (Pestka et al, 1995).

Kits based on PCR, RT-PCR and other related techniques are rarely used for mycotoxin detection. Commercial immunoassay kits have been developed for a wide array of toxins that have performed well, both in laboratory and field (Keoltzov and Tanner, 1990; Dorner et al, 1993; Azer and Cooper 1991). The kits available are mainly membrane-based assays in the form of dipstick, enzyme-linked immunofiltration or flow-through devices and lateral flow or capillary migration (Schneider et al, 2004). In all these tests, specific antibodies are immobilized on to a membrane creating a reaction zone for all subsequent reaction steps. In the end a specific colour development on the membrane, which is dependent on the presence/absence of the mycotoxin is visually evaluated against a negative control. Trichothecene detection kits that are now commercially available for safety verification are listed in Table 1.6.

Table 1.6. Immunological Test Kits for the Analysis of Trichothecenes

Sl. No.	Test Kit	Analyte(s)	Form	Level	Comapany
1.	EZ-Screen	T-2	ELISA: Multisite card	12.5 ppb	DiAGnostix, Inc., Burlington, NC 27215
2.	Agri-screen	DON	ELISA: Microwell	1000 ppb	Neogen Corp., Lansing, MI 48912
		T-2	,,	500 ppb	”
3.	Veratox	DON	,,	300 ppb	”
		T-2	,,	50 ppb	”
4.	EZ-Quant DON 0.5 Test Kit	DON	ELISA: Microtitre plate	0.2 ppm	DIAGNOSTIX, Mississauga, ON, Canada L4Z 1N8
5.	EZ-Quant DON High Sensitivity Test Kit	DON	ELISA: Microtitre plate	0.025 ppm	”
6.	T-2 Toxin TTOO00101	T-2	Flow through kit	50 ppb, 100ppb, 200 ppb	Toxi-Test N.V., Harelbekestraat 72, B-9000 Ghent, BELGIUM
7.	-	DON	Rapid Portable Field Test	100 ppb, 200 ppb, 500 ppb, 1000 ppb	”
8.	Ridascreen	T-2	ELISA: Microtitre plate	3.5 ppb	R-BIOPHARM GmbH, Darmstadt, Deutschland
9.	Ridascreen EXPRESS	DON	ELISA: Microtitre plate	0.5, 1, 2,5 ppm	”
10.	*Myco DON*	DON	ELISA: Microtitre plate	0.25 ppm	Strategic Diagnostics Inc. Europe Business Park, London Road, Hook, Hampshire
11.	ImmunoscreenDON "GOLD"	DON	ELISA: Microtitre plate	0.05 ppm	Tecna S.r.l.Trieste, Italy
12.	DONTest HPLC	DON	IAC	0.1 ppm by HPLC	VICAM, Pleasant Street, Watertown, USA
13.	DONTest-TAG	DON	IAC	0.5 ppm by fluorometry	”
14.	T-2 TAG	T-2	IAC	0.15 ppm by fluorometry	,,
15.	QuickTox™ Kit DON	DON	Dipsticks	2 ppm	EnviroLogix Industrial Parkway, Portland, USA
16.	QuantiTox™ Trichothecenes Plate Kit	Trichothecene Mycotoxins	ELISA: Microwell	0.14 ppb	

1.5.9. Occurrence of *Fusarium* Toxins World Wide

Food, a complicated matrix composed of a wide array of ingredients including proteins, carbohydrates, fats and additives serves as energy source not only to humans and livestock, but also to a large number of microorganisms. The detection of foodborne microorganisms becomes difficult due to the complexity of the matrix in which they are embedded and the interfering substances. *Fusarium* toxins are reported worldwide from food and feed industries resulting in acute and chronic mycotoxicosis, both in animals and humans. Though trichothecene contamination rates second only to aflatoxins, cereal grains particularly wheat-either raw or processed- and its products are the major sources of DON, NIV and T-2 contamination worldwide. Type A toxins such as T-2, HT-2 and DAS are not frequently encountered and occurs in very low levels. They are more toxic than the type B trichothecenes and in most cases the intake levels are higher than the t-TDI levels (discussed in section 4.1.2. Tolerable Daily Intake Level). The intake levels of DON and NIV are far below the t-TDI for adult population group whereas for young children the intake of DON is either close to TDI or in most cases exceeded.

DON is the most widely distributed *Fusarium* toxin and is commonly encountered in mycotoxicosis outbreaks in several different countries (Bottalico and Perrone, 2002). 3-acetyl and 15-acetyl derivatives of the toxin are concomitantly found along with DON. The commodities with which DON has been associated include wheat, oats, corn and barley (Schothorst and van Egmond 2004; Placinta et al, 1999). Other trichothecenes such as HT-2, NIV and ZEA have also been reported in wheat (Schollenberger et al, 2002; 2005a; 2006). Different trichothecenes have been encountered in other food and feed items like barley (Ryu et al, 1996; Yoshizawa, 1997; Bottalico and Perrone, 2002), potato tubers (Latus-Zietkiewicz et al, 1995; Schollenberger et al, 2005a), maize (Wang et al, 1995a; Lauren et al, 1996; Ryu et al, 1996; Ritieni et al, 1997; Shetty and Bhat, 1997), sorghum (Ayalew et al, 2006), red pepper (Schollenberger et al, 2005a), oat kernels/grains (Hietaniemi and Kumpulainen, 1991; Langseth and Elen, 1996), chilly (Patel et al, 1996; Prasad et al, 2000), sunflower seeds (Schollenberger et al, 2005a; Rafaj et al, 2000), sunflower meal for feed (Rafaj et al, 2000) and hazelnuts (Schollenberger et

al, 2005a). In India trichothecenes have been reported from wheat and their by-products, maize and sorghum (Rukmini and Bhat, 1978; Bhavanishankar and Shantha, 1987; Bhat et al, 1989; Janardhana et al, 1999; Lincy et al, 2008). Worldwide distribution of the various trichothecenes in various commodities across the world is collated in Table 1.7.

Toxigenic *Fusarium* strains have also been isolated from a variety of commodities like pumpkins (Elmer, 1996); sunflower seed and nuts (Jimènez et al, 1991), bananas (Chakrabarti and Ghosal, 1986; Jimènez et al, 1997), infected wheat ears (Parry et al, 1995; Kosiak et al, 1997; Tóth, 1997; Pancaldi and Alberti, 2001), peanuts and sesame (Reddy and Reddy, 1994), rye grains (Fadl-Allah et al, 1997), amaranth (Bresler et al, 1991), coconuts (Zohri and Saber, 1993), maize (Desjardins et al, 2000), millet (Wilson et al, 1993), asparagus, legumes, onion and garlic (Snowdon, 1991) and coffee (Serani et al, 2007).

Maximum levels of T-2 and DON have been reported from Poland. DON levels as high as 927 mg/kg in maize and T-2 levels up to 24 mg/ kg in barley have been reported (Table 1.7). Up to 800 µg/kg level of T-2, 100 µg/kg of NIV and 8.38 mg/kg of DON have been reported in mould damaged wheat from the Kashmir valley of India that resulted in mycotoxicosis (Bhat et al, 1989). T-2 toxin levels up to 0.064 have been reported recently in sorghum (Lincy et al, 2008). Other than the report of Lincy et al (2008) DAS has been detected from Poland, Croatia and Nigeria (Table 1.7).

In a glance Table 1.7 shows that the reports on trichothecene contamination of sorghum are remnants of the occurrence data for other small grains and cereals, worldwide. In the current study we have focused on trichothecene production by isolates of *Fusarium* from sorghum and other food commodities. We have also screened a number of samples collected from the local market for the presence of trichothecenes.

1.5.10. Control Strategies for Prevention of *Fusarium* Infection

The strategies for prevention of *Fusarium* infection and trichothecene production should be implicated right from the field up to the consumption level in order to minimize or prevent trichothecenes from entering the human and animal food chains. To achieve this goal it is necessary that measures be taken both in the pre- and post-harvest periods during cultivation.

The control measures for pre-harvest period are mainly aimed at preventing *Fusarial* attack and the factors of choice include selection of resistant crop variety, good agronomic practices, weather conditions during flowering, type and amount of fungicides used and moisture content at harvest (Larsen et al, 2004). Intensity of FHB is related not only to the nature of trichothecenes produced, but also to the wheat genotype and effect of environment on host-trichothecene-pathogen interaction (Wong et al, 1995). Use of high yielding crop varieties that are resistant to *Fusarium* can inhibit both disease progression and toxin production (Vasanthi and Bhat, 1998). Chinese wheat varieties that were naturally resistant to FHB contained fewer levels of DON in grain in comparison with use of susceptible Canadian varieties (Wong et al, 1995). Resistance to trichothecenes may be induced by the introduction of *tri 101*, the gene coding for 3-*O*-acetyltransferase. Transgenic barley, rice and wheat, expressing the gene *tri 101*, have been shown to accumulate less amount of DON than that of the wild type (Manoharan et al, 2006; Kimura et al, 2006; Ohsato et al, 2007; Okubara et al, 2002). McCormick et al (1999) and Kimura et al (1998a) have reported of resistance of yeast cells to trichothecenes after transformation with *tri 101*. The gene *tri 101* which is located outside the core cluster contributes to the self protection of the producer *Fusarium* and the transformants by converting the toxins to their less toxic metabolites (McCormick et al, 1999). Appropriate agronomic practices include deep ploughing (to remove residual fungal material from the surface), tillage (mixing of the crop debris with the top 10-30 m soil) and crop rotation [(to break the continuous production of infectious material) (Larsen et al, 2004; Jouany, 2007)]. DON content of the grain decreased with the application of fungicides tebuconazole and metconazole in conventional farming systems (Bizrele et al, 2002). A highly controversial strategy for control of trichothecenes is the

introduction of non producing strains to displace the producing stains in the field or during storage (Desjardins et al, 1993). Competitive exclusion of mycotoxin occurs by the field release of non producing strains that are more competitive and hence outgrow the producer strains. However the toxin producing fungi would eventually kill the non producing fungi rendering this procedure ineffective. Transgenic plants carrying *Bt* gene (*Bt* toxin from *Bacillus thuringiensis*) contained reduced *Fusarium* toxins than their isogenic counterparts. In *Bt* maize hybrids decreased *Fusarium* ear rot symptoms and less accumulation of fumonisins were observed (Munkvold et al, 1997) whereas the *Bt* gene had only slight effect on *Fusarium* infection and trichothecene contamination in *Bt* maize (Magg et al, 2002, Schaafsma et al, 2002).

The control strategies for post-harvest period are mainly aimed at preventing mycotoxin accumulation after the fungal attack and include methods such as proper drying of the grains and sufficient storage capacity, hygiene at storage and management of the stored grain in short to medium term (Larsen et al, 2004). An important measure to be taken immediately after harvest is the efficient separation of diseased material from healthy grain in case the grain shows signs of fungal infection or disease symptoms (Larsen et al, 2004). Another critical factor in controlling fungal attack or mycotoxin accumulation is moisture management at harvest. Storage of grain at low moisture content (<14.5 %) together with regular sampling for accurate moisture determination play an important role in preventing fungal spoilage and mycotoxin production. Drying the grain to safe moisture levels and storage of crops in moisture free condition are important methods to avoid mycotoxin contamination. Insufficient cover or protection of the commodity during storage or transport and thereby getting exposed to the vagaries of monsoon may also lead to the development of mold contamination (Larsen et al, 2004).

Food commodities which are not severely contaminated can be subjected to various detoxification methods provided that the method assures complete breakdown of the toxin molecules without the formation of potential residual product. A simple method to reduce the contamination levels of grain is sorting which is elimination of infected and physically damaged kernels from the healthy ones (Jouany, 2007). Density segregation is a commonly used method which enables the separation of moldy grains from the healthy

ones (Placinta et al, 1999). Washing with water under pressure or 15 % sodium chloride has shown reduction up to 18 % and 23 % respectively in DON contaminated wheat (Vasanthi and Bhat, 1998). Considerable degree of decontamination has also been noticed during processing stages such as milling, baking etc. Presence of DON has been observed in the bran fraction during the milling of cereals (Vasanthi and Bhat, 1998). Up to 75 % reduction in DON content was observed after milling (Placinta et al, 1999). House et al (2003) have observed 34 % reduction in DON and ZEA levels after dehulling the grain. Dilution of the contaminated grain with other components has also been employed, especially when used as ingredients in compound feed (Placinta et al, 1999). Irradiation with Gamma rays and electron beam has significantly reduced *Fusarium* infection and toxin accumulation in barley (Aziz and Moussa, 2004; Kottapalli et al, 2003).

The reason behind the ineffective control of mycotoxin contamination in food or feed is the uneven distribution of the toxin in the sample. Hence regular samplings along with the use of proper analytical techniques are highly essential for the timely detection and control of establishment of toxigenic fungi in the crop or assay of toxin in the food commodities.

The study of literature available on trichothecenes and *Fusarium* highlighted the following points.

1. Most of the reports show that *Fusarium* infected and thereby trichothecene contaminated cereals and grains are the major source of trichothecene toxicosis. Deoxynivalenol, the most frequently encountered trichothecene toxin, is the major contaminant detected in wheat and barley (Table 1.7). Other trichothecenes are also reported from different food and feed commodities, the consumption of which results in acute toxicosis. *Fusarium* infection and trichothecene contamination results in a poor quality product which have been a serious problem faced by the cereal growers world wide.

   India is one among the major producers of cereals and grains worldwide. Very few studies have reported on trichothecenes in Indian foods and in most cases

no attempt has been made to identify the *Fusarium* species associated with it. Also there are only limited reports on analysis of samples from the Indian market. An effort was therefore made to understand the type of toxins or the nature of *Fusarium* species prevailing in India

2. The amount of toxin encountered in food is often below the limits required for conventional methods of detection. Also the morphological features of *Fusarium* vary considerably with changing environmental conditions resulting in features that are unreliable for authentication of species. Use of morphological features was replaced by DNA based method for specific detection of *Fusarium*. Chemotype specific and species specific PCR methods have been developed for the detection of trichothecegenic *Fusaria* (Table 1.5). There are only few studies using group specific PCR for the direct detection of toxigenic *Fusarium* from food materials.
3. Immunoassays such as ELISA, RIA etc, that make use of antibodies developed against the different trichothecene toxins or their derivatives, have been developed for the detection of trichothecenes (1.5.8.2. Immunochemical Methods). These antibodies are very specific to the particular toxin for which it is made. However, antibodies to the common enzymes in the metabolic pathway for these toxins may be expected to allow their simultaneous detection in food sample. It appeared that immuno detection of the enzymes involved in trichothecene synthesis would allow for the detection of pathogenic fungi, using methods that are more universal than that of PCR.
4. The literature has reference to the enhanced synthesis of trichothecenes in the presence of high glucose and low nitrogen (1.5.7.1. Physico-Chemical Factors Controlling Trichothecenes Production). However the cis acting elements on the promoters of *tri* genes controlling this has not yet been identified and this became a point of focus in this thesis.

In view of the above aspects a set of objectives were proposed for the present study, which can be listed as

1. Isolation and screening of trichothecene producing *Fusarium* species
2. Cloning of *tri 5* gene involved in trichothecene biosynthesis
3. Analysis of promoters for genes involved in trichothecene biosynthesis

Chapter 2.

Materials and Methods

All chemicals and reagents were of molecular biology grade or higher and were obtained from standard chemical suppliers. All microbiological media and molecular biology reagents were autoclaved at 121 ºC for 20 min or filter sterilized using Millipore disposable filters [0.2 µm (Acrodisc® Syrige filter, Pallman Corporation, MI, USA) or 0.4 µm (Millipore) as appropriate] in case of heat sensitive ingredients. Standard procedures described by Sambrook and Russell (2001) or manufacturer's instructions (for commercial kits) were followed for all molecular biological experiments.

2.1. Fungal Strains

Fusarium isolates used in this study were procured from different sources (3.2.1. Screening of *Fusarium* Isolates for Trichothecenes). The cultures were maintained on Potato Dextrose Agar (PDA) under refrigerated conditions. The standard culture (NCIM 651) a trichothecene producing *Fusarium* was obtained from National Collection of Industrial Micro-organisms, Pune, India.

2.1.1. *Fusarium* Isolation and Maintenance

Requirements

1. Potato Dextrose Agar (plates and slants)

   2.4 g of PDB was dissolved in 100 ml distilled water. 1.5 g agar was added and autoclaved (pH between 5-5.5).
2. Sterile water (10 ml and 9 ml tubes)
3. Scalpel
4. Nichrome wire
5. Dissection microscope

Procedure

To 10 ml sterile water tubes a small scrape of mycelia or 1g of food material was added and mixed properly to get a uniform suspension. Successive serial dilutions were prepared by transferring 1 ml of the suspensions to fresh 9 ml tubes (1:10 dilution obtained at each transfer), until 1-10 conidia were observed in a drop of the suspension when viewed under microscope at 100 X magnification. 100 µl of the suspensions were plated on to PDA, starting from the increasing dilutions and the plates were incubated at

28 ºC overnight for already growing cultures or for a period of 3-5 days for washings from food materials. Bacterial contamination during isolation of *Fusarial* cultures from food materials were avoided by including streptomycin at a concentration of 50 µg/ml in the medium while plating. Isolation of germlings in the plates was carried out under dissection microscope where the hyphae appeared as small threads on the agar surface. Using a sterile scalpel the agar piece bearing the hyphae was excised off and was placed on to fresh PDA plate with the help of sterile nichrome wire. The plates were incubated at 28 ºC for 3-5 days till a profuse mat like growth was observed on the agar surface. The purified *Fusarium* isolates were subcultured on to PDA slants and maintained.

2.2. Detection of Trichothecene Producing *Fusarium*

2.2.1. Extraction of Trichothecenes Produced by *Fusarium* Culture

Requirements

1. GYEP media
   5 % glucose
   0.1 % yeast extract
   0.1 % peptone
2. Corn
   Corn grits which were autoclaved twice prior to inoculation

Procedure

Production of trichothecenes by the *Fusarium* isolates was investigated. For toxin analysis by TLC and HPLC, inoculations were made by transferring mycelia from one week old cultures to previously autoclaved 250 ml flasks containing 50 g partially ground rice provided with a moisture content of 30-40 %. The inoculated rice was incubated at 28 ºC. For analysis of T-2 toxin, 20 ml of GYEP broth in 150 ml conical flaks were inoculated with mycelia from one week old cultures of *Fusarium* isolates and incubated at 28 ºC for two weeks.

2.2.2. Toxin Extraction from Rice Cultures

Requirements

1. Acetonitrile:water (84:16)
2. Chloroform:methanol (1:1)

3. Separating funnel
4. Whatman No.1 filter paper

Procedure

Rice cultures (50 g) were extracted with 500 ml acetonitrile:water and filtered through Whatman No.1 filter paper. 1 g celite was added to the filtrate and was shaken in a rotary shaker for 30 min. The extract was filtered through Whatman No. 1 filter paper. The filtrate was mixed with equal volume of chloroform:methanol in a separating funnel. Lower phase was evaporated to dryness and was stored at -20 °C until use.

2.2.3. Purification of Trichothecenes

Requirements

1. Purification column
2. Sodium chloride (NaCl)
3. Aluminium oxide
4. Hexane
5. Eluent solution

   Chloroform:Methanol:Acetone - 6:3:1
6. Anhydrous sodium sulphate

Procedure

A 10 ml microtip was used as the purification column. The lower end of the tip was plugged with cotton. 1 g of NaCl was packed into the column followed by the addition of 2 g of aluminium oxide after which 1 g of NaCl was packed again. The sample was dissolved in a minimum volume (1 ml) of acetonitrile:water and was loaded on to the column. The column was washed with 10 ml hexane to remove oils and pigments produced by *Fusarium*. Hexane was completely drained off and the column was eluted with the eluent solution, chloroform:methanol:acetone (20 ml/ sample). The eluted out toxin was passed through anhydrous sodium sulphate. The purified samples were concentrated by evaporation and stored at -20 °C.

2.2.4. Thin Layer Chromatography (TLC)

Requirements

1. Standard toxins T-2 toxin and DON were purchased from Sigma (St. Louis, MO, USA).
2. Glass plates, 20 cm × 20 cm and 5 mm width
3. TLC plate spreader (Camag)
4. Hexane
5. Silica gel
6. Capillary tube
7. UV chamber
8. Mobile Phase - TEF

   Toluene:Ethyl acetate:90% Formic acid - 6:3:1
9. *para*-anisaldehyde dye solution (For 17 ml)

   p-anisaldehyde - 0.001 ml

   Methanol - 14 ml

   Acetic acid - 2 ml

   Sulphuric acid - 1 ml

Procedure

(i) Preparation of TLC Plate

The glass plates were wiped with hexane. 5 g silica per plate was weighed out and mixed well with double the quantity of water in a stoppered conical flask for 1 min. Thin layer plates of 0.3 mm thickness were made using a TLC plate spreader. These plates were air dried at room temperature for 2-3 h and were activated by incubating at 110 ºC for 1 h. (The plates were activated again at 110 º C for 20 min, if they were used some other day). The plates were cooled to 40 ºC before spotting.

(ii) Analysis of Trichothecenes

TLC was carried out following the procedure of Scott et al (1970). The concentrated toxin samples were reconstituted in chloroform before spotting on to TLC plates. Using a capillary tube, 10 µl of the extracted toxin was spotted 2 cm above from

the bottom edge of the plate along with the standard toxin. The plates were then developed for a distance of 15-20 cm in the solvent system in a TLC tank that has been previously saturated with the mobile phase. The plates were dried in an oven at 80 °C for 20 min and observed under UV light (245 nm). The plates were sprayed with freshly prepared mixture of *p*-anisaldehyde. Again the plates were oven dried at 80 °C for 10-20 min for colour development. The plates were observed under visible light or UV light at 245 nm and characteristic spots were marked.

2.2.5. Detection of T-2 Toxin using Gas Chromatography (GC)

T-2 toxin was extracted from GYEP broth culture filtrates of *Fusarium* isolates as described by Tag et al (2001) and concentrated by heat evaporation. GC analysis was carried out following the method of Tag et al (2001). 5-10 µl of the toxin reconstituted in ethyl acetate was injected into the Gas Chromatograph (Shimadzu Gas Chromatograph GC-15A provided with a FID detector). GC analyses were performed on a SE-30 column (3 m x 3 mm id, mesh size 80/100, ageing temperature: 280 °C) packed with polydimethyl siloxane. Injector and detector temperatures were 260 °C and 270 °C respectively. The oven temperature was programmed from 100 °C (held for one min) to 280 °C (held for 10 min) at the rate of 10 °C/min. Nitrogen was passed through the column at the rate of 1 ml/min. Retention time of the toxin from the fungal isolates was compared with that of the standard. Spiking of sample with standard was routine.

2.2.6. Detection of DON using High Performance Liquid Chromatography (HPLC)

A modified method of Martins and Martins (2001) was followed. The toxin was reconstituted in a small volume of acetonitrile:water (84:16) and injected into HPLC attached with C-18 reverse phase column (Li Chrospher 100). The mobile phase used was acetonitrile:water in the ratio 84:16 at the rate of 1 ml/min. The column was maintained at 28 ºC. LC-10 AT Liquid Chromatograph (Shimadzu) provided with a SEL-10A *VP* Shimadzu UV-VIS detector was used and the results were recorded at 218 nm. Retention time of the toxin from fungal isolates was compared with that of the standard. Spiking of sample with standard was routine.

2.2.7. Specific Detection of Type A Trichothecenes using HPLC

2.2.7.1. Toxin Extraction

Requirements

1. 20 British standard mesh (BSM) sieve
2. Standard toxins (T-2, HT-2, DAS, DON, NIV procured from Sigma (St. Louis, MO, USA).
3. Acetonitrile: water (84:16)
4. Hexane
5. Separating funnel
6. Whatman No.1 filter paper
7. Dichloromethane
8. Chloroform: methanol (90:10)
9. 4-Dimethylaminopyridine (DMAP) (Merck Schuchardt OHG, Hohenbrunn, Germany)
10. 10. Coumarin-3-carboxylic acid (Sigma-Aldrich Chemie, Steinheim, Germany)
11. Thionyl chloride (Sigma-Aldrich Chemie, Steinheim, Germany)
12. Phosphate buffer (50 mM)

    0.2 g/L Potassium dihydrogen orthophosphate (KH_2PO_4)

    2.9 g/L Disodium hydrogen phosphate (Na_2HPO_4)

    8.5 g/L NaCl

    0.2 g/L Potassium chloride (KCl)

    The components were weighed and dissolved in double distilled water. pH of the buffer was adjusted to 7.5 and the volume was made up to 1 L.

13. Acetonitrile:water (65:35) containing 0.75 % acetic acid

Procedure

100 g of each sample was dried at 45 ºC for 48 h and ground to fine powder to pass through 20 BSM sieve. The samples were analyzed for type A toxins such as T-2, HT-2 and DAS. Extraction of the toxins was carried out following the modified procedure of Jimènez et al (2000). The ground food samples were mixed thoroughly. 10 g

each of the samples were blended with 50 ml of acetonitrile/water using a high-speed blender for 5 min and filtered through Whatman no. 1 filter paper prior to toxin analysis. The filtrate was defatted with 25 ml hexane, extracted with 30 ml of dichloromethane and finally concentrated to dryness under vacuum at 40 ºC. The residue was redissolved in 3 ml of chloroform/methanol prior to cleanup and derivatization (Mateo et al, 2002). All experiments were performed in duplicates.

2.2.7.2. Toxin Derivatization

Derivatization of the extracted toxins was carried out following the procedure of Mateo et al (2002). DMAP (10 µl) was added to the evaporated samples followed by the addition of 10 µl of coumarin reagent. The mixture was heated at 80 ºC for 20 min, cooled in ice water and redissolved in 0.4 ml toluene. Phosphate buffer was added to the vials and vortexed vigorously for phase separation. The upper organic phase was transferred to a fresh vial and evaporated to dryness under a slow stream of nitrogen. The concentrated toxin extracts were reconstituted in 75 µl of acetonitrile:water. The final concentration was adjusted by appropriate dilution whenever necessary in order to maintain the mycotoxins levels within the linear range of calibration.

2.2.7.3. High Performance Liquid Chromatography (HPLC)

HPLC was carried out for the identification and characterization of type A trichothecenes following the procedure of Mateo et al (2002). The concentrated toxin extracts were reconstituted in 75 µl of acetonitrile:water (65:35) containing 0.75 % acetic acid. The final concentration was adjusted by appropriate dilution whenever necessary in order to maintain mycotoxin levels within the linear range of calibration. The solution was filtered through 0.20 µm filter and injected into the HPLC system (SPD-10A VP dual mode) which consisted of a Waters 600 pump connected to a Waters 474 Scanning Fluorescence detector and a Digital Celebris 590 PC (Millenium Software was used). Chromatographic separations were performed on a C-18 reversed-phase column (stainless steel LiChrospher 100, 250-4 mm, 5-mm particle size) provided with a C-18 guard column (4-4 mm, 5 mm particle size). The mobile phase was relatively polar consisting of acetonitrile:water (65:35) containing 0.75 % acetic acid at a flow rate of

1 ml/min (isocratic). The excitation and emission wavelengths were set at 292 and 425 nm respectively, with a slit width of 18 nm for detection of fluorescence.

2.2.8. Gas Chromatography-Mass Spectrometry (GC-MS)

Requirements

1. Autoclaved rice (partially ground)
2. Acetonitrile
3. Zinc acetate
4. Celite 5
5. Ammonium sulpahte
6. Separating funnel
7. Hexane
8. Dichloromethane:chloroform (1:1)
9. Charcoal:alumina column (1:4) prepared in a 10 ml microtip
10. Chloroform:methanol (1:4).

Procedure

Inoculations were carried out by transferring mycelia from one week old cultures to previously autoclaved, partially ground rice, in 250 ml flasks, provided with 30 % moisture. The flasks were incubated at 28 °C for two weeks. The procedure for extraction of toxin as described by Onji et al (1998) was followed. 10 g of two week old cultures in rice were extracted with 50 ml acetonitrile and filtered. A 20 ml volume of the filtrate was mixed with equal volume of 10 % zinc acetate and allowed to stand at room temperature for 15 min. 10 g celite 545 was added to the mixture which was filtered and the filtrate was mixed with 3 g of ammonium sulphate in a separating funnel. The aqueous layer was re-extracted with 30 ml acetonitrile, mixed with double the volume of hexane and was allowed to stand for 10 min for phase separation. The lower phase was mixed with equal volume of dichloromethane-chloroform and passed through charcoal-alumina column (1:4) for toxin purification. The toxin was eluted out in 3 ml of chloroform-methanol (1:4). The eluate was evaporated to dryness and stored at 4 °C. The residue was finally dissolved in 1 ml of acetone prior to injection onto GC-MS instrument.

GC-MS was carried out following the modified procedure of Onji et al (1998). 2-5 µl of the toxin extract was injected into the Gas Chromatograph (TurboMass Gold Mass Spectrometer provided with a MS detector, Perkin Elmer Instruments). GC separations were performed on a SE-30 column (30 m x 0.25 id x 0.25 µm film thickness). Column oven temperature was maintained at 120 °C for 0.2 min, programmed from 120 to 250 °C at the rate of 20 °C /min. Nitrogen carrier gas was used at a constant flow rate of 1 ml/min. Injector and detector temperatures were 260 and 270 °C, respectively. The mass conditions were as follows: full scan mode; ionization energy: 70 eV: ion source temperature: 150 °C and interface temperature: 150 °C.

2.3. Polymerase Chain Reaction (PCR)

2.3.1. DNA Isolation

Mycelia were obtained after inoculating 20 ml PDB (HiMedia, Mumbai, India) in 100 ml flasks by transferring mycelia from one week old cultures and incubated at 28 °C for 3-4 days. Mycelia was collected by filtration and washed in sterile deionized water. DNA isolation was performed following the modified method of Lee et al (2001).

Requirements

1. Mortar and pestle
2. Potato Dextrose Broth (PDB- HiMedia Mumbai, India)
3. Fungal lysis buffer
   50mM EDTA (pH 8)
   50mM Tris (pH 8)
   3% SDS
   1% β-mercapto ethanol
4. Phenol equilibrated with 0.1M Tris-HCl, (pH 8): chloroform:isoamyl alcohol (25:24:1)
5. 70 % ethanol (Prepared from 95 % pure distilled ethanol)
6. TE buffer (pH 8)
   10 mM Tris- HCl (pH 8)
   1 mM EDTA (pH 8)

Procedure

Approximately 100 mg of mycelia was ground in 500 µl lysis buffer that has been preheated at 65 °C for 90 min. The contents were transferred to sterile micro-centrifuge tube and vortexed. The tubes were incubated for 90 min in a water bath set at 65 °C with intermittent vortexing of the tubes at intervals of 30 min. Equal volume of phenol:chloroform:isoamyl alcohol was added and the tubes were centrifuged at 10,000 rpm for 15 min. The supernatant was transferred to a fresh micro-centrifuge tube. Phenol:chloroform:isoamyl alcohol extraction was repeated 3-4 times which helped in minimizing protein contamination to a great extent. 200 µl of 1M NaCl and 800 µl of ice cold ethanol were added and incubated overnight at -20 °C for precipitation of DNA. The tubes were centrifuged at 10,000 rpm for 15 min. The pellet was washed in 70 % ice cold ethanol, finally suspended in suitable volume of TE buffer and stored at -20 °C thereafter. The quality of DNA preparation was checked by 0.8 % agarose gel electrophoresis (as described in section 2.3.2).

2.3.2. Agarose Gel Electrophoresis

Rquirements

1. Agarose (SRL, Mumbai, India)
2. 50 X TAE buffer pH 7.2 (1 L)

   Tris base - 242 g

   Glacial Acetic Acid - 57.1 ml

   0.5 M EDTA (pH 8) - 100 ml

   The buffer was made up to 1 L, autoclaved and stored at room temperature.
3. Gel casting boat
4. DNA loading dye (6 X)

   Xylene cyanol - 0.25 %

   Bromophenol Blue - 0.28 %

   Glycerol - 30 %

   Stored at 4 °C.
5. Mini gel apparatus and Power supply (Bangalore Genei, India)
6. Ethidium bromide stock solution (10 mg/ml)

10 mg of ethidium bromide was dissolved in 1 ml sterile deionized water. The solution was stored in a micro centrifuge tube wrapped in aluminium foil, at 4 °C. The solution was used at a working concentration of 0.5 µg/ml.

7. Gel Documentation unit (Herolab transilluminator, GmbK Laborgeräte, Ludwig and Chemidoc, BIO-RAD Laboratories, Italy)

Procedure

The boat was sealed with adhesive tape and comb was placed for the wells. Appropriate amount of agarose was weighed out and dissolved in 1 X TAE buffer, by heating in an oven. The mixture was cooled to 50 ºC and poured into the sealed boat. After the gel was polymerized properly, the comb and the adhesive tapes were removed and the gel was placed in electrophoresis tank containing sufficient volume of 1 X TAE buffer. 5 µl aliquot of the DNA preparation was mixed with 2 µl of the loading dye and was loaded into the well. Electrophoresis was carried out at 50 V till the dye reached 3/4th of the gel. The gel was removed from the tank and stained by soaking in ethidium bromide solution for 5 min. Finally the gel was destained in distilled water for 10 min before examination on a UV transilluminator and the image was documented using a gel documentation system.

2.3.3. Polymerase Chain Reaction (PCR)

Requirements

1. PCR thermocycler (Gene Amp PCR system 9700, Perkin-Elmer, USA)
2. Template DNA- genomic DNA isolated from *Fusarium* isolates.
3. Forward primer
4. Reverse primer
5. Taq DNA polymerase* (Bangalore Genei, India)
7. dNTP mix (2.5 mM each of dATP, dGTP, dTTP and dCTP; Bangalore Genei, India)
8. 10 X reaction buffer (15 mM $MgCl_2$, 0.1 % gelatin; Bangalore Genei, India)
9. Nuclease free water
10. 100 bp DNA marker (Bangalore Genei, India)

*Taq DNA polymerase was used in PCR for screening of *Fusarium*.

XT-Taq was used in PCR for amplification of the fragments to be used for sequencing.

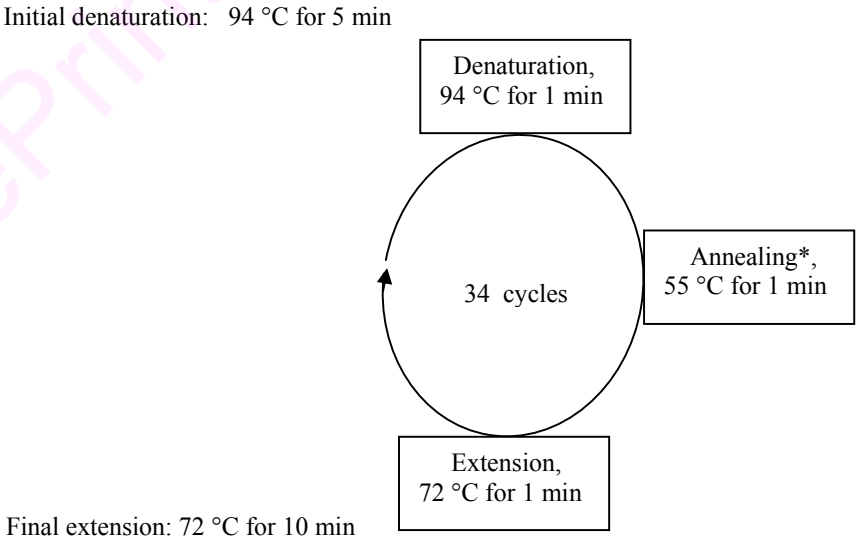
Procedure

PCR was performed with a 25 µl final reaction mixture containing the different components listed in the following table. The components were mixed properly before loading on to the PCR machine. The series of events taking place in the PCR reaction are schematically represented in Fig. 2.1.

Components	**Volume (µl)**	**Final concentration**
Nuclease free water*	17.7	-
10 X reaction buffer	2.5	1 X
dNTP mix	0.5	2.5 mM
Taq DNA polymerase	0.3	1 U
Forward primer	1	0.1 µM
Reverse primer	1	0.1 µM
Template DNA	2	~50 ng

*Volume of water was adjusted to make up to 25 µl final reaction volume

Fig. 2.1. PCR Reaction Cycle



(*Depends on the melting temperature of the primers)

A 10 µl aliquot of the PCR product was analyzed by agarose gel (1.5 %) electrophoresis as described in section 2.3.2. The size of the PCR amplicons was checked by comparing with a 100 bp DNA ladder (Bangalore Genei, India). The authenticity of the amplicon was again checked with nested PCR (same reaction conditions as described above) using the diluted primary amplicon as the template DNA.

2.4. Morphology and Conidial Characteristics

Requirements

1. Banana Leaf Agar (BLA)

2 g agar was added to 100 ml tap water and autoclaved. This is called Tap Water Agar (TWA). Banana leaf was washed properly, cut into small pieces and were autoclaved separately. During plating, sterile banana leaf pieces were placed in the petri plate using sterile forceps. TWA was poured over the leaf pieces in such a way that the pieces were immersed in the media. (For better results, the plates should be incubated for 1-2 h at 4 °C prior to inoculation for the leaf extract to diffuse into the agar)

2. Sterile distilled water
3. Brush
4. Slides and Cover slips
5. Needle
6. Glycerol
7. Phase Contrast Microscope (Olympus BX40 Olympus Optical Co. Ltd., Japan)

Procedure

Cultural characters like colony colour, nature of mycelial growth and pigment production were studied from one week old cultures on Potato Dextrose Agar. Mycelial suspension of each isolate (100 µl) was plated onto low nutrient Banana Leaf Agar and incubated at 28 °C temperature for 1-2 weeks to allow profuse sporulation. Spores were wiped out using a brush into an micro-centrifuge tube. About 40 µl of this suspension was taken on a slide and mixed with 20 µl glycerol. Using a needle, a cover slip was placed carefully over the spore suspension. Excess fluid was wiped out using tissue paper and the slides were observed under a Phase Contrast Microscope.

2.5. DNA Sequencing

Gene sequencing was carried out by dideoxy chain termination method of Sanger et al (1977). The reaction was carried out in an automatic DNA sequencer (ABI prism, Applied Biosystems, USA) with fluorescent dideoxy chain terminators at the Department of Biochemistry, University of Delhi, South Campus, New Delhi.

2.6. Cloning of PCR Product

Requirements

1. Luria Bertani (LB) medium (Ready to use LB broth and LB agar purchased from HiMedia, Mumbai, India or prepared using different media components purchased from Himedia, India)

   10 g/L Bacto-tryptone

   5 g/L Bacto-Yeast extract

   10 g/L NaCl

   pH of the media was adjusted to 7-7.2, autoclaved and maintained at room temperature.
2. pTZ57R/T vector (InsT/A Cloning kit, MBI Fermentas, Lithuania)
3. Components of PCR reaction mix (section 2.3.3)
4. GenElute PCR Clean-Up Kit (Sigma, USA)

2.6.1. Bacterial Strains and Plasmids

Escherichia coli (*E. coli*) strains DH5α (*supE*44 Δ*lac*U169(ǿ80-*lacZ*ΔM15)*hsdR*17 *recA*1 *endA*1 *gyrA*96thi-1 *relA*1) and BL21 (*hsdS gal*(λ*c* I*ts*857 *ind*1 Sam7 *nin*5 *lac*UV5-T7 gene1) were used as cloning and protein expression hosts, respectively. The cultures were maintained at 4 °C on LB agar medium.

2.6.2. Amplification of Gene Fragment

Amplification of specific gene from the genomic DNA of *Fusarium* was carried out by PCR using the gene specific primers following the procedure as described in section 2.3.3.

2.6.3. Cloning into T-tailed Vector (pTZ57R/T)

2.6.3.1. Purification of PCR Products

For purification, the PCR amplicons were obtained in bulk and pooled together. PCR amplicons were purified using GenElute PCR Clean-Up Kit (Sigma, USA) following manufacturers instructions. The purified PCR product was eluted out in 50 µl elution buffer (provided with the kit) and stored at -20 °C.

2.6.3.2. 'A' Tailing of PCR Product

The vector pTZ57R/T has been designed by the manufacturers in such a way that the multiple cloning site (MCS) of the vector was digested at the *EcoRV* site to create 'T' tail/overhang at its 3' ends. For easy and efficient ligation, the PCR products have to be prepared in such a way that they have an 'A' tail/overhang at their 3'ends.

Requirements

1. dATP (Bangalore Genei, India)
2. XT-Taq system (Bangalore Genei, India)
3. Sterile DNase free water
4. GenElute PCR Clean-Up Kit (Sigma, USA)
5. PCR amplification system

Procedure

A-tailing of the purified PCR amplicon was carried out by the method of Kobs (1997). To 5 µl of purified PCR fragment, 1 µl of *Taq* DNA polymerase reaction buffer (1X) was added. dATP to final concentration of 0.2 mM and 5 Units (2 µl) of *Taq* DNA polymerase (Bangalore Genie, India) were added to the reaction. The reaction volume was made up to 10 µl and the samples were incubated at 70 °C for 20 to 30 min. To remove the residual dATP present in the reaction mixture, the A-tailed PCR product was again purified using GenElute PCR Clean-Up Kit (Sigma, USA). The product was reconstituted in 30 µl deionized water and stored at -20 °C.

2.6.3.3. Ligation of A-Tailed PCR Product into T-tailed Vector

The A-tailed PCR product was T/A cloned by ligating to pTZ57R/T vector using InsT/A Clone PCR Product Cloning Kit (MBI Fermentas, Lithuania).

Requirements

1. Plasmid vector pTZ57R/T DNA*
2. 10 X Ligase Buffer* (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8)
3. PEG 4000 solution* (10 X 50 % w/v PEG 4000 solution)
4. T4 DNA Ligase*, 5 U/µl (Prepared in 20 mM Tris-HCl, pH 7.5; 1 mm DTT, 50 mM KCl, 0.1 mM EDTA and 50 % glycerol).
5. Deionized water*
6. A-tailed PCR product

* Supplied with the kit

Procedure

The following components were pipetted into a thin-walled 0.2 ml PCR reaction tube.

Components	Volume (µl)
Plasmid vector pTZ57R/T DNA	2.0
Purified PCR fragment	10.0
10X Ligase Buffer	3.0
PEG 4000 solution	3.0
T4 DNA Ligase, 5U/µl	1.0
Deionized water	11.0
Final volume	30

The reaction components were mixed by brief spinning and the samples were incubated at 22 °C for overnight. Heating the reaction mixture at 65 °C for 10 min inactivated the enzyme.

2.7. Transformation of *E. coli*

Transformation of *E. coli* DH5α and BL21 strains were carried out following the procedure of Sambrook and Russell (2001).

Requirements

1. LB broth (2 ml and 50 ml)
2. LB agar
3. SOB

   20 g/L Bacto-tryptone

   5 g/L Bacto-Yeast extract

   0.6 g/ L NaCl

   0.19 g/L KCl

   10 mM Magnesium sulphate (added from 1 M stock)

   10 mM Magnesium chloride (added from 1 M stock)

   The first four components and the magnesium salts were autoclaved separately and then mixed to constitute the SOB medium.

4. SOC medium

   To 1 ml of the basal SOB medium, 7 µl of filter-sterilized glucose solution (50 % w/v) was added.

5. 0.1 M $CaCl_2$ stock solution

   1.47 g of $CaCl_2$ was dissolved in 100 ml of deionized water. The solution was sterilized by filtration and stored at -20 °C.

6. Ampicillin stock solution

   Ampicillin resistance was used as the selection marker. Ampicillin (HiMedia, Mumbai, India) at a working concentration of 100 µg/ml was used. The solution was prepared by dissolving 100 mg ampicillin in 1 ml deionized water and sterilized by filtration. The solution was stored at 4 °C.

7. 0.1 M IPTG (isopropyl-β-D- thiogalactopyranoside) stock solution

   0.12 g of IPTG was dissolved in 5 ml of deionized water. The solution was filter-sterilized and stored as aliquots at -20 °C.

8. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) stock solution

   100 mg of X-Gal was dissolved in 2 ml of N, N'-dimethylformamide (DMF). The solution was stored at -20 °C in a micro centrifuge tube wrapped in aluminum foil.

9. Sterile polypropylene tube

10. Sterile micro-centrifuge tubes and micro-tips
11. Water bath

Procedure

2.7.1. Preparation of Competent Cells using $CaCl_2$

Competent cell preparation was carried out following the procedure of Sambrook and Russell (2001). A single colony of *E. coli* (DH5α strain) was picked up from a plate, freshly grown for 16-20 h at 37 °C and transferred into 2 ml sterile LB broth in a test tube. The tube was incubated at 37 °C overnight in a shaker incubator at 180 rpm. An aliquot of 200 µl of the overnight culture was added as inoculum to 50 ml sterile LB broth in 500 ml conical flask and incubated at 37 °C in a shaker incubator at 180 rpm. The OD_{600} of the culture was determined periodically to monitor cell growth. When the OD_{600} reached 0.40-0.50, the cells were transferred aseptically to 50 ml sterile polypropylene tube. The culture was cooled by storing the tube on ice for 10 min. The cells were recovered by centrifugation at 4000 rpm for 10 min at 4 °C. The medium was decanted from the cell pellet and the pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$. The tube was stored on ice for 10 min. The cells were recovered by centrifugation at 4000 rpm for 10 min at 4 °C. The supernatant was decanted and the tubes were kept in an inverted position for 1 min to allow the last traces of supernatant to drain away. The cell pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ and cells were stored at 4 °C overnight.

2.7.2. Transformation of Competent Cells

Transformation of *E. coli* was carried out by $CaCl_2$ method (Sambrook and Russel, 2001). About 100 µl suspensions of competent cells were added to sterile micro-centrifuge tubes. Plasmid DNA (~50 ng) or 2 to 5 µl of ligation mixture was added to each tube. The contents of the tubes were mixed by gently swirling and the tubes were stored on ice for 30 min. Competent cells that received plasmid DNA and without plasmid DNA were used as controls. The tubes were transferred to water bath set at 42 °C for 90 seconds so that the cells were subjected to heat shock. The tubes were rapidly transferred to ice and the cells were allowed to chill for 1-2 min. 800 µl of SOC medium

was added to each tube and the cultures were incubated for 45 min at 37 °C in a shaker incubator at 180 rpm.

2.7.3. Selection of Transformants

The transformants were selected on ampicillin containing LB agar plates. 30 µl each of X-Gal and IPTG were spread on the agar surface (before plating bacteria) for colour selection. 75-100 µl aliquots of the transformed bacterial cells were plated on to the agar surface. Control *E. coli* competent cells were plated on to LB plates containing ampicillin, X-Gal-IPTG and those devoid of them. The plates were incubated at 37 °C for 15-17 h.

2.8. Isolation of Plasmid DNA

The plasmids were isolated from the transformed *E. coli* cells by alkali lysis method (Birnboim and Doly, 1979)

Requirements

1. LB broth
2. Sterile micro-centrifuge tubes
3. Solution I

   50 mM glucose

   25 mM Tris-Cl (pH 8)

   10 mM EDTA (pH 8)
4. Solution II

   0.2 N NaOH (freshly prepared from 10 N NaOH stock solution)

   1 % SDS (Prepared freshly before use)
5. Solution III

   60 ml Potassium acetate (5 M)

   11.5 ml Glacial acetic acid

   28.5 ml Distilled water

   The resulting solution was 3 M and 5 M with respect to potassium and acetate, respectively.
6. Phenol:chloroform:isoamyl alcohol (25:24:1)
7. Iso-propyl alcohol

8. 70 % ethanol (prepared from 95 % pure distilled ethanol)
9. TE buffer (see section 2.3.1)

Procedure

Single colony of appropriate strains were inoculated into 2 ml of LB broth in test tubes containing required antibiotic and grown overnight at 37 °C in a shaker incubator at 180 rpm. 1.5 ml of the overnight cultures was transferred to a 1.5 ml micro centrifuge tube and the cells were harvested by centrifugation at 10,000 rpm for 2 min. After discarding the supernatant, 100 µl of solution I was added and vortexed vigorously to obtain a homogenous suspension. The samples were kept on ice for 5 min. About 200 µl of freshly prepared alkaline solution (solution II) was added to the tubes and mixed gently by inverting the tubes several times until the lysed cell suspension became clear. The samples were kept on ice for 5 min, 150 µl of ice-cold potassium acetate solution (solution III) was added. The contents of the tubes were mixed by inverting the tubes gently and were centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a fresh tube, equal volume of phenol:chloroform:isoamyl alcohol was added and vortexed thoroughly. Centrifugation at 10,000 rpm for 10 min separated the two phases. The upper aqueous phase was transferred to fresh tube and double the volume of iso-propyl alcohol was added. The tubes were kept at -20 °C for 1 h to overnight for precipitation of DNA. The tubes were centrifuged at 10,000 rpm for 10 min and the supernatant was discarded. The pellet was washed with 300 µl of 70 % ethanol and air-dried. The pellet was dissolved in 20 µl of TE buffer. The plasmids were checked by 0.8 % agarose gel electrophoresis (along with control plasmid for selection of the recombinant plasmid in the case of ligation samples). The plasmids solutions were stored at -20 °C for further use.

2.9. Confirmatory Tests for Identification of the Recombinant Plasmids

2.9.1. PCR

Presence of inserted gene in the recombinant plasmid was confirmed using PCR (section 2.3.3), using the gene specific primers and diluted plasmid DNA as template.

2.9.2. Restriction Digestion of Plasmid DNA

Requirements

1. Restriction enzymes (MBI Fermantas, Lithuania)
2. TY^+ Tango 10 X buffer (MBI Fermantas, Lithuania)
3. Nuclease-free water

Procedure

The gene that was cloned in pTZ57R/T was excised off from the vector using specific restriction enzymes. The following constituents were added in a micro centrifuge tube in the order mentioned.

Constituents	Single digestion	Double digestion
	Volume (µl)	
Nuclease-free water	12.0	11.0
10 X buffer*	2.0	2.0
Plasmid DNA	5.0	5.0
Restriction enzyme 1	1.0	1.0
Restriction enzyme 2	-	1.0
Final volume	20.0	20.0

*In case of single digestion the enzyme specific buffer was used.
TY+ Tango 10 X buffer in 1X or 2X concentration (after comparing compatibility of the two enzymes in requisite buffer) was used for double digestion.

The contents of the tube were mixed gently by pipetting and the tube was centrifuged briefly at 10, 000 rpm to collect the contents at the bottom of the tube. The reaction was carried out by incubating the tubes in 37 °C water bath for 4-8 h. The

samples were analyzed by 1.5 % agarose gel electrophoresis along with 100 bp DNA ladder (section 2.3.2).

2.9.3. Checking the Direction of Insertion of the Gene in pTZ57R/T

The recombinant plasmid was screened by PCR for checking the direction of the inserted fragment with respect to the *lac Z* promoter of pTZ57 vector. PCR was carried out using M13 forward and reverse primers in combination with gene specific forward and reverse primers and diluted plasmid DNA as template (procedure described in section 2.3.3).

2.9.4. Sequencing the Cloned Gene

DNA sequencing of the gene fragment cloned in pTZ57R/T was carried out using M13 forward universal sequencing primer as described in section 2.5.

2.9.5. Analysis of Nucleotide Sequences

The nucleotide sequences of the cloned genes were analyzed using various computer programs, such as nucleotide BLAST (Basic Local Arithmetic Search Tool; Altschul et al, 1997) and Dialign 2 (Morgenstern, 1999).

2.10. Sub-Cloning of Gene Fragment into Intermediate/Expression Vector

2.10.1. Purification of the Gene and Vector Fragments

The recombinant plasmid was multiplied in *E. coli* DH5α from which the gene fragment was excised off using specific restriction enzymes (MBI Fermantas, Lithuania) following the procedure as described earlier (section 2.9.2). The samples were analyzed by 1.5 % agarose gel electrophoresis along with 100 bp DNA ladder (section 2.3.2). The released fragment was separated on agarose gel, excised off from the gel and purified using QUIAquick gel extraction kit (Quiagen, Germany) following the manufacturers instructions. The purified fragments were suspended in 30 µl elution buffer (provided in the kit) and checked by 1.5 % agarose gel electrophoresis (section 2.3.2).

Vector was also treated with the same enzymes and incubated at 37 °C overnight. The vector fragment was purified by phenol:chloroform:isoamyl alcohol (25:24:1) extraction followed by isopropanol precipitation and was re-suspended in deionized nuclease free water.

2.10.2. Ligation

The following components were pipetted into a thin-walled 0.2 ml PCR reaction tube.

Components	Volume (µl)
Plasmid vector	1.0
Purified gene fragment *	10.0
10X Ligase Buffer	3.0
PEG 4000 solution	3.0
T4 DNA Ligase, 5U/µl	1.0
Deionized water	12.0
Final volume	30

*Concentration of the insert fragment should be ~8 times more than that of the vector.

The reaction components were mixed by brief spinning and the samples were incubated at 22 °C for overnight. Heating the reaction mixture at 65 °C for 10 min inactivated the enzyme.

2.10.3. Transformation and Plasmid Isolation

Transformation of *E. coli* DH5α with the ligation mixture and plasmid isolation was carried out following the procedure of Sambrook and Russell (2001) as described earlier (sections 2.7. and 2.8.).

2.10.4. Screening of the Recombinant Plasmids

The recombinant plasmids were screened and checked by PCR and insert release using selected restriction enzymes along with control plasmid, following the procedures as described in sections 2.9.1 and 2.9.2. Recombinant as well as the control plasmids were isolated in bulk and maintained at -20 °C.

2.10.5. Transformation of Recombinant Plasmids in *E. coli* BL21

Competent cell preparation, transformation, plasmid isolation and screening of the recombinants were carried out following the procedures as described in sections 2.7., 2.8., 2.9.1. and 2.9.2.

E. coli BL21, *E. coli* BL21 cells bearing control vector and the same recombinant construct were maintained on ampicillin containing LB agar plates.

2.11. Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out following the procedure of Laemmli (1970).

Requirements

1. Separating gel buffer

   1 g Sodium dodecyl sulphate

   45.4 g Tris buffer

   Dissolved in 450 ml double distilled water, pH adjusted to 8.9 with 1 N HCl and made up to 500 ml. Stored at 4 °C.

2. Stock acrylamide for separating gel

   30 g Acrylamide

   0.4 g Bisacrylamide

   Dissolved in 50 ml of double distilled water and made up to 100 ml. The Solution was filtered through Whatman No.1 filter paper and stored at 4 °C in dark brown bottle.

3. Stacking gel buffer

   0.40 g Sodium dodecyl sulphate

   6.06 g Tris buffer

   Dissolved in 190 ml of double distilled water, pH adjusted to 6.8 with 1 N HCl and made up to 200 ml.

4. Stock acrylamide for stacking gel:

   30 g Acrylamide

   0.4 g Bisacrylamide

   Dissolved in 30 ml of double distilled water and made up to 50 ml. The solution was filtered through Whatman No.1 filter paper and stored at 4 °C in dark brown bottle.

5. Tank Buffer

   5.76 g Glycine

   1.2 g Tris buffer

   0.4 g Sodium dodecyl sulphate

   Dissolved in 400 ml double distilled water and pH adjusted to 8.3 with 1 N HCl

6. Sample Buffer-10 ml (5 X)

   60 mm Tris HCl, pH 6.8

   25 % Glycerol

   2 % sodium dodecyl sulphate

   14.4 mM β-Mercaptoethanol

   0.1 % Bromophenol blue

7. Ammonium persulfate [(APS) (freshly prepared)]

   Ammonium persulfate (100 mg/ml) was prepared by dissolving 100 mg APS in 1 ml of deionized water.

8. TEMED (N,N,N',N'-Tetramethyl ethylene daiamine)

9. *n*-Butanol

Procedure

2.11.1. Preparation of Separating Gel (6 ml)

The following constituents were added in the given order.

Components	Volume
Separating gel buffer	3 ml
Acrylamide separating gel	3 ml
APS	30 µl
TEMED	30 µl

The components were mixed well and poured between two clean glass plates. The surface was layered with 5 ml of *n*-butanol and allowed to polymerize for 30 min. After polymerization n-butanol was removed and the gel surface was rinsed with water. The comb was inserted carefully into gel sandwich leaving 1-2 mm from the gel surface.

2.11.2. Preparation of Stacking Gel (5 ml)

The following constituents were added in the given order.

Components	Volume
Stacking gel buffer	1.75 ml
Acrylamide stacking gel	0.5 ml
Water	2.25 ml
APS	40 µl
TEMED	40 µl

The components were mixed well and the solution was poured over the separating gel. The gel was allowed to polymerize for 30 min after which the comb was carefully pulled out and the wells formed were marked properly.

2.11.3. Sample Preparation

To the sample (200 µl of the *E. coli* total cell extract or any other sample) sample buffer was added (to get 1 X sample buffer in a mixture), vortexed thoroughly and boiled for 1 min. The sample was cooled and centrifuged at 8000 rpm for 10 min to collect the supernatant. 15-20 µl of sample was loaded (depending on the protein concentration) into the wells along with protein size marker.

2.11.4. Electrophoresis Conditions

The gel was run at 50 mA constant current until the tracking bromophenol blue dye reached the end of the gel (about 3-4 h).

2.11.5. Staining and Destaining of the Gel

The gels were stained in 0.05 % (w/v) Coomassie brilliant blue R-250 in acetic acid:methanol:water (10:25:65 % v/v), for 0.5-18 h and destained repeatedly in the same solution without dye (methanol can be replaced with ethanol).

2.11.6. Documentation of Gel

After proper destaining, the gel was documented in a gel documentation unit (Chemidoc, UNIVERSAL HOOD II, BIO-RAD Laboratories, Italy).

2.12. Over-Expression of the Recombinant Protein in *E. coli*

Requirements

1. IPTG
2. LB broth (2ml tubes and 50 ml flasks)
3. Ampicillin (100 μg/ml)
4. Lysis buffer
   100 mM EDTA
   100 mM β-mercaptoethanol
   10 mM Phenyl Methyl Sulfonyl Fluoride (PMSF) dissolved in isopropanol
5. Sonicator
6. Dialysis tubing (Sigma-Aldrich, USA)
7. Bradford's Reagent (see section 2.14)
8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) components (see section 2.11)

Procedure

Expression of the cloned gene in recombinant plasmid was conducted in *E. coli* BL21. Overnight cultures were prepared at 37 °C by inoculating 2 ml LB broth with single colonies of *E.* coli BL21 bearing the recombinant plasmid. 200 μl of the culture was used to inoculate 50 ml LB broth containing ampicillin and incubated at 37 °C in a shaker incubator at 180 rpm till an OD_{600} of 0.8-1 was obtained. Induction of the *lac Z* promoter for over-expression of the cloned gene was carried out under IPTG induction for an incubation period of 12 h. Cells were harvested by centrifugation at 4000-6000 rpm for 10 min. The pellet was washed twice in deionized water, resuspended in lysis buffer and lysed by sonication. Sonication was repeated twice for 1 min while incubated on ice with an interval of 1 min. The supernatant collected after centrifugation at 10,000 rpm for 10 min were electrophoretically separated under denaturing conditions using 15 % (w/v) polyacrylamide slab gels (SDS-PAGE) with a 3 % (w/v) stacking gel. The supernatant was mixed with equal volume of sample buffer and were loaded in each lane.

Electrophoretic separations were carried out at 80 V for 4-5 h. Separated components were stained for proteins using Coomassie Brilliant Blue R-250.

2.13. Protein Purification by Gel Elution

The cell extracts of *E. coli* BL21 bearing recombinant plasmid was subjected to 15 % SDS PAGE (section 2.12.) along with cells bearing the vector itself which served as a negative control. Gel lanes, one each, having the cell lysates of control and recombinant were cut carefully and stained with Coomassie brilliant blue R-250 for 3-4 h. The remaining unstained portion of the gel was stored at 4 °C. The stained gel, after destaining, was carefully placed near the unstained gel. A portion of the unstained gel carrying the over-expressed protein was properly cut off and transferred to separating gel buffer. The gel pieces were placed in a dialysis tubing (12 KDa cut off) with adequate quantity of separating gel buffer and clamped properly. The dialysis tubing was placed perpendicular to the current flow inside the tank. The protein was electrophoretically eluted out from the gel pieces at 30 mA for 30 min using separating gel buffer as the tank buffer. The eluted out protein inside the dialysis tube was collected and dialysed against deionized water at 4 °C for 48 h. The purified protein was concentrated by lyophilization and stored at -20 °C. Purity of the protein was checked using 15 % SDS-PAGE and the protein concentration was analyzed using Bradford's method (1976).

2.14. Estimation of Protein

Protein estimations were carried out following the method of Bradford (1976).

Requirements

1. Coomassie Brilliant Blue G250
2. Bovine serum albumin (BSA) stock solution

BSA (100 µg /ml) was prepared by dissolving 100 µg APS in 1 ml of distilled water.

3. 95 % Ethanol
4. 85 % Phosphoric acid
5. Bradford Reagent

50 mg Coomassie Brilliant Blue G250

25 ml 95 % Ethanol

50 ml 85 % Phosphoric acid

Reagent was prepared by dissolving Coomassie Brilliant Blue G-250 in 95 % ethanol, followed by addition of phosphoric acid and the volume was made up to 500 ml.

Procedure

(i) Standard Curve

Bovine serum albumin (BSA) was chosen as the standard. Known concentrations of standard ranging from 1-10 µg in a total volume of 100 µl were pipetted out into clean test tubes. 100 µl deionized water was used as blank. 1 ml of Bradford reagent was added to each tube and mixed well by vortexing. The tubes were kept at room temperature for 30 min for colour to develop. The samples were transferred to cuvette and using reagent blank to zero the spectrophotometer. OD measurements were taken at 595 nm and a standard graph was plotted.

(ii) Analysis of Sample

Appropriate dilutions of the samples were prepared. 10 µl of the samples were pipetted out into clean test tubes and made up to 100 µl with distilled water. 1 ml of Bradford reagent was added and mixed thoroughly. The tubes were kept at room temperature for 30 min for colour development. OD was measured at 595 nm. Experiments were conducted in duplicates. Protein concentration for each sample was calculated using the standard curve equivalent.

2.15. Immunization of Poultry

Requirements

1. Antigen
2. Poultry
3. Phosphate buffered saline (PBS) 50 mM, pH 7.5 (section 2.2.7.1)
4. Freund's incomplete adjuvant (Sigma-Aldrich, USA)
5. Freund's complete adjuvant (Sigma-Aldrich, USA)
6. Sterile injection syringe and needle

Procedure

Twenty week old poultry was injected intramuscularly, at multiple sites with 225 µg of the immunogen that was dissolved in 500 µl of 50 mM PBS, emulsified in 500 µl

of Freund's complete adjuvant. The poultry received booster injections every 4 weeks after initial immunization with the same amount of antigen, emulsified in Freund's incomplete adjuvant, for a period of 4 months.

2.16. Isolation of IgY from Avian Eggs

Requirements

1. Phosphate buffered saline (PBS) 50 mM, pH 7.5 (Buffer preparation as described in section 2.2.7.1)
2. Chloroform
3. Poly ethylene glycol 6000 (PEG 6000)

Procedure

Eggs were collected from the immunized poultry everyday. The yolk was separated from the egg white and washed in sterile water. To the yolk 50 mM PBS (40 ml/yolk) was added and stirred in a magnetic stirrer for 30 min at room temperature.

Chloroform (10 ml/yolk) was added to the solution and stirring was continued for another 30 min. The solution was then centrifuged at 8000-10000 rpm for 30 min. The supernatant was centrifuged at the same conditions to obtain a clear supernatant. To the supernatant PEG 6000 (14 % w/v) was added and stirred in a magnetic stirrer for 30 min at room temperature. The precipitate was separated by centrifugation at 8000-10000 rpm for 30 min at room temperature. The pellet was dissolved in 50 mM PBS (10 ml/yolk) and stored frozen in small aliquots for further use.

2.17. Immunoblotting- Western Blot

Western Blot i.e, electro transfer of the separated components in SDS-PAGE to nitrocellulose (NC) membrane was carried out following the procedure of Towbin et al (1979).

Requirements

1. Nitrocellulose membrane [(NC) (Sigma-Aldrich, USA)]
2. Semi-dry blotter (Nova Blot, Pharmacia Biotech, Sweden)
3. Electrophoresis power supply (Bangalore Genei, India)

4. Blocking agent

Freshly prepared 2 % gelatin, prepared in TBS-T buffer (prepared as described in section 2.17.6) was used as the blocking agent.

5. Amido black
6. Tris Buffered Saline-Tween (TBS-T) buffer (50 mM) pH 7.2

    6.057 g/L Tris buffer

    8 g/L NaCl

    0.1 % Tween-20

The Tris buffer salt was weighed and dissolved in 800 ml of distilled water. The pH was adjusted to 7.2 using 1 N HCl. NaCl and Tween-20 were added to the solution and the volume was made up to 1 L with distilled water.

7. Primary antibody
8. Secondary antibody
9. 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (BCIP/NBT) (Bangalore Genei, India)

Procedure

The antigen preparations were separated on 15 % SDS-PAGE following the procedure as descried in section 2.11. The separated components were electro transferred to nitrocellulose (NC) membrane at the rate of 0.8 mA per cm^2 of membrane for 2 h in a semi-dry blotter. After transfer, part of the membrane was stained with amido black and the remaining membrane was blocked with 2 % gelatin for 2-3 h followed by washing the membrane 3-4 times, 10 min each with TBS-T buffer. The membrane was incubated with appropriate dilution of primary antibody at 37 °C for 2 h. After washing 3-4 times (10 min each) with TBS-T buffer, the membrane was incubated with appropriate dilution of secondary antibody for 1 h. The membrane was again washed 3-4 times with TBS-T buffer. The substrate BCIP/NBT was added to the membrane and was allowed to stand for 5-10 min for color to develop. The membrane was finally rinsed twice with deionized water to stop the reaction

2.18. Enzyme Linked Immuno Sorbent Assay (ELISA)

The indirect ELISA was according to the method of Engvall and Perlmann (1971) with modifications.

Requirements

1. Microtitre plate
2. Antigen dilutions
3. Primary antibody
4. Secondary antibody
5. 0.1 % TBS-T buffer (50 mM) pH 7.2 (Buffer preparation as described in section 2.17.6)
6. Blocking solution

   0.2 % bovine serum albumin (BSA) prepared in TBS-T buffer (prepared as described in section 2.17.6) was used as the blocking agent.
7. Diethanolamine buffer pH 9.8

   10 ml Diethanolamine

   0.2 g Sodium azide

   0.1 g Magnesium Chloride ($MgCl_2.6H_2O$)

   10 ml of the diethanolamine was dissolved in 100 ml of deionized water to obtain a 10 % solution. 97 ml of this solution was dissolved in 800 ml of distilled water. Sodium azide and $MgCl_2$ were added. The pH of the solution was adjusted to 9.8 using 1 M HCl. The volume of the solution was made up to 1 L and was stored at 4 $^{\circ}$C.
8. *para*-Nitro Phenyl Phosphate (*p*-NPP)

   1 mg/ml of *p*-NPP dissolved in diethanolamine buffer was used as the substrate for the assay.
9. 3 M NaOH
10. ELISA reader (Microplate Spectrophotometer- SPECTRA MAX 340, Molecular Devices, Sunnyvale, USA).

Procedure

The wells of the microtitre plate were coated with 100 µl of suitable dilutions of the antigen and kept overnight at 4 ºC for stabilization. The contents of the wells were

decanted and the wells were washed with TBS-T buffer 3-4 times. The wells were then blocked with 100 µl of BSA for 2 h at 37 ºC. Excess of blocking agent was removed by washing the wells 3-4 times with 0.1 % TBS-T solution. Suitable dilution of primary antibody (100 µl/well) was coated on to the wells of the plate and incubated at 37 ºC for 2 h. The wells were washed with TBS-T solution 3-4 times to remove excess of the antibody. 100 µl of suitable dilution of secondary antibody was added to each well and the plate was incubated at 37 ºC for 1 h. The non-reacted or excess antibody was removed by washing the wells with TBS-T solution 3-4 times. The substrate for Alkaline Phosphatase, *para*-Nitro Phenyl Phosphate (*p*-NPP) was added to the wells and incubated for 30 min for the colour to develop. The reaction was stopped using 50 µl of 3 M NaOH. Colour intensity was measured at 405 nm using an ELISA reader.

2.19. *Agrobacterium*-Mediated *Fusarium* Transformation

2.19.1. Transformation of *Agrobacterium*

Transformation of *Agrobacterium* was carried out following the freeze thaw method of An et al (1988).

Requirements

1. LB broth (5 ml and 50 ml)
2. LB agar
3. 20 mM $CaCl_2$ stock solution

0.294 g of $CaCl_2$ was dissolved in 100 ml of deionized water. The solution was sterilized by filtration and stored at -20 °C.

4. Kanamycin stock solution

Kanamycin resistance was used as the selection marker. Kanamycin (HiMedia, Mumbai, India) at a working concentration of 50 µg/ml was used. The solution was prepared by dissolving 100 mg of kanamycin in 1 ml deionized water and sterilized by filtration. The solution was stored at 4 °C.

5. Sterile polypropylene tube
6. Sterile micro-centrifuge tubes and micro-tips
7. Water bath

Procedure

Agrobacterium strain EHA 105 was grown overnight in 5 ml of LB broth at 28 °C. 2 ml of the overnight culture was added to 50ml of YEP broth in a 500 ml flask and was incubated in a shaker incubator at 180 rpm until the culture attained to an OD_{600} of 0.5 to 1. The culture was chilled on ice for 15 min. The cells were harvested by centrifugation at 4000 rpm for 10 min. The supernatant was discarded. The pellet was resuspended in 1 ml of 20 mM $CaCl_2$ solution (ice cold) and 100 µl aliquots were dispensed into pre-chilled micro-centrifuge tubes. Plasmid DNA (1µg) was added to the tubes and the tubes were then frozen by immersing in liquid nitrogen. For transformation the cells were thawed by incubating the tubes in 37 °C water bath for 5 min. 1ml of LB medium was added to the tubes and incubated at 28 °C for 2 to 4 h in a shaker incubator at 180 rpm. The tubes were centrifuged for 1 min at 4000 rpm and the cells were resuspended in 100 µl of LB broth. 100 µl aliquots of the transformed bacterial cells were plated on to LB agar surface and the plates were incubated at 28 °C. (Transformed colonies should appear in 2-3 days).

Note.

1. The cells can be frozen in liquid nitrogen and stored at -80 °C. The frozen cells can be used for future transformation experiments. Add about 1 µg of DNA to the frozen cells.
2. The plasmid can be either CsCl-banded DNA or a crude preparation isolated by a rapid small scale preparation.
3. Quick freezing is the most important factor of the procedure.

2.19.2. Transformation of *Fusarium* with *Agrobacterium*

Requirements

1. MM Salts for induction medium (IM)

   3.625 g KH_2PO_4

   5.125 g K_2HPO_4

   0.375 g NaCl

   1.25 g $MgSO_4.7H_2O$

   0.165 g $CaCl_2.2H_2O$

   0.0062 g $FeSO_4.7H_2O$

1.25 g $(NH_4)_2SO_4$

Dissolved each salt one at a time in 1 L of deionized water and stored at room temperature (Do not autoclave. The final solution typically contains a small amount of white precipitate).

2. 1 M MES (Stock Solution)

19.52 g of MES was dissolved in 80 ml of distilled water. pH was adjusted to 5.3 with 5 M KOH. The solution was filter sterilized and stored at -20 °C. (The salt if precipitated on thawing should be kept in a water bath at 65 °C for a while followed by vortexing to dissolve the salt.

3. 5 M KOH

7.013 g of KOH was dissolved in 25 ml of distilled water and stored at room temperature.

4. 10 mM Acetosyringone (AS)

0.01962 g of AS was added to 10 ml sterile water and stirred for 1 h. pH was adjusted to 8 with 5 M KOH. The solution was filter sterilized and stored at -20 °C.

5. 100 mg/l cefotoxime

The solution was prepared by dissolving 100 mg of cefotoxime in 1 ml deionized water and sterilized by filtration. The solution was stored at -20 °C.

6. 100 mg/ml augmentin

Prepared by dissolving 100 mg augmentin in 1 ml of deionized water and was sterilized by filtration.

7. M-100 Trace Element Solution

30 mg H_3BO_3

70 mg $MnCl_2.4H_2O$

200 mg $ZnCl_2$

20 mg Na_2MoO_4

50 mg $FeCl_3.6H_2O$

200 mg $CuSO_4.5H_2O$

The salts were dissolved in 500 ml deionized water and was stored at room temperature.

8. M-100 salt solution

16 g KH_2PO_4

4 g Na_2SO_4

8 g KCl

2 g $MnSO_4.7H_2O$

1 g $CaCl_2$

8 ml M-100 Trace Element Solution

The components were dissolved in 750 ml of deionized water and the final volume was made up to 1 L.

9. Induction Media

80 ml of 1 X MM salt solution

0.36 g of glucose (10 mM)

1 ml of glycerol (0.5 %)

The components were dissolved in 192 ml of deionized water and autoclaved. The solution was cooled to 50 °C, added 8 ml of 1 M MES (40 mM in the final solution).

10. Induction Media Plates

160 ml of 1 X MM salt solution

0.36 g of glucose (5 mM)

2 ml of glycerol (0.5 %)

6 g agar

The components were dissolved in 384 ml of deionized water and autoclaved. The solution was cooled to 50 °C, added 16 ml of 1 M MES (40 mM in the final solution).

11. Induction Media Plates with and without Acetosyringone (AS)

The components of IM plates were dissolved in 384 ml of deionized water and autoclaved. 8 ml of 10 mM acetosyringone and 8 ml of 1 M MES were added after cooling the media to 50 °C.

12. M-100 plates with 500 μg/ml cefotoxime, 300 μg/ml augmentin and 150 μg/ml hygromycin

10 g Glucose

3 g KNO_3

62.5 ml M-100 salt solution

15 g agar

The ingredients were dissolved in 750 ml deionized water, made up to a final volume of 1 L and autoclaved. 5 ml of cefotoxime, 3 ml of augmentin and appropriate volume of hygromycin (depending on the concentration of commercial stock) were added at the time of preparing plates.

13. Sterile filter paper discs

Whatman No. 1 filter cut to approporiate size was triple sterilized before use

Procedure

Agrobacterium mediated *Fusarium* transformation was carried following the method of Covert et al (2001). Pre-inoculum was prepared by inoculating 7 ml of LB (containing 50 μg/ml of kanamycin) with single colony of *Agrobacterium* (EHA 105) and the tubes were incubated in a shaker incubator at 29 °C overnight. OD_{660} of the culture was checked and the cells were diluted with IM (containing 200 μM AS) to a final volume of 20 ml to achieve an OD of 0.15. The *Agrobacterium* cells were incubated for 4 h with shaking at 29 °C and 250 rpm. The final OD should range from 0.6-0.8. Shortly before treating the *Agrobacterium* cells the *Fusarium* spores were harvested from one week old cultures on PDA plates using 5 ml of sterile water and were transferred to sterile polypropylene tubes. The spores were washed with distilled water and harvested by centrifugation at 8000 rpm for 10 min. The spores were diluted with IM to attain a final sore concentration of 10^5-10^6. Sterile filter papers were placed on IM and IMAS plates with sterile forceps. 100 μl of the diluted spores was mixed with 100 μl of *Agrobacterium* cells (OD_{660} = 0.6-0.8) and the mixture was spread evenly on the filter paper placed on IM and IMAS plates. (Care was taken to avoid spreading of the fluid outside the filter paper). The co-culture plates were incubated at 29 °C for 2 days after which the filter papers were transferred to M-100 plates containing 500 μg/ml cefotoxime, 300 μg/ml augmentin and 150 μg/ml hygromycin. The plates were incubated at 27 °C. Putative transformants were visisble after 3-5 days as rapidly growing circular colonies. The potential transformants were transferred to fresh M-100 plates (containing

500 μg/ml cefotoxime, 300 μg/ml augmentin and 150 μg/ml hygromycin) and the cultures after incubation were stored at 4 °C.

Chapter 3.

Isolation, Screening and Characterization of Trichothecene Production in *Fusarium*

3.1. Introduction

Fusarium, growing mainly on small-grain cereals such as barley, corn, oats, rye, wheat and sorghum in different parts of the world has been reported to produce tetracyclic sesquiterpenes, the trichothecenes. Fusarium head blight (FHB), caused by *F. graminearum*, is a devastating disease of wheat and barley that accounts for heavy economic loss to both growers and the industry. Leslie and Summerell (2006) have provided information on identification of species and the corresponding toxins produced by them, listing more than 35 *Fusarium* species with confirmed toxigenicity. More than 70 species of *Fusarium* have been identified and characterized by them on the basis of morphological characters. Among these only a small number of species are known to be pathogenic. The remaining less pathogenic or opportunistic *Fusarium* species are able to accumulate considerable amounts of mycotoxins during the infection process or when establishing itself in a food material (Table 1.2). Therefore the aetiological characterization of FHB is difficult since the clinical effects could be brought about by the synergistic action of both predominant and opportunistic *Fusarium* species.

Contamination of food materials with trichothecenes have been reported world wide (1.5.9. Occurrence of *Fusarium* Toxins World Wide). Most of the reports are with regard to contamination of cereals especially wheat, carrying often, considerably high levels of DON (Table 1.7). Other trichothecenes such as T-2, DAS, HT-2, NIV etc has also been reported from across the world though less frequently and not in high amounts. Sorghum is one of the world's staple foods, consumed widely in India. It is also an important raw material in feed industry. Other than the few reports from India and Ethiopia, most of the data for occurrence of trichothecenes in sorghum report their contamination with fumonisin producers (Leslie et al, 2005). Recently Lincy et al (2008) have identified contamination of T-2 and DAS in sorghum samples from India (section 4.3.3. Quantification of Trichothecenes). Bhavanishankar and Shantha (1987) and Rukmini and Bhatt (1978) have noted the presence of trichothecenes in sorghum in India.

A wide array of analytical techniques is available for the analysis of trichothecenes in food materials (1.5.8. Detection of Trichothecenes). Among the various techniques discussed in the chemical methods, TLC is the easiest and fastest method for

the screening of a given set of samples where more number of samples are involved. Not withstanding the ease by which TLC provides information regarding contamination of food with toxins, highly sensitive methods such as HPLC, GC in combination with mass spectrometry are widely preferred when accuracy and specificity is required.

The genus *Fusarium* is characterized by the presence of macro- and microconidia and chlamydospores and theses are used as the major identifying features (1.3. Morphological Characters). Secondary characters such as pigmentation and mycelial growth are also other important cultural characters that play a major role in identification. Determination of species and taxonomic identification has been a matter of debate for *Fusarium* researchers since their identification. This is because the changing environmental factors such as climatic conditions, geographical features, growth media etc can incite variations in the genetic make up of these fungi which are highly reflected in their morphological features. A direct consequence of confusion in taxonomy is confusion over species-mycotoxin associations. *Fusarium* isolates producing a particular toxin have been given different names as a result of the different taxonomic systems used, or simply as a result of misidentification. As a result conventional methods of classification and identification procedures have been revised repeatedly. A *Fusarium* classification system proposed by Joffe (1974) is explained in section 1.4.1 (Conventional Taxonomic Identification Based on Morphology). Taxonomic identification of *Fusarium* based on morphological features has in the recent past been replaced by molecular techniques (1.4.2. Molecular Taxonomy of *Fusarium*). The reliability of these methods needs confirmation.

In this chapter, work on the screening for trichothecene producers among *Fusaria* isolated from sorghum and other Indian foods has been presented. To this end a combination of techniques including PCR, TLC, GC, HPLC and GC-MS have been employed. Morphological and molecular methods have been used to characterize these isolates to the species level.

3.2. Materials and Methods

3.2.1. Screening of *Fusarium* Isolates for Trichothecenes

Of the 167 *Fusarium* isolates used in this study, 115 isolates from sorghum were kindly provided by International Crop Research Institute for Semi Arid Tropics (ICRISAT), Hyderabad. Other strains were isolated from different food materials such as maize, pigeon pea, coffee, ginger and vegetables such as beans (*Phaseolus vulgaris*), bitter gourd (*Momordica charantia*), chocho (*Sechium edule*), cauliflower (*Brassica oleracea botrytis*) and red chilli (*Capsicum annum*). The cultures were purified by single spore culture and maintained on potato dextrose agar (PDA, HiMedia, Mumbai, India) at 4 °C. NCIM 651 (identified as *F. asiaticum* in this study), a DON producer, obtained from National Collection of Industrial Micro-organisms, Pune, India, was used as standard culture.

In this study, isolation and purification of the *Fusarium* isolates were carried out following the single-spore sub-culturing method. The isolated germlings from the PDA plates, after serial dilution and plating, were transferred on to fresh PDA slants and maintained at 4 ºC.

3.2.2. PCR Assay for Gene *tri 5*

3.2.2.1. Design of Oligonucleotide Primers for PCR Amplification of *tri 5*

The gene *tri 5* is 1192 bp in length with two exons of length 469 and 658 bp respectively, and one intron of length 50-65 bp. The complete *tri 5* gene sequence of different trichothecene producers available in the NCBI databank were compared using Dialign 2 (Morgenstern, 1999), a program which gives base to base alignment of the given sequences. The *tri 5* gene sequences of various trichothecene producing *Fusaria* were highly conserved (99 %) at the exons. Variations were noticed at the introns and the region upstream to *tri 5* as have been reported also by Desjardins et al (1993).

Primers T5GF2 and T5GR1 for screening of *Fusarium* isolates were picked up from the highly conserved regions of exon 2 (Fig. 3.1). The primers span across ~652 bp of the exon. A third primer T5GR2 priming within the region of amplification of the first two primers was selected as a nested primer amplifying a ~379 bp fragment when used in

combination with T5GR1. Wobble base pairs were included in the primer sequences wherever sequence differences across the different species were observed.

Fig. 3.1. Schematic Diagram of *tri 5* Gene Showing the Priming Sites of Oligonucleotides

3.2.2.2. EF and ISSR Primers Used

EF1 and EF2 primers were used to amplify ~650 bp fragment, a part of the EF-1α gene of *Fusarium* isolates (Fig. 1.2) Inter Simple Sequence repeats (ISSR)-PCR was performed using ISSR random primers to identify the variations among the different trichothecene producing *Fusarium* isolates.

Oligonucleotide sequences of the primers used in the study are listed in Table 3.1.

PCR amplification of the *tri 5* gene fragment was carried out following the procedure as described in section 2.3.3. For the ISSR primers, amplification was carried out in 25 µl reaction mixture following the same PCR conditions with the difference that specific annealing temperatures of 40, 42 and 50 °C were used for the primers ISSR 810, ISSR 811 and ISSR 826, respectively. PCR amplification of EF-1α gene of the *Fusarium* isolates was carried out at an annealing temperature of 52 °C.

Table 3.1. List of Primers Used in the Study

Sl. No.	Oligo name	5'-sequence-3'**	Length (bp)
1.	T5GF2	5'-ACCATCCTCCATTCACCAC-3'	19
2.	T5GR2	5'-CACACCTCACCCTCCTTCT-3'	19
3.	T5GR1	5'-TYACTCCACTAGCTCAATTG-3'	20
4.	EF1*	5'-ATGGGTAAGGARGACAAGAC-3'	20
5.	EF2*	5'-GGARGTACCAGTSATCATGTT-3'	21
6.	ISSR 810	5'-GAGAGAGAGAGAGAGAT-3'	17
7.	ISSR 811	5'-GAGAGAGAGAGAGAGAC-3'	17
8.	ISSR 826	5'-ACACACACACACACACC-3'	17

*Primer sequences adapted from O' Donnel et al (1998b)

** Y: C+T, R: A+G, S: G+C

3.2.3. Screening of the Isolates for Production of Trichothecenes

The *Fusarium* isolates procured from different sources were primarily screened for the ability for trichothecene production by TLC, following the procedures as described in section 2.2.4. Production of T-2 and DON by the *Fusarium* isolates was further confirmed by GC (section 2.2.5) and HPLC (section 2.2.6) methods, respectively. Characterization of trichothecene production in selected isolates was carried out by GC-MS as described in section 2.2.8.

3.2.4. Gene Sequencing

The PCR amplified fragments of EF-1α gene of the *Fusarium* isolates were sequenced using EF1 primer following the procedure as described in section 2.5. The *tri 5* gene fragment amplified from the genomic DNA of a trichothecene producer was sequenced after cloning in pTZ57R/T (procedure described in detail in chapter 5).

3.2.5. Phylogenetic Analysis

Sequences of the EF1-PCR fragments were searched against those in the FUSARIUM-ID v. 1.0 database (Geiser et al, 2004) using BLAST (Altschul et al, 1997).

Neighbour Joining (NJ) analysis was performed using the heuristic search options with 1000 parsimony bootstrap replications on the *Molecular Evolutionary Genetics Analysis* version 3.1 software [(*MEGA3.1*); Kumar et al, 2004] on DNA. Indels were coded as single events.

3.3. Results and Discussion

3.3.1. Purification of *Fusarium* Isolates

Fusarium strains were isolated and purified from the various food commodities. As also reported by Leslie and Summerell (2006), genetic variance among the *Fusarium* cultures may not be reflected significantly in their morphological features that a uninucleate origin of the culture is essential. Food materials normally harbor numerous fungi and bacteria along with various *Fusarium* species. Even the growing cultures are likely to contain mixed cultures of other species and so purification of the cultures is very essential in the process of identification.

3.3.2. Trichothecene Production in *Fusarium* Isolates

3.3.2.1. TLC

Trichothecene production was monitored in 167 *Fusarium* isolates. A list of isolates positive for trichothecene production is provided in Table 3.2. At least one of the toxins T-2, DON, DAS or NIV were detected in the toxin extracts of 26.9 % (n = 45, 86.7 % of which were from sorghum) of the isolates whereas the extracts from the remaining 73.1 % (n = 122) of the isolates contained no trichothecene toxins. T-2 toxin produced pink colour in white light and blue fluorescence after UV exposure of TLC plates (raw data presented in appendix, Fig.A.1.A). DON appeared yellow/ orange in white light and brown in UV light (raw data presented in appendix, Fig.A.1.B). NIV was gray colour in white light and turned brown after exposure to UV. DAS was a pink coloured streak at the point of loading. After UV exposure the DAS producing isolates appeared as pink to red colored streaks on the TLC plate.

Among the trichothecene producers 51.1 % (n = 23) of the isolates were found to produce DON, 24.5 % (n = 11) produced T-2, 8.9 % (n = 4) produced T-2 and DON, 2.2 % (n = 1) produced T-2 and DAS, 2.2 % (n = 1) produced T-2, DON and DAS, 6.7 % (n = 3) produced DAS and 4.4 % (n = 2) produced NIV. Out of the 115 isolates from sorghum, 34 % (n = 39) were trichothecene producers.

Table 3.2. List of Isolates that Produced Trichothecenes

SL. No.	Name of Isolate [a]	Source	Toxin produced [b]	SL. No.	Name of Isolate [a]	Source	Toxin produced [b]
1.	ICR-PQ-10	Sorghum	T-2, DAS	24.	FM 302	Sorghum	DON
2.	ICR57	”	NIV	25.	FM 306	”	DON
3.	ICR1	”	T-2, DON	26.	FM 550	”	DON
4.	ICR-PQ-11	”	T-2	27.	FM 553	”	T-2
5.	ICR-PQ-13	”	T-2, DON	28.	FM 242	”	DON
6.	ICR-PQ-9	”	DON	29.	FM 246	”	DON
7.	ICR103	”	T-2	30.	FM 247	”	DON
8.	ICR15	”	DON	31.	FM 006	”	DON
9.	ICR18	”	DON	32.	FM 303	”	DON
10.	ICR-PQ-2	”	T-2, DAS, DON	33.	FM 307	”	T-2
11.	ICR106(1)	”	DAS	34.	FM 311	”	DON
12.	ICR61	”	DON	35.	FM 556	”	T-2
13.	ICR50	”	T-2, DON	36.	FM 243	”	T-2
14.	ICR4	”	DAS	37.	FM 244	”	T-2
15.	ICR11	”	T-2, DON	38.	FM 245	”	DON
16.	ICR110(1)	”	NIV	39.	FM 299	”	T-2
17.	ICR62	”	DAS	40.	Isolate 1	Beans	DON
18.	ICR-PQ-12	”	DON	41.	Isolate 2	Chilly	DON
19.	ICR113	”	T-2	42.	Isolate 3	Maize	DON
20.	ICR-PQ-4	”	DON	43.	Isolate 4	Maize	DON
21.	ICR8	”	T-2	44.	Isolate 5	Maize	T-2
22.	ICR96	”	DON	45.	Isolate 6	Chow chow	DON
23.	ICR-PQ-15	”	DON	46.	Std culture	NCIM	DON

[a] Isolates 1-39 provided by ICRISAT which were isolated from sorghum, isolates 40-45 isolated from local vegetables and isolate 46, a standard culture (NCIM 651) obtained from NCIM, Pune, India. [b] All isolates were positive for PCR. Toxins were analyzed by GC (T-2) or HPLC (DON) as appropriate.

In this study, TLC was used as the primary screening method for detection of trichothecene producing *Fusarium*. TLC is the least sensitive among the various other analytical techniques used for detection of toxins. Because it allows simultaneous analysis of multiple numbers of samples even without derivatization, the method has been widely used as a primary analytical technique where more samples are involved.

3.3.2.2. PCR for Detection of Trichothecene Producing *Fusaria*

All 167 isolates were subjected to PCR using T5GF2-T5GR1 primers, resulting in the amplification of ~652 bp outside the intron region of *tri 5* gene from the genomic DNA of all trichothecene producers irrespective of the different species or the type of toxin they produced (Fig. 3.2). Semi-nested primer PCR was carried out with T5GF2-T5GR2 primers using the amplicon of T5GF2-T5GR1 PCR as template DNA that resulted in the amplification of ~379 bp fragment.

PCR was carried out with other genera of fungi to check the specificity and reliability of the primers. Amplification was observed only from DNA isolated from the trichothecene producers and not from DNA isolated from other fungi (Fig. 3.3).

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Fig. 3.2. PCR Amplification of 652 and 379 bp Fragments of *tri 5* from *Fusarium*

Lanes. 1 and 2: ICR PQ-12; 3 and 4: ICR50; 5: 3 Kb DNA ladder; 6 and 7: ICR-PQ-2; 8 and 9: Isolate 1; 10 and 11: ICR61.

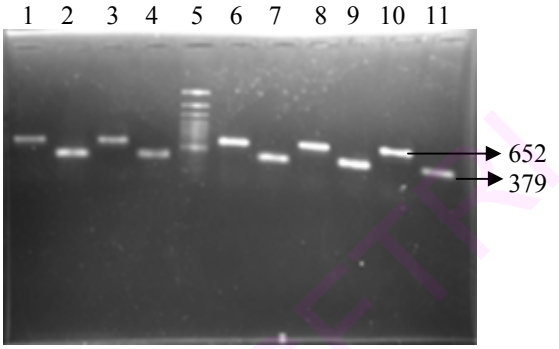
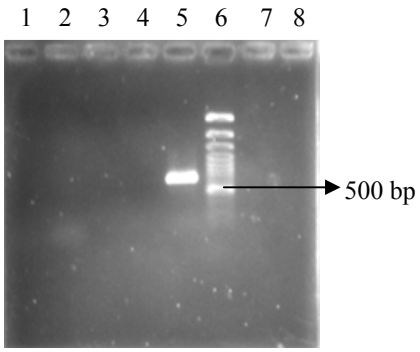


Fig. 3.3. Amplification of 652 bp *tri 5* Fragment from Different Genera of Fungi

Lanes. 1: *Rhizopus*; 2: *Aspergillus flavus*; 3: *Penicillium*; 4: *Aspergillus niger*; 5: *Fusarium* (NCIM 651); 6: 3-Kb DNA ladder; 7: *Fusarium chlamydosporum*; 8: Negative control (primer alone, without template).



The gene *tri 5*, the first in the trichothecene biosynthetic pathway, is a common function in all trichothecene producers and therefore serves as the primary target for most of the detection methods. A number of studies have used PCR for the detection of group and species specific identification of trichothecegenic *Fusarium* (section 1.5.8.3 and Table 1.5). Niessen and Vogel (1998) have been the pioneers in the use of PCR for detection of trichothecegenic *Fusarium*. PCR assays based on primers for *tri 5* have been reported by Doohan et al (1999), Edwards et al (2001), Schnerr et al (2001) and Halstensen et al (2006a).The method developed by Niessen and Vogel (1998) using primers specific for *tri 5* that detected potential trichothecene producing *Fusaria* in contaminated cereals and malts. In this chapter we report the use of PCR for the detection of trichothecegenic *Fusaria* in culture (while however the direct detection of such fungi from food is reported in chapter 4, section 4.3.2. Screening of Food Samples for the Presence of Trichothecenes). We have used a semi nested primer (that amplified a 379 bp fragment of *tri 5*) in addition to authenticate the PCR.

3.3.2.3. Sequencing of *tri 5*

The sequence of 652 bp fragment of *tri 5* obtained after sequencing using M13 forward primer is provided in Fig. 3.4.A. The sequence was homologous (100 %) to the *tri 5* gene sequence of *F. asiaticum* strain NRRL 6101 (AY102604). Multiple alignment of the sequences with that of other *Fusarium* species also indicated greater homology of the *tri 5* gene fragment with that of *F. asiaticum* (Fig. 3.4.B).

Fig. 3.4.A. Sequence of *tri 5* from NCIM 651

T5GF2 FOR PRIMER

GGTACCTCGCGAATGCATCTAGATT**AGGGATGCTTGATTGAGCAG**TATAACTTTGGTGGATTC
CCAGGATCTGATGACTACCCTCAATTCCTTCGACGCATGAATGGCCTGGGTCATTGTGTTGGG
GCTTCTCTATGGCCCAAGGAGCTGTTCGACGAGCGAAAGAATTTCCTCGAAATCACGACAGCC
GTTGCCCAGATGGAGAACTGGATGGTTTGGGTCAACGATCTCATGTCATTCTACAAGGAATTC
GACGATGAGCGTGACCAGATCAGTCTGGTCAAGAACTTTGTCACCTGCCATGAGATCACTCTC
GATGAAGCCTTGGAGAAGCTCACGCAGGAAACTCTGCACTCGTCCAAGCAGATGGTTGCTGTC
TTCGCGGACAAGGACCCTCAGGTGATGGACACGATTGAGTGTTTCATGCACGGCTACGTTACG
TGGCATTTATGCGACGCCAGATACCGCCTCCATGAGATTTATGAAAAGGTCAAGGATCAGGAT
ACAGAGGACGCTAAGAAGTTCTGCAAGTTCTTTGAGCAGGCAGCCAATGTCGGCGCCGTTGCA
CCCTCGGAGTGGGCTTATCCACA

Fig. 3.4.B. Multiple Sequence Alignment showing Sequence Identity of *tri 5* Gene of Common Trichothecene Producers

```
NCIM 651                ------AGGG ATGCTTGATT GAGCAGTATA ACTTTGGTGG ATTCCCAGGA
F.asiaticum             TTTTTGAGGG ATGCTGGATT GAGCAGTATA ACTTTGGTGG ATTCCCAGGA
F.culmorum              TTTTTGAGGG ATGCTGGATT GAGCAGTACA ACTTTGGAGG TTTCCCAGGA
F.pseudograminearum     TTTTTGAGGG ATGCTGGATT GAGCAGTAGA ACTTTGGAGG ATTCCCAGGA
F.sporotrichioides      TTTTCGAGGG ATGCTGGATC GAACAGTAGA ACTTTGGAGG ATTTCCAGGA
F.poae                  TTTTTGAGGG ATGCTGGATC GAGCAGTAGA GCTTTGGAGG TTTCCCAGGA
F.cerealis              TTTTCGAGGG ATGCTGGATT GAGCAGTAGA ACTTTGGAGG ATTCCCGGGA
F.langsethiae           TTTTCGAGGG ATGCTGGATC GAACAGTAGA ACTTTGGAGG ATTTCCAGGA
F.kyushuense            TTTTTGAAGG ATGCTGGATC GAGCAGTAGA ACTTTGGAGG ATTTCCAGGA
M.roridum               TCTTCGAGGG TTGCTGGATC GAGCAGTAGA ACTTCCACGG CTTCCCCGGC
T.harzianum             TTTTCCAAGG CTGCTGGATT GAGCAGCAGA ACTTCAAGGG CTTCCGTGGC
S.chartarum             TCTTTGAAGG TTGCTGGATT GAGCAATATA ACTTCCATGG CTTCCCAGGC

NCIM 651                TCTGATGACT ACCCTCAATT CCTTCGACGC ATGAATGGCC TGGGTCATTG
F.asiaticum             TCTGATGACT ACCCTCAATT CCTTCGACGC ATGAATGGCT TGGGTCATTG
F.culmorum              TCTGATGACT ACCCTCAATT CCTTCGTCGT ATGAACGGTT TGGGTCATTG
F.pseudograminearum     TCTGATGACT ACCCTCAATT CCTTCGACGC ATGAACGGTT TGGGTCACTG
F.sporotrichioides      TCTCATGACT ACCCTCAGTT TCTTCGACGT ATGAACGGCT TGGGTCATTG
F.poae                  TCCCATGACT ATCCTCAGTT TCTTCGACGC ATGAATGGCT TGGGTCACTG
F.cerealis              TCTGATGACT ACCCCCAATT CCTTCGTCGT ATGAACGGTT TGGGTCACTG
F.langsethiae           TCTCATGACT ACCCTCAATT TCTTCGACGT ATGAACGGCT TGGGTCATTG
F.kyushuense            TCCCATGATT ATCCTCAGTT TCTTCGACGC ATGAACGGCT TAGGCCATTG
M.roridum               TCTTATGACT TCCCCGGCTT CCTCCGTCGC ATGAACGGTC TTGGCCACTG
T.harzianum             TCCAGTGACT ACCCTGGCTT CCTCCGCCGT ATCAACGGCC TGGGCCATTG
S.chartarum             TCCTTTGACT ATCCTGGGTT CCTTCGTCGT ATGAATGGAC TAGGACACTG

NCIM 651                TGTTGGGGCT TCTCTATGGC CCAAGGAGCT GTTCGACGAG CGAAAGAATT
F.asiaticum             TGTTGGGGCT TCTCTATGGC CCAAGGAGCT GTTCGACGAG CGAAAGAATT
F.culmorum              TGTTGGGGCT TCTCTATGGC CCAAGGACCT GTTCGACGAG CGAAAGAACT
F.pseudograminearum     TGTTGGGGCT TCTCTATGGC CCAAGGACCT GTTCGATGAG AGGAAGCATT
F.sporotrichioides      TGTCGGGGCT TCTCTATGGC CCAAGGAGCA GTTCAATGAG CGAAGTCTAT
F.poae                  TGTCGGGGCT TCTTTGTGGC CCAAGGAGCA GTTTGATGAA CGAAGTCTAT
F.cerealis              TGTTGGGGCT TCTCTATGGC CCAAGGACCT GTTCGACGAG CGAAAGAATT
F.langsethiae           TGTCGGGGCT TCTCTATGGC CCAAGGAGCA GTTCAATGAG CGAAGTCTAT
F.kyushuense            TGTCGGGGCT TCTTTGTGGC CCAAGGAGCA GTTTGATGAG CGAAGTCTGT
M.roridum               TGTTGGAGGA TCTCTGTGGC CCAAGGAGCT TTTCGACGAG CAGAAGCACT
T.harzianum             TGTCGGCTCT TCAATCTGGC CTATTGAACT TGTGGATGAG GAAGAGCACT
S.chartarum             TGTCGGAGGA TCTTTGTGGC CAAAGGAAAA CTTCAACGAG CAGGAGCATT

NCIM 651                TCCTCGAAAT CACGACAGCC GTTGCCCAGA TGGAGAACTG GATGGTTTGG
F.asiaticum             TCCTCGAAAT CACGACAGCC GTTGCCCAGA TGGAGAACTG GATGGTTTGG
F.culmorum              TCCTCGAAAT CACGACAGCC GTTGCCCAGA TGGAGAACTG GATGGTTTGG
F.pseudograminearum     TCCTTGAAAT CACATCAGCC GTTGCCCAGA TGGAGAACTG GATGGTTTGG
F.sporotrichioides      TCCTTGAGAT TACATCAGCC ATTGCCCAGA TGGAGAACTG GATGGTCTGG
F.poae                  TCCTTGAAAT CACATCAGCC ATTGCTCAGA TGGAGAACTG GATGGTCTGG
F.cerealis              TCCTCGAAAT CACGACAGCC GTTGCCCAGA TGGAGAACTG GATGGTTTGG
F.langsethiae           TCCTTGAGAT TACATCAGCC ATTGCCCAGA TGGAGAACTG GATGGTCTGG
F.kyushuense            TTCTTGAAAT TACATCAGCC ATTGCCCAGA TGGAGAACTG GATGGTTTGG
M.roridum               TCCTTGAGAT TACCAGCGCC GTTGCTCAGA TGGAGAACTG GATGGTGTGG
T.harzianum             TCCTCGAAAT TACCACAGCA ATCGCCCAGA TGGAGAACTG GATGGTCTGG
S.chartarum             TCTTGGAAAT CACCAGCGCC ATCGCCCAAA TGGAAAACTG GATGGTTTGG

NCIM 651                GTCAACGATC TCATGTCATT CTACAAGGAA TTCGACGATG AGCGTGACCA
F.asiaticum             GTCAACGATC TCATGTCATT CTACAAGGAA TTCGACGATG AGCGTGACCA
F.culmorum              GTCAATGATC TCATGTCGTT CTACAAGGAA TTCGACGATG AGCGTGACCA
F.pseudograminearum     GTCAACGATC TCATGTCGTT CTACAAGGAA TTCGACGATG AGCGTGACCA
F.sporotrichioides      GTCAATGATC TTATGTCATT CTACAAGGAA TTCGACGATG AGCGTGACCA
F.poae                  GTCAACGATC TTATGTCATT CTACAAGGAA TTCGACGATG AGCGTGACCA
F.cerealis              GTCAATGATC TCATGTCGTT CTACAAGGAA TTCGACGATG AGCGTGATCA
F.langsethiae           GTCAATGATC TTATGTCATT CTACAAGGAA TTCGACGATG AGCGTGACCc
F.kyushuense            GTCAATGATC TTATGTCGYT CTACAAGGAA TTCGACGATG AGCGTGACCA
M.roridum               GTTAACGACT TGATGTCTTT CTACAAGGAG TTTGATGACC CTCGTGACCA
S.chartarum             GTTAACGACC TCATGTCCTT CTACAAGGAG TTCGACGACC CTCGCGACCA
```

The bases in red colour indicate the sequence variation of *tri 5* of NCIM 651 from that of the other trichothecegenic *fungi. F.asiaticum* (AY102604), *F.culmorum* (AY102602), *F.pseudogramiearum* (AY102582), *F. sporotrichioides* (AY130293), *F.poae* (AY130294), *F.cerealis* (AY102574), *F.lngsethiae* (AF449793), *F.kyushuense* (AF44989), *M.roridum* (AF009416), *S.chartarum* (AF329103)

3.3.2.4. HPLC Detection of DON

The culture filtrates of isolates that appeared positive after TLC analysis were characterized further by HPLC. A total of 27 isolates (60 % of the 45 toxin positive isolates) were thus identified as DON producers. The peak specific for DON was obtained at 3.4 min [(± 0-0.4) (raw data presented in appendix, Fig. A.2)]. Spiking of samples with pure toxin resulted in increased peak area. Results of the effort at detecting DON in the culture filtrates of the 46 isolates of *Fusarium* tested are given in Table 3.3.

Table 3.3. Isolates Positive for DON by HPLC

SL. No.	Name of Isolate	DON production	SL. No.	Name of Isolate	DON production
1.	ICR-PQ-10	-	24.	FM 302	+
2.	ICR57	-	25.	FM 306	+
3.	ICR1	-	26.	FM 550	+
4.	ICR-PQ-11	-	27.	FM 553	-
5.	ICR-PQ-13	+	28.	FM 242	+
6.	ICR-PQ-9	+	29.	FM 246	+
7.	ICR103	-	30.	FM 247	+
8.	ICR15	+	31.	FM 006	+
9.	ICR18	+	32.	FM 303	+
10.	ICR-PQ-2	+	33.	FM 307	-
11.	ICR106(1)	-	34.	FM 311	+
12.	ICR61	+	35.	FM 556	-
13.	ICR50	+	36.	FM 243	-
14.	ICR4	-	37.	FM 244	-
15.	ICR11	+	38.	FM 245	+
16.	ICR110(1)	-	39.	FM 299	-
17.	ICR62	-	40.	Isolate 1	+
18.	ICR-PQ-12	+	41.	Isolate 2	+
19.	ICR113	-	42.	Isolate 3	+
20.	ICR-PQ-4	+	43.	Isolate 4	+
21.	ICR8	-	44.	Isolate 5	-
22.	ICR96	+	45.	Isolate 6	+
23.	ICR-PQ-15	+	46.	Std culture	+

3.3.2.5. GC Detection of T-2

Production of T-2 was characterized by GC. 14 isolates (31.1 % of the 45 toxin positive isolates) were identified as producers of T-2 (Table 3.4). T-2 specific peak was obtained at 20.4 min [(± 0-0.4) (raw data presented in appendix, Fig. A.3)]. Spiking of samples with pure toxin resulted in increased peak area.

Table 3.4. Isolates Positive for T-2 by GC

SL. No.	Name of Isolate	T-2 production	SL. No.	Name of Isolate	T-2 production
1.	ICR-PQ-10	+	24.	FM 302	-
2.	ICR57	-	25.	FM 306	-
3.	ICR1	+	26.	FM 550	-
4.	ICR-PQ-11	+	27.	FM 553	+
5.	ICR-PQ-13	-	28.	FM 242	-
6.	ICR-PQ-9	-	29.	FM 246	-
7.	ICR103	+	30.	FM 247	-
8.	ICR15	-	31.	FM 006	-
9.	ICR18	-	32.	FM 303	-
10.	ICR-PQ-2	-	33.	FM 307	+
11.	ICR106(1)	-	34.	FM 311	-
12.	ICR61	-	35.	FM 556	+
13.	ICR50	-	36.	FM 243	+
14.	ICR4	+	37.	FM 244	+
15.	ICR11	-	38.	FM 245	-
16.	ICR110(1)	-	39.	FM 299	+
17.	ICR62	-	40.	Isolate 1	-
18.	ICR-PQ-12	-	41.	Isolate 2	-
19.	ICR113	+	42.	Isolate 3	-
20.	ICR-PQ-4	-	43.	Isolate 4	-
21.	ICR8	+	44.	Isolate 5	+
22.	ICR96	-	45.	Isolate 6	-
23.	ICR-PQ-15	-	46.	Std culture	-

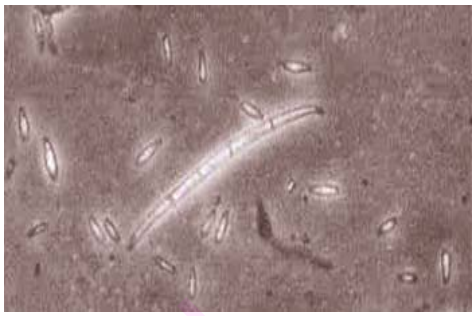
3.3.3. Variation Among the Isolates of *Fusarium*

The morphological characteristics of the colonies formed by toxin positive isolates in PDA, such as colony colour and nature of mycelial growth were noted. Growth features of fungal spores on BLA such as the type of conidia present and their shape were studied (Fig. 3.5). The differences which were observed in colony as well as spore morphology of the different isolates are listed in Table A.1.

Fig. 3.5. Macro- and Microconidia from *Fusarium* Isolates

ICR61

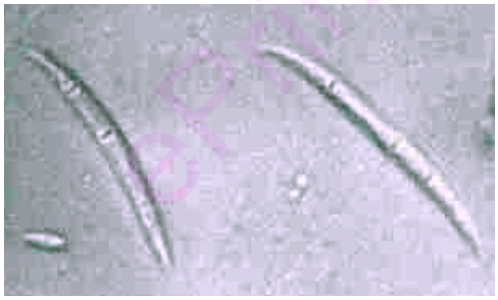
ICR50



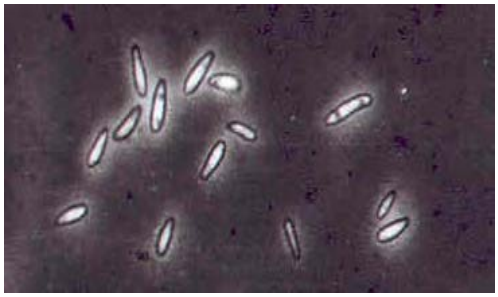
ICR-PQ-12

ICR-PQ-2

Standard culture

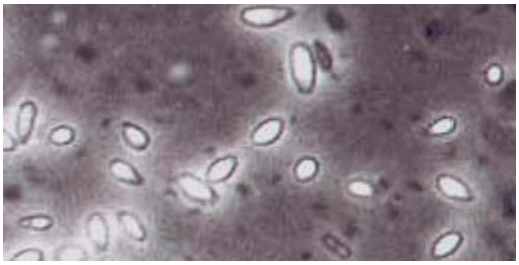


FM550

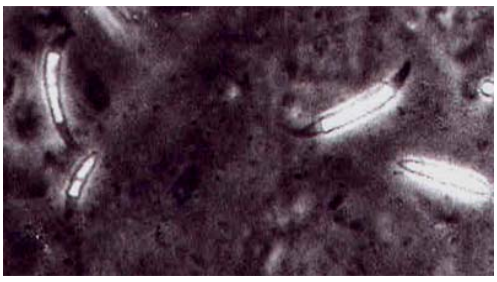


FM311

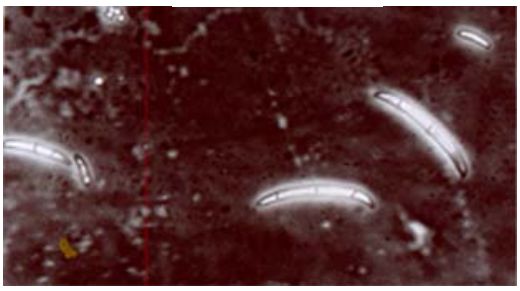
FM242



FM246

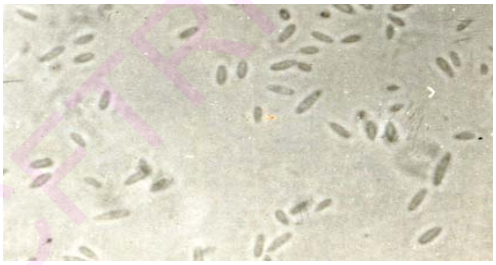


Isolate 6

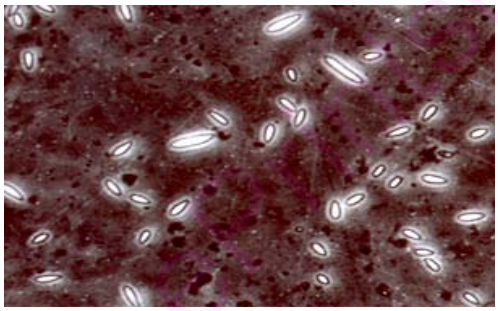


Isolate 1

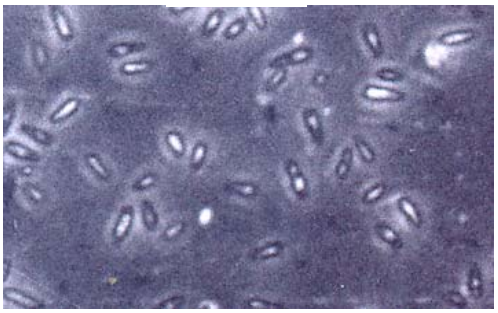
ICR57



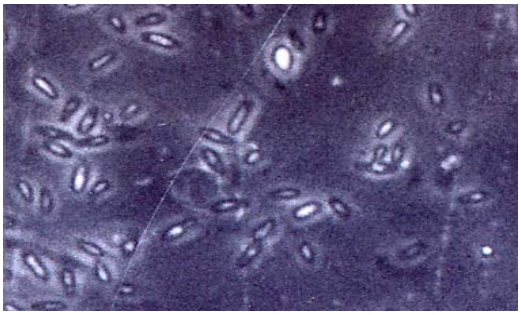
ICR-PQ-13



ICR11



ICR1



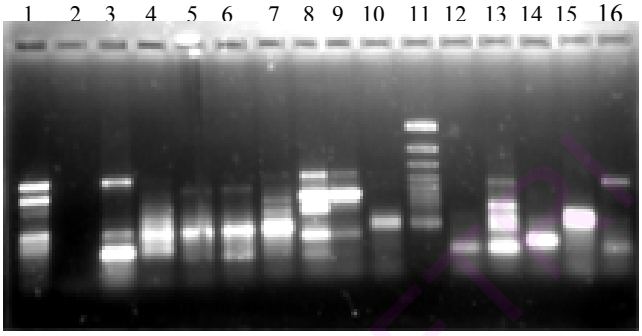
Based on different criteria, the 45 isolates positive for trichothecene production were classified into 13 groups from which one isolate each were selected for detailed studies profiling the toxin produced by them and their identification to the species level.

Possible genetic variability among the isolates was analyzed using three ISSR-PCR using the primers ISSR 810, ISSR 811 and ISSR 826. Results were scored on the basis of number of fragments amplified, their presence or absence across the isolates and repeatability of amplification. Band pattern obtained after PCR amplification revealed marked difference between the isolates, for all the three primers (Fig. 3.6). PCR with ISSR 810 and 811 amplified different fragments from few of the isolates only. ISSR 826 was selected and used for further analysis due to the consistency in the band pattern obtained after PCR amplification using the primer. A fragment of size ~400-bp was amplified from almost all the *Fusarium* isolates whereas fragments of sizes ~1.2-Kb and 800-bp were found common to few of the isolates with ISSR 826. The study thus revealed polymorphism between and within the different *Fusarium* species, as reported also by Mishra et al (2003; 2004) where they used ISSR markers to investigate genetic variation among *Fusarium graminearum* and *Fusarium culmorum* isolates from Canada and worldwide, respectively. They could notice remarkable variations both between and within the populations. None of the ISSR markers used in this study could classify the isolates with respect to toxin production nor could they determine differences to the species level.

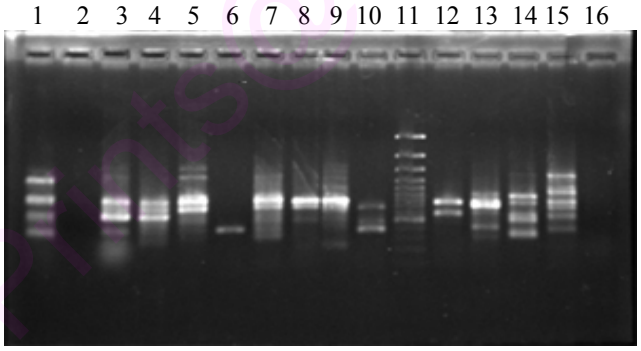
Fig. 3.6. PCR Amplification from *Fusarium* Isolates using Inter Simple Sequence Repeats (ISSR) Primers

Lanes 1: ICR61; 2: FM246; 3: ICR57; 4: ICR1; 5: ICR-PQ-13; 6: ICR11; 7: ICR50; 8: ICR-PQ-2; 9: ICR-PO-12; 10: FM242; 11: 3-Kb Marker; 12: FM 550; 13: FM311; 14: Standard culture; 15: Isolate 1; 16: Isolate 6.

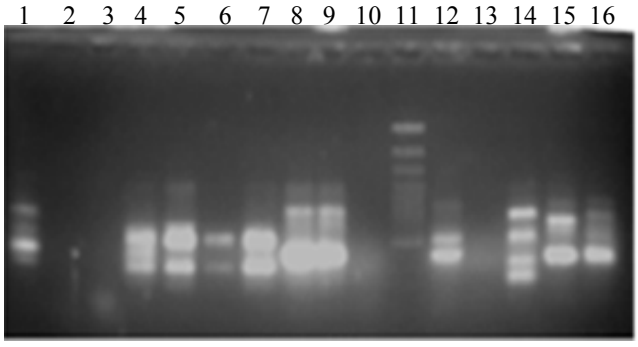
A. Amplification using ISSR 826



B. Amplification using ISSR 811



C. Amplification using ISSR 810



3.3.4. GC-MS for Characterization of Toxin Production

Toxin extracts from the 13 isolates of this study and the standard culture NCIM 651 were analyzed using GC-MS for characterization of trichothecene production. The culture filtrate of 13 isolates indicated the presence of trichothecenes (raw data presented in appendix, Fig. A.5). Table 3.5 shows the mass spectrometric signature that allows the definite identification of trichothecenes in the test samples. 10-13 mass fragments characteristic of the different trichothecenes were observed. 207 was the 100 % peak in almost all cases. By this method DON was detected in the culture filtrates of 12 isolates of the study, confirming the findings that have been observed in HPLC (provided in section 3.3.2.4. HPLC Detection of DON). The isolates ICR61, FM311 and ICR-PQ-2 produced Fus-X along with DON whereas the isolate FM246 secreted NIV along with DON. NIV was detected in the culture filtrate of isolate ICR57 and traces of DAS and NIV in isolate ICR1. Standard culture secreted DON in culture filtrate.

Table 3.5. Ions monitored for *Fusarium* Trichothecenes Determination by GC-MS

Trichothecene	Parent ion	Daughter ions used for identification
NIV	312	312, 253, 235, 205, 177, 187, 159, 149, 121, 105
DON	296	296, 281, 275, 259, 235, 207, 181, 165, 147, 129
DAS	366	366, 282, 273, 266, 249, 243, 236, 254, 224, 208, 196, 184
Fus-X	354	355, 325, 308, 284, 281, 264, 256, 241, 207, 191, 185, 171

3.3.5. Characterization of *Fusarium* Species using Morphology, EF-1α Gene Sequences, Phylogenetic and Molecular Evolutionary Analyses and Toxin Production

Growth pattern on PDA varied from profuse velvety mycelia to sparsely growing cotton thread like mycelia (Table A.1). Conidial features, studied after growth on BLA, revealed clear differences in the structure, shape and number of septa of macro- and microconidia. Few isolates produced profuse macroconidia along with septate or aseptate microconidia whereas in some isolates, majority of the which were from sorghum, only microconidia were observed (Table A.1).

Thirteen isolates, positive for *tri 5*-PCR and ability to produce trichothecenes, were identified to species level, following a study of colony and spore features and the sequences of the EF-1α gene. Specific primers for EF-1α gene amplified a distance of ~650 bp of the gene which spanned across 3 introns. These introns are highly informative, allowing identification to the species level. The sequences of EF-1α gene from each of the 13 *Fusarial* isolates (query) and the standard culture NCIM 651 were compared to those from various species of *Fusarium* (subject). Similarity of the query sequence to those present in the database was the criteria used for the identification of species (Fig. 3.7).

Phylogenetic and molecular evolutionary analyses carried out also eventually makes use of sequence variations in the intronic region of EF-1α gene of the different taxas. NJ analysis of *MEGA* version 3.1 (Kumar et al, 2004) was used in this study for analyzing the similarity level of the isolates in relation to the already existing toxigenic species of *Fusarium*. Totally 23 taxas were considered out of which 13 were EF1 sequences from the present study (query), one was the standard culture NCIM 651 and the rest were EF1 sequences of *Fusarium* species showing homology to the query sequence. The EF1 gene sequence datasets consisted of maximum of 624 characters. All the informative characters and indels were concentrated in the first two introns. Bootstrap analysis of the sequences showed 271 parsimonious informative sites and 339 variable sites. The overall mean distance between the taxa or within the groups was 0.119. NJ analysis divided the datasets into two major clusters, one consisting mainly of *Fusarium*

species included in the *Giberella fujikuroi* complex and the other consisting of trichothecene producing species and relatives, respectively. Most of the taxas were concentrated in the former while the latter consisted of only three taxas (from this study) that grouped into two clades. The standard culture NCIM 651 showed complete sequence similarity to that of *F. asiaticum.* The results of NJ analysis of the sequences from the 13 isolates of the current study and the standard culture NCIM 651 are given in Fig. 3.8.

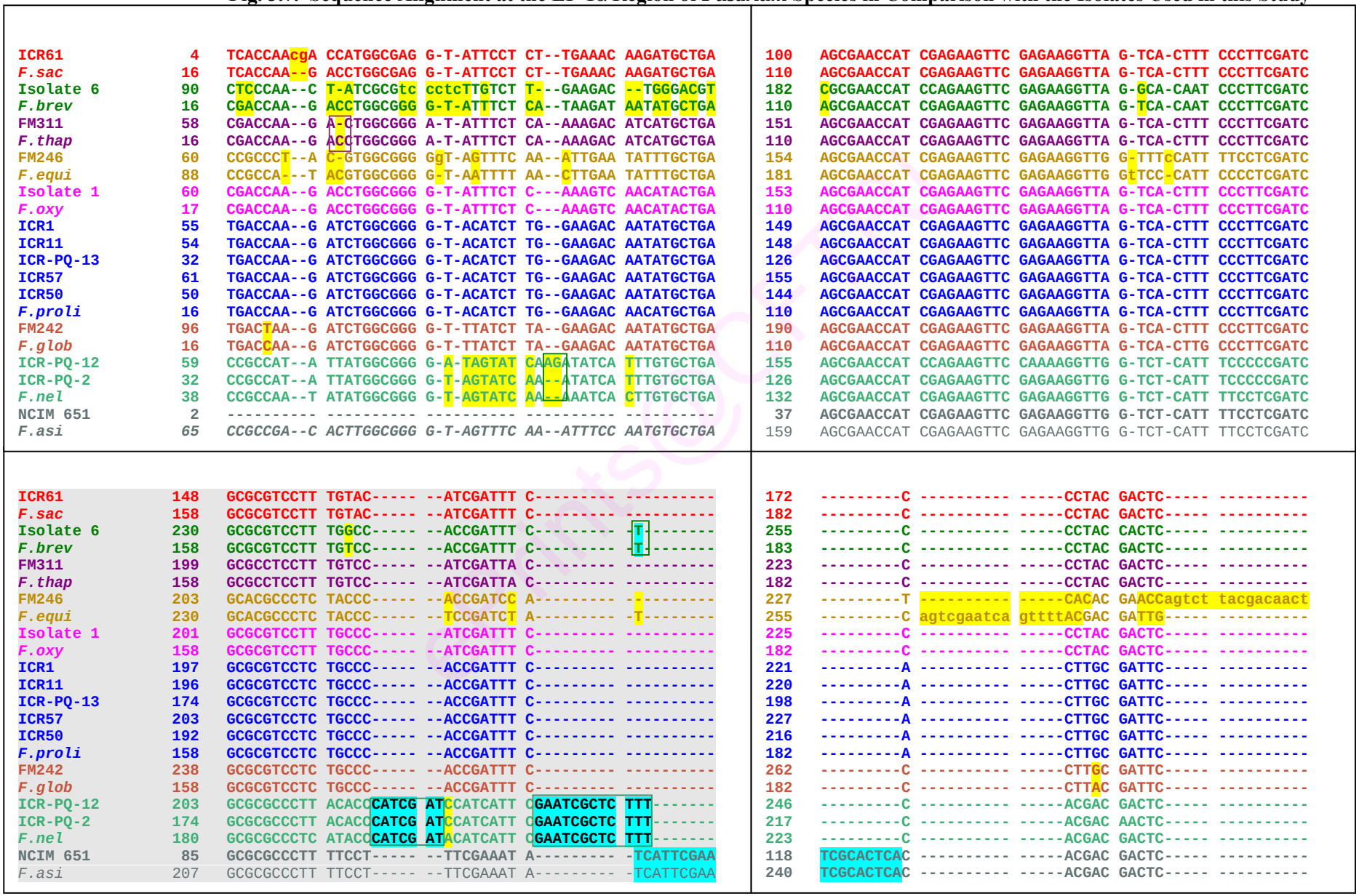
All the isolates which produced only microconidia were grouped as *F. proliferatum* in which the isolates ICR1, ICR11, ICR-PQ-13, ICR50, ICR57, FM242 and FM311 were categorized. The EF1 sequences for part of the gene which was amplified by the primers EF1-EF2 from ICR1, ICR11, ICR-PQ-13, ICR50 and ICR57 were completely identical to that of *F. proliferatum*, a species included in Liseola section (1.5.5.3.1) of *Fusarium*. These isolates formed a single clade along with *F. proliferatum* in molecular phylogenetic analysis also [(boot strap value of 63 %) (Fig. 3.8)]. The isolates ICR11, ICR-PQ-13, ICR50 and ICR57 were identified as producers of DON with the exception of isolate ICR1 which secreted NIV and DAS in the culture filtrate (Table 3.6). There is a general consensus that *Fusarium* growing on sorghum mainly belongs to *Giberella fujikuroi* species complex which comprises of *Fusarium* species included in section Liseola. (Leslie et al, 2005). Members of this group are generally known to produce fumonisins (section 1.5.5.3.1, Table 1.2). However, trichothecene production has been associated with isolates of *F. verticillioides* (=*F. moniliforme*) isolated from India and Spain (Ramakrishna et al, 1989; Cantalejo et al, 1999).

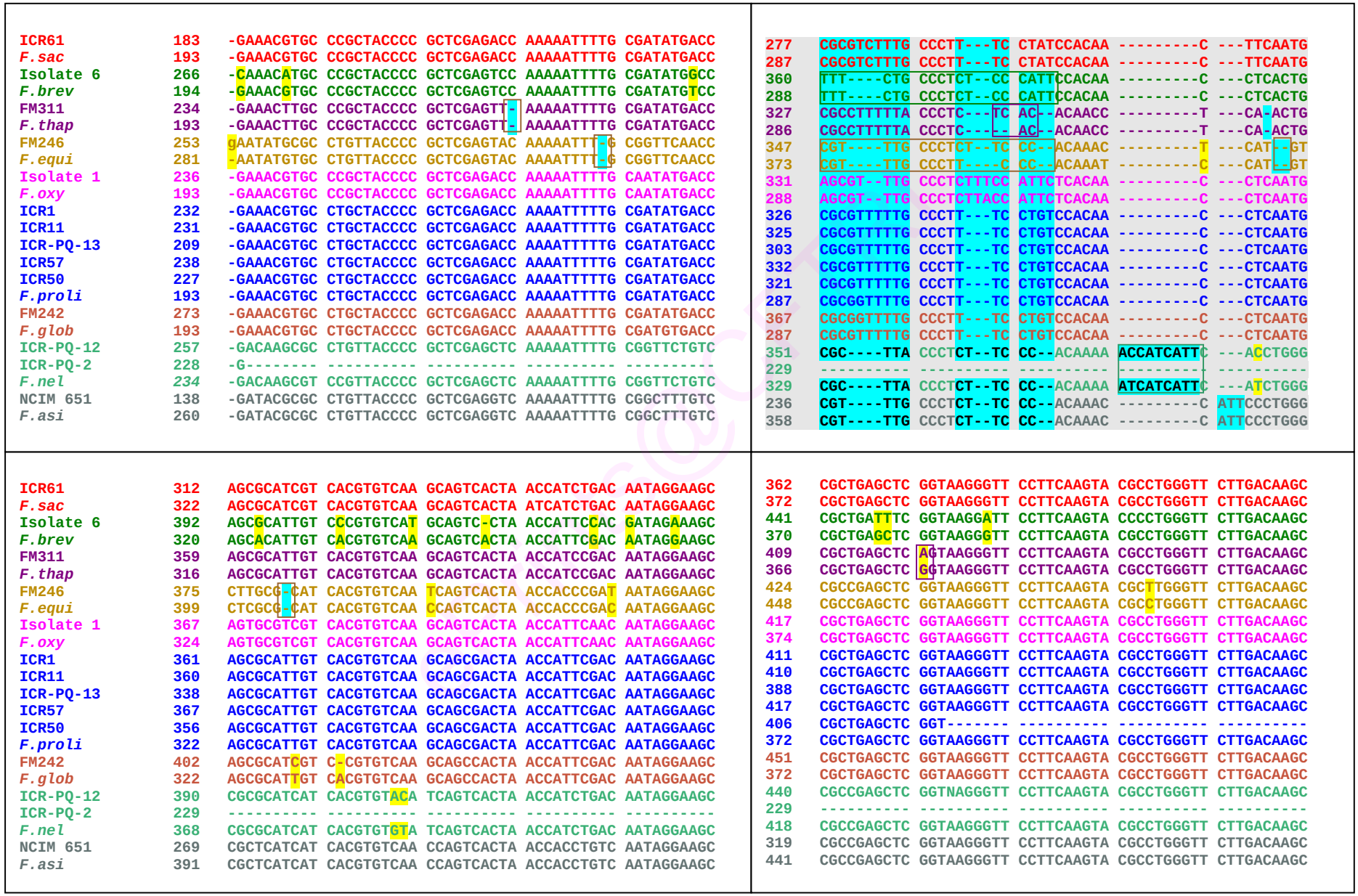
The EF1 sequence from FM242 was identical to that from *F. globosum* and *F. proliferatum*. Multiple sequence alignment underlined this similarity of sequence between FM242 and *F. proliferatum* whereas variations in the form of substitutions (bases 101, 267, 410, 532, 534, 537, 544, 546, 548, 551 of FM242) and deletions (bases 414, 504, 538, 539 of FM242) were observed in the sequence of FM242 when compared with that of *F. globosum* (Fig. 3.7). The isolate FM242, a DON producer like the rest of the *F. proliferatum* group, clustered together with *F. proliferatum* in phylogenetic analysis also, rather than to its BLAST hit "*F. globosum"*. This may be possible because *F. globosum* is a close relative of *F. proliferatum* and is classified in the Liseola section

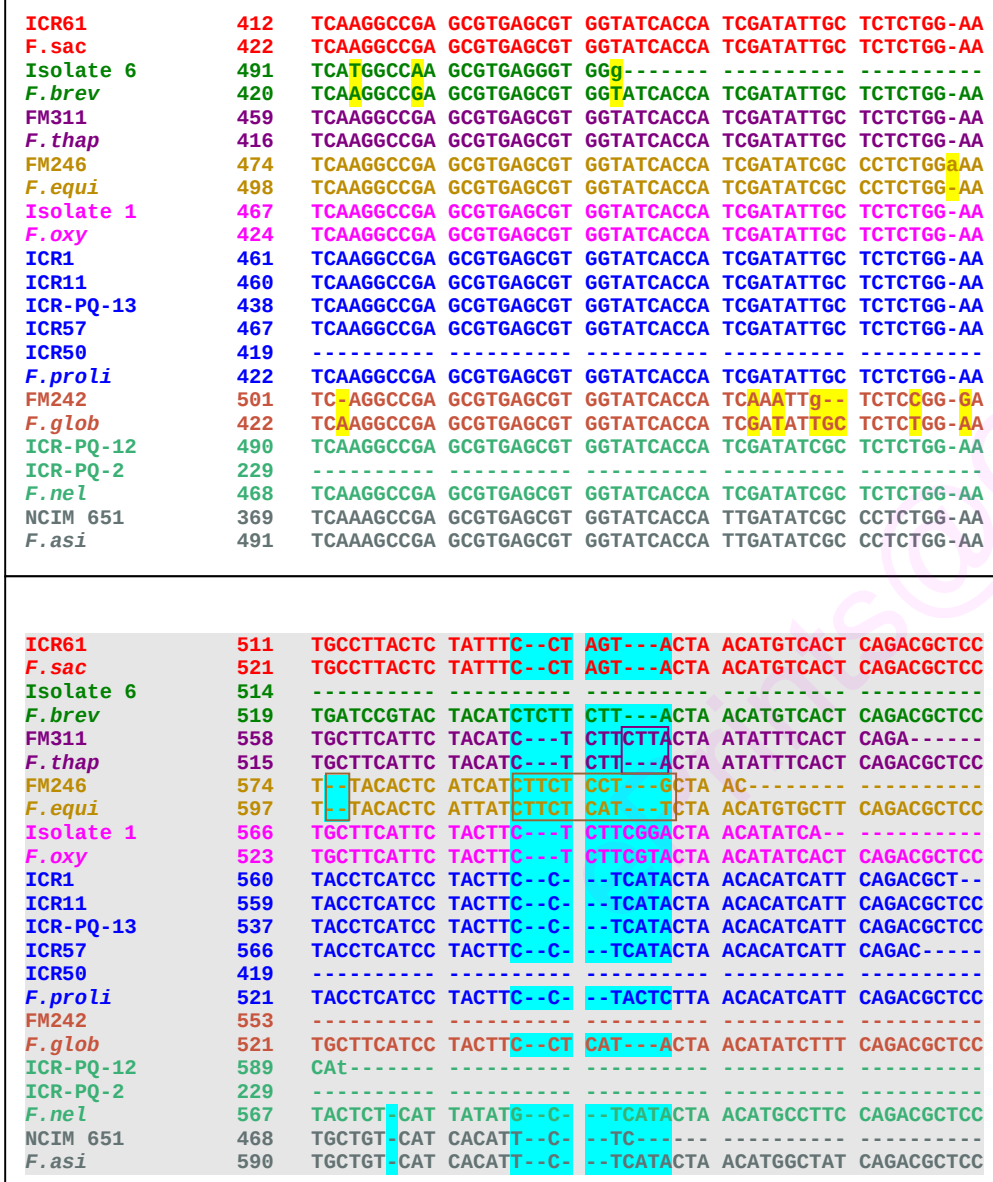
of *Fusarium* (Fandohan et al, 2003). In this study, the isolate FM242 was finally identified as *F. proliferatum.*

Isolate 6 was identified by the production of microconidia thereby classifying it as *F. proliferatum.* Inspite of the differences observed between the EF1 sequence of Isolate 6 and *F. brevicatenulatum* (bases 90-183 of Isolate 6) in the multiple sequence alignment, the segments 230-255 and 361-378 (sequences given in box against Isolate 6 in Fig. 3.7) appeared characteristic for *F. brevicatenulatum* which was also observed in the sequence of Isolate 6. Phylogenetic studies also identified the isolate as *F. brevicatenulatum* which is a fumonisin producer that has been grouped with Liseola [(Table 1.2) (Leslie and Summerell, 2006)]. DON was detected in the culture filtrate of isolate 6 (Table 3.6).

Fig. 3.7. Sequence Alignment at the EF-1α Region of *Fusarium* Species in Comparison with the Isolates Used in this Study




ICR61 183 -GAAACGTGC CCGCTACCCC GCTCGAGACC AAAAATTTTG CGATATGACC
F.sac 193 -GAAACGTGC CCGCTACCCC GCTCGAGACC AAAAATTTTG CGATATGACC
Isolate 6 266 -CAAACATGC CCGCTACCCC GCTCGAGTCC AAAAATTTTG CGATATGGCC
F.brev 194 -GAAACGTGC CCGCTACCCC GCTCGAGTCC AAAAATTTTG CGATATGTCC
FM311 234 -GAAACTTGC CCGCTACCCC GCTCGAGTT- AAAAATTTTG CGATATGACC
F.thap 193 -GAAACTTGC CCGCTACCCC GCTCGAGTT- AAAAATTTTG CGATATGACC
FM246 253 gAATATGCGC CTGTTACCCC GCTCGAGTAC AAAAATTT-G CGGTTCAACC
F.equi 281 -AATATGTGC CTGTTACCCC GCTCGAGTAC AAAATTTT-G CGGTTCAACC
Isolate 1 236 -GAAACGTGC CCGCTACCCC GCTCGAGACC AAAAATTTTG CAATATGACC
F.oxy 193 -GAAACGTGC CCGCTACCCC GCTCGAGACC AAAAATTTTG CAATATGACC
ICR1 232 -GAAACGTGC CTGCTACCCC GCTCGAGACC AAAATTTTTG CGATATGACC
ICR11 231 -GAAACGTGC CTGCTACCCC GCTCGAGACC AAAATTTTTG CGATATGACC
ICR-PQ-13 209 -GAAACGTGC CTGCTACCCC GCTCGAGACC AAAATTTTTG CGATATGACC
ICR57 238 -GAAACGTGC CTGCTACCCC GCTCGAGACC AAAATTTTTG CGATATGACC
ICR50 227 -GAAACGTGC CTGCTACCCC GCTCGAGACC AAAATTTTTG CGATATGACC
F.proli 193 -GAAACGTGC CTGCTACCCC GCTCGAGACC AAAAATTTTG CGATATGACC
FM242 273 -GAAACGTGC CTGCTACCCC GCTCGAGACC AAAAATTTTG CGATATGACC
F.glob 193 -GAAACGTGC CTGCTACCCC GCTCGAGACC AAAAATTTTG CGATGTGACC
ICR-PQ-12 257 -GACAAGCGC CTGTTACCCC GCTCGAGCTC AAAAATTTTG CGGTTCTGTC
ICR-PQ-2 228 -G-------- ---------- ---------- ---------- ----------
F.nel 234 -GACAAGCGT CCGTTACCCC GCTCGAGCTC AAAAATTTTG CGGTTCTGTC
NCIM 651 138 -GATACGCGC CTGTTACCCC GCTCGAGGTC AAAAATTTTG CGGCTTTGTC
F.asi 260 -GATACGCGC CTGTTACCCC GCTCGAGGTC AAAAATTTTG CGGCTTTGTC
277 CGCGTCTTTG CCCTT---TC CTATCCACAA ---------C ---TTCAATG
287 CGCGTCTTTG CCCTT---TC CTATCCACAA ---------C ---TTCAATG
360 TTT----CTG CCCTCT--CC CATTCCACAA ---------C ---CTCACTG
288 TTT----CTG CCCTCT--CC CATTCCACAA ---------C ---CTCACTG
327 CGCCTTTTTA CCCTC---TC AC--ACAACC ---------T ---CA-ACTG
286 CGCCTTTTTA CCCTC---TC AC--ACAACC ---------T ---CA-ACTG
347 CGT----TTG CCCTCT--TC CC--ACAAAC ---------T ---CAT--GT
373 CGT----TTG CCCTT----C CC--ACAAAT ---------C ---CAT--GT
331 AGCGT--TTG CCCTCTTTCC ATTCTCACAA ---------C ---CTCAATG
288 AGCGT--TTG CCCTCTTACC ATTCTCACAA ---------C ---CTCAATG
326 CGCGTTTTTG CCCTT---TC CTGTCCACAA ---------C ---CTCAATG
325 CGCGTTTTTG CCCTT---TC CTGTCCACAA ---------C ---CTCAATG
303 CGCGTTTTTG CCCTT---TC CTGTCCACAA ---------C ---CTCAATG
332 CGCGTTTTTG CCCTT---TC CTGTCCACAA ---------C ---CTCAATG
321 CGCGTTTTTG CCCTT---TC CTGTCCACAA ---------C ---CTCAATG
287 CGCGGTTTTG CCCTT---TC CTGTCCACAA ---------C ---CTCAATG
367 CGCGGTTTTG CCCTT---TC CTGTCCACAA ---------C ---CTCAATG
287 CGCGTTTTTG CCCTT---TC CTGTCCACAA ---------C ---CTCAATG
351 CGC----TTA CCCTCT--TC CC--ACAAAA ACCATCATTC ---ACCTGGG
229 ---------- ---------- ---------- ---------- ----------
329 CGC----TTA CCCTCT--TC CC--ACAAAA ATCATCATTC ---ATCTGGG
236 CGT----TTG CCCTCT--TC CC--ACAAAC ---------C ATTCCCTGGG
358 CGT----TTG CCCTCT--TC CC--ACAAAC ---------C ATTCCCTGGG
ICR61 312 AGCGCATCGT CACGTGTCAA GCAGTCACTA ACCATCTGAC AATAGGAAGC
F.sac 322 AGCGCATCGT CACGTGTCAA GCAGTCACTA ATCATCTGAC AATAGGAAGC
Isolate 6 392 AGCGCATTGT CCCGTGTCAT GCAGTC-CTA ACCATTCCAC GATAGAAAGC
F.brev 320 AGCACATTGT CACGTGTCAA GCAGTCACTA ACCATTCGAC AATAGGAAGC
FM311 359 AGCGCATTGT CACGTGTCAA GCAGTCACTA ACCATCCGAC AATAGGAAGC
F.thap 316 AGCGCATTGT CACGTGTCAA GCAGTCACTA ACCATCCGAC AATAGGAAGC
FM246 375 CTTGCG-CAT CACGTGTCAA TCAGTCACTA ACCACCCGAT AATAGGAAGC
F.equi 399 CTCGCG-CAT CACGTGTCAA CCAGTCACTA ACCACCCGAC AATAGGAAGC
Isolate 1 367 AGTGCGTCGT CACGTGTCAA GCAGTCACTA ACCATTCAAC AATAGGAAGC
F.oxy 324 AGTGCGTCGT CACGTGTCAA GCAGTCACTA ACCATTCAAC AATAGGAAGC
ICR1 361 AGCGCATTGT CACGTGTCAA GCAGCGACTA ACCATTCGAC AATAGGAAGC
ICR11 360 AGCGCATTGT CACGTGTCAA GCAGCGACTA ACCATTCGAC AATAGGAAGC
ICR-PQ-13 338 AGCGCATTGT CACGTGTCAA GCAGCGACTA ACCATTCGAC AATAGGAAGC
ICR57 367 AGCGCATTGT CACGTGTCAA GCAGCGACTA ACCATTCGAC AATAGGAAGC
ICR50 356 AGCGCATTGT CACGTGTCAA GCAGCGACTA ACCATTCGAC AATAGGAAGC
F.proli 322 AGCGCATTGT CACGTGTCAA GCAGCGACTA ACCATTCGAC AATAGGAAGC
FM242 402 AGCGCATCGT C-CGTGTCAA GCAGCCACTA ACCATTCGAC AATAGGAAGC
F.glob 322 AGCGCATTGT CACGTGTCAA GCAGCCACTA ACCATTCGAC AATAGGAAGC
ICR-PQ-12 390 CGCGCATCAT CACGTGTACA TCAGTCACTA ACCATCTGAC AATAGGAAGC
ICR-PQ-2 229 ---------- ---------- ---------- ---------- ----------
F.nel 368 CGCGCATCAT CACGTGTGTA TCAGTCACTA ACCATCTGAC AATAGGAAGC
NCIM 651 269 CGCTCATCAT CACGTGTCAA CCAGTCACTA ACCACCTGTC AATAGGAAGC
F.asi 391 CGCTCATCAT CACGTGTCAA CCAGTCACTA ACCACCTGTC AATAGGAAGC
362 CGCTGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
372 CGCTGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
441 CGCTGATTTC GGTAAGGATT CCTTCAAGTA CCCCTGGGTT CTTGACAAGC
370 CGCTGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
409 CGCTGAGCTC AGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
366 CGCTGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
424 CGCCGAGCTC GGTAAGGGTT CCTTCAAGTA CGCTTGGGTT CTTGACAAGC
448 CGCCGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
417 CGCTGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
374 CGCTGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
411 CGCTGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
410 CGCTGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
388 CGCTGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
417 CGCTGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
406 CGCTGAGCTC GGT------- ---------- ---------- ----------
372 CGCTGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
451 CGCTGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
372 CGCTGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
440 CGCCGAGCTC GGTNAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
229 ---------- ---------- ---------- ---------- ----------
418 CGCCGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
319 CGCCGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC
441 CGCCGAGCTC GGTAAGGGTT CCTTCAAGTA CGCCTGGGTT CTTGACAAGC



EF-1α sequences from isolates used in this study and that of related *Fusarium* species is depicted in same colour. The intron region is marked in grey. The differences in sequence between isolate from this study and its BLAST counterpart are highlighted in yellow. Sequence differences across the different species of *Fusarium* are highlighted in blue.

[*F. sac*: *F. sacchari* (434), *F. brev*: *F. brevicatenulatum* (447), *F.thap*: *F. thapsina* (442), *F. equi*: *F. equiseti* (568), *F. oxy*: *F. oxysporum* (513), *F. proli*: *F.proliferatum* (432), *F. glob*: *F. globosum* (427), *F. nel*: *F. nelsonii* (562), *F.asi*: *F. asiaticum* (381)]

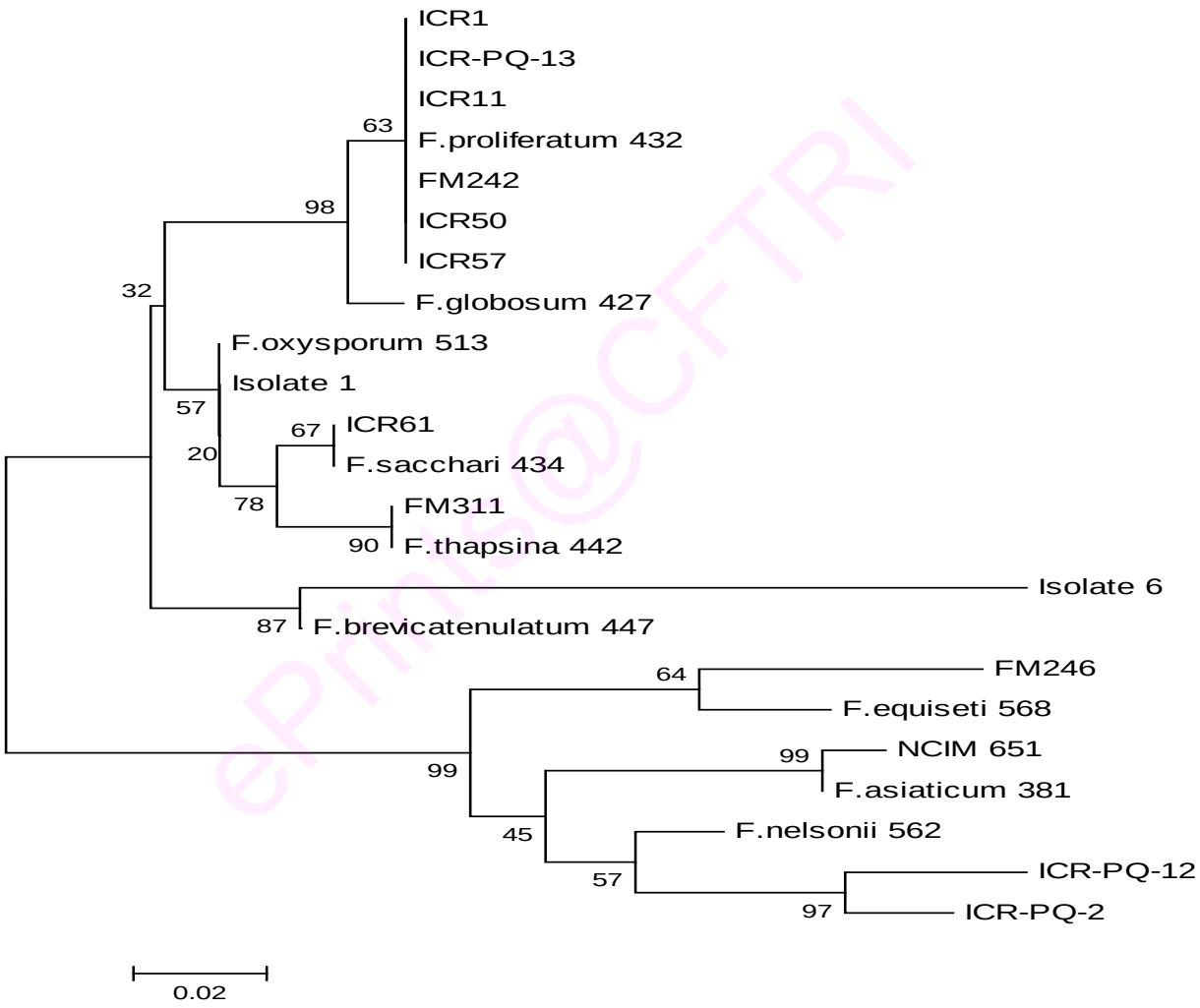
The EF-1α gene sequence of FM311 which produced microconidia alone when grown on banana leaf agar and the toxins DON and Fus-X in the culture filtrate, was identical to that of *F. thapsina* as assessed while using BLAST and molecular phylogeny analysis [(strong bootstrap support of 90 %) (Table 3.6)]. The EF1 sequence of FM311 was homologous to that of *F. thapsina* in multiple sequence alignment except in the segments marked in the boxes in Fig. 3.7. The species of *F. thapsina* included in the Liseola section is a known fumonisin producer [(Table 1.2) (Fandohan et al, 2003)].

ICR61 was identified as *F. sacchari* by all three methods (BLAST, multiple sequence alignment, molecular phylogeny). As described earlier this species belongs to the Liseola section which contains fumonisin producers. The production of DON by the isolate ICR61 is in agreement with the report of Štyriak et al (1994) that has isolated *F. sacchari*, from broiler feed, capable of synthesizing DON.

In the NJ analysis the bootstrap value of isolates ICR-PQ-2 and ICR-PQ-12 was significantly higher between themselves (97 %) than when compared with *F. nelsonii* (57 %). However the EF-1α sequence alignment agreed completely with the identification of ICR-PQ-2 and ICR-PQ-12 as *F. nelsonii*, a species belonging to Arthrosporiella section. The EF1 sequences of the isolates ICR-PQ-12 and ICR-PQ-2 revealed segments which were characteristic for *F. nelsonii* (marked in box, Fig. 3.7). These segments of sequences were present mainly in the intron regions and served as an identification marker for *F. nelsonii*. The isolates of the present study also produced morphological features characteristic of *F. nelsonii* when grown on BLA. ICR-PQ-2 and ICR-PQ-12 were characterized for production of DON (Table 3.6). Few of the *Fusarium* species included in Arthrosporiella have been identified as producers of type A trichothecenes (Table 1.2). Little is known about the ecology and pathology of *F. nelsonii*, which has not hitherto been reported to produce any toxin (Leslie and Summerell, 2006). *F. nelsonii* has been reported to be similar to *F. semitectum* which has been reported earlier to produce T-2 and DAS (Rukmini and Bhat, 1978; Molto et al, 1997).

Fig. 3.8. NJ Consensus Tree for Translation Elongation Factor-1α Sequences

(Accession numbers of the EF-1α sequences in FUSARIUM-ID v. 1.0 database are given after the *Fusarium* species)



Isolate 1 produced macroconidia characteristic for *F. oxysporum*. The identification was confirmed by the EF1 sequence data which grouped the isolate with *F. oxysporum* after employing both sequence alignment and molecular phylogenetic analysis (Fig. 3.7 and 3.8). *F. oxysporum* is the only single species included in section Elegans which has been reported to produce NIV, T-2, HT-2 and DAS toxins by isolates from Korea, Spain, Argentina and India (Ghosal et al, 1976; Lee et al, 1986; Molto et al, 1997; Cantalejo et al, 1999). *F. oxysporum* from this study was identified as a DON producer (Table 3.6).

Isolate FM246 was identical to *F. equiseti* after comparison of spore morphology and EF1 sequence. Sequences characteristic of *F. equiseti* were observed in the intron region of the isolate FM246 though variations in the form of substitutions and deletions (60-253 of FM246) were observed in the exons of FM246 (Fig. 3.7). This agreed with the grouping of FM246 and *F. equiseti* in a single clade with a bootstrap support of 64 % in the phylogenetic analysis. The section Gibbosum to which *F. equiseti* has been included is known to contain both type A and type B trichothecene producers. The isolate FM246 revealed to be a producer of DON. This is in concordance with the reports of *F. equiseti* as a trichothecene producer (DON, DAS and NIV) by Molto et al (1997) and Moss and Thrane (2004), respectively. Table 3.6 summarizes the results obtained in this study.

Fumonisins and zearalenone have been implicated with the occurrence of toxicosis, mainly in maize and its products, from different parts of India (Bhavanishankar and Shantha, 1987; Sinha, 1990; Bhat et al, 1997; Janardhana et al, 1999). Analysis of sorghum samples from the Deccan Plateau revealed the presence of only fumonisins (Bhat et al, 1989; 1997). The studies of Rukmini and Bhatt (1978) from Andhra Pradesh have claimed T-2 production by *F. incarnatum* isolated from infected sorghum. In addition, Ghosal et al (1976) had earlier reported of trichothecene production by *F. oxysporum* isolated from safflower seeds in India. In a parallel study we have detected considerable amounts of trichothecene in market samples of sorghum [(Lincy et al, 2008) (Chapter 4. Detection of Trichothecenes in Food and Feed)]. This is probably the first detailed study of *Fusarium* isolated from India, where the presence of different toxigenic *Fusarium* isolates were identified from different food materials, mainly sorghum.

Sorghum is one of the staple millets consumed in Asia and Africa whereas in India it stands second only to rice in the rate of consumption. The occurrence of trichothecene producers at the rate of 34 % among the 115 *Fusarium* isolates from Indian sorghum indicates the possibility of large scale contamination of the grain in the country with these toxins and so demands proper screening of food commodities for the detection of these toxins and the fungi producing these toxins.

Fig. 3.6. Description of Strains Selected for Characterization and Identification to the Species Level

Sl. No.	Name of Isolate	Colony morphology		Spore Morphology	Species (Morphology)	Species (EF-1α)	Toxin produced (GC-MS)
		From above	From below				
1.	ICR57	Pinkish white, cotton thread like mycelia	No characteristic colour	Only microconidia present	*F. proliferatum*	*F. proliferatum*	NIV
2.	ICR1	Pinkish white, cotton thread like mycelia	’’	’’	”	”	DAS, NIV
3.	ICR-PQ-13	White turning to pink with age, floccose mycelia	Pink to carmine red	’’	”	”	DON
4.	ICR50	White turning to pink with age, powdery appearance of mycelia	No characteristic colour	’’	”	”	DON
5.	ICR11	White turning to pink with age, floccose mycelia	Pink to carmine red	’’	”	”	DON
6.	ICR-PQ-2	Yellow to orange, profuse velvety mycelia	No characteristic colour	Macro- and mesoconidia present	*F. nelsonii*	*F. nelsonii*	DON, Fus-X
7.	ICR61	White, cotton thread like mycelia	’’	Both macro and two types of microconidia are present	*F. sacchari*	*F. sacchari*	DON, Fus-X
8.	ICR-PQ-12	Pinkish yellow to orange, profuse velvety mycelia	Carmine red	Macro- and mesoconidia present	*F. nelsonii*	*F. nelsonii*	DON
9.	FM 246	Orangish white, floccose	White	Both macro and two types of microconidia are present	*F. equiseti*	*F. equiseti*	DON, NIV
10	FM 242	White , powdery appearance of mycelia	No characteristic colour	Only microconidia present	*F. proliferatum*	*F. proliferatum*	DON
11.	FM 311	White, turning to violet with age	’’	’’	”	*F. thapsina*	DON, Fus-X
12.	Isolate 1	Pinkish white, profuse velvety growth	Pink	Only macroconidia present	*F. oxysporum*	*F. oxysporum*	DON
13.	Isolate 6	White, floccose mycelia	No characteristic colour	’’	*F. brevicatenulatum*	*F. brevicatenulatum*	DON
14.	NCIM 651	Pinkish white, profuse velvety growth	Pink	’’	*F. asiaticum*	*F. asiaticum*	DON

3.4. Conclusions

Isolates of *Fusarium* were procured from different sources, purified and maintained on PDA. The isolates were primarily screened for the production of trichothecenes by TLC. Around 45 isolates were identified as positives. PCR assay of the isolates resulted in the amplification of the 652 bp *tri 5* fragments from the genomic DNA of the TLC positive isolates for trichothecene production. Production of T-2 and DON was further characterized using GC and HPLC methods, respectively. 14 isolates that secreted T-2 and 27 isolates that synthesized DON were identified. ISSR-PCR assay and study of morphological characters of the trichothecene positive isolates revealed marked variations existing among them. The isolates were grouped into 13 major types based on their morphological features among which one isolate each were selected for further analyses. GC-MS analysis of the toxin extracts of the 13 selected isolates resulted in the identification of 8 isolates producing DON, three isolates producing DON and Fus-X, one isolate secreting DON and NIV, one isolate producing DAS and NIV and one isolate producing NIV. Identification of the 13 isolates to species level was carried out using the colony and spore morphology. The isolates were identified as six *F. proliferatum,* two *F. nelsonii* and one each of *F. sacchari*, *F. oxysporum*, *F. equiseti*, *F. thapsina* and *F. brevicatenulatum* after EF-1α sequence comparison. Molecular phylogenic analyses revealed concordant results which underlined the identification of species. Here we present a detailed study of *Fusarium* isolated from India, where we report the presence of different toxigenic *Fusarium* isolates. Production of trichothecenes by *F. nelsonii, F. brevicatenulatum* and *F. thapsina* has not been reported hitherto. Further work on the identity of species is needed with respect to the isolates classified here as *F. proliferatum*.

A sequel to this series of identification and characterization of trichothecegenic *Fusarium* is continued in the next chapter where the analysis of market samples of food commodities have been investigated for the presence of trichothecenes or trichothecegenic *Fusaria*.

Chapter 4.

Detection of Trichothecenes in Market Samples of Food and Feed

4.1. Introduction

The first Food and Agriculture Organization (FAO), World Health Organization (WHO) and United Nations Environmental Program (UNEP) conference on mycotoxins in 1997 reported the occurrence of seven mycotoxins in significant amounts in naturally contaminated food and feed (FAO, 1997). The toxins included aflatoxin, ochratoxin A, patulin, zearalenone, trichothecenes, citrinin and penicillic acid (Jelinek et al, 1989). The FAO estimated losses of 1000 million tons of foodstuffs every year worldwide due to mycotoxin contamination (Prema, 2004).

4.1.1. Legislative Limits for Trichothecenes in Food and Feed

Different countries have set legislative limits which is the permissible level of toxins in different food commodities which when consumed will not exceed beyond the tolerable daily intake (TDI) levels that has been derived for the different age groups of humans or livestock. DON being the most commonly reported toxin among the trichothecenes worldwide, most of the regulation limits or tolerance guidelines available are for these toxins. The European Union (EU) Commission formed in 1995 is a union of 15 countries such as Austria, Belgium, Denmark, Finland, France, Germany, Greece, Ireland, Italy, Luxembourg, Netherlands, Portugal, Spain, Sweden and the United Kingdom (Prema, 2004). The Commission has issued joint regulatory limits for DON in raw cereals and their refined products for their member states (Table 4.1).

Table. 4.1. Limits of DON Proposed by EU Commission

(Adapted from Larsen et al, 2004)

Food type	Permissible level (µg/kg)
Unprocessed durum wheat and corn	2000
Other unprocessed cereals	1500
Whole meal wheat flour, bran and pasta (dry)	750
Maize based breakfast cereals and snacks	500
Cereals for infants and ingredients used in their manufacture thereof	100

Other than the EU commission, other nations have also laid down mandatory limits for few toxins for all food materials in general. Regulatory limits that have been proposed for DON by Canada, Soviet Union and United States range from 500-2000 µg/kg for products meant for human consumption. The German Government has proposed advisory levels of DON in grain-based foodstuffs and bread or related products to be 500 µg/kg and 350 µg/kg, respectively (Schollenberger et al, 2005a). Up to 1000 µg/kg levels of DON are permitted in cereals in Russia whereas limits of up to 500 µg/kg has been specifically proposed for rye alone (Prema, 2004). Austria has proposed maximum admissible levels of 6 µg/kg of DON in wheat (Prema, 2004). In India the Prevention of Food and Adulteration Act (PFA, 2000) has proposed tolerance limit of 1000 µg/kg for DON in cereals in general. Russia is the only country where legal limits for T-2 toxin have been proposed and the maximum permissible level suggested is 100 µg/kg for all cereals (Prema, 2004). Owing to the potent toxicity of T-2 toxin and its derivatives, ingestion of even low levels of these toxins are required to elicit carcinogenic, immunotoxic and haematotoxic effects in mammalian cells.

Animals, particularly ruminants, are able to metabolize many of the mycotoxins that are commonly encountered in their food materials. Also, some of the metabolites are not carried over to their products like milk or meat so that the advisory limits proposed for them are always higher than what is proposed for humans. USA advisory directives for concentration of DON in feed materials (grain and by-products) offered to cattle and chicken and pig are 10 mg/kg and 5mg/kg respectively (Trucksess et al, 1995). DON levels up to 12 mg/kg have been proposed by EU commission in maize and its bye-products (Table 4.2). Permissible levels of various trichothecenes in animal feed or feed ingredients are provided in Table 4.2.

Table 4.2. Regulatory Limits Proposed for Trichothecenes in Animal Feed Ingredients

Country	Mycotoxin	Animal feed products	Regulatory limits
Europe*	DON	Cereals and cereal products with the exception of maize	8 mg/kg
”	”	Maize by-products	12 mg/kg
Serbia and	Trichothecenes	Feed for chickens, pigs and calves	300 µg/kg
Montenegro**	”	Feed for swine, ox and poultry	600 µg/kg
Ukraine**	DON	Combined feed for all animals	1000 µg/kg
”	T-2	Combined feed for layers and broilers	200 µg/kg
”	T-2	Combined feed for calves and cattle	50 µg/kg
Columbia**	ZEA	Sorghum	1000 µg/kg
Cuba**	DON	All feeds	300 µg/kg
China**	T-2	Complete feed	80 µg/kg
”	DON	”	500 µg/kg
Japan**	DON	Compound feeds	1000 µg/kg
”	DON	Feed for all livestock excluding calves < 3 months	200 µg/kg
”	DON	Feed for calves < 3 months	4000 µg/kg
USA**	DON	Grain and grain by-products for all animals	5000 µg/kg
”	”	Feed for cattle and chicken***	10 mg/kg
”	”	Feed for pig***	5mg/kg
Canada**	T-2	Feed for swine and poultry	1000 µg/kg
”	DON	Feed for cattle and poultry	5000 µg/kg
”	HT-2	Feed for cattle and poultry	100 µg/kg
”	DAS	Feed for swine	2000 µg/kg
”	DAS	Feed for poultry	1000 µg/kg
Iran**	T-2	Complete feed for sheep, goat and cattle	100 µg/kg
”	DON	Complete feed for sheep, goat and cattle	5000 µg/kg
Israel**	DON	All grains	1000 µg/kg
”	T-2	”	100 µg/kg
”	DAS	”	200 µg/kg

*EU Commission Recommendation of 17th August 2006 (2006/576/EC)

**DIRECTIVE 2002/32/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 7 May 2002

*** Trucksess et al, 1995

4.1.2. Tolerable Daily Intake Level (TDI)

Hazard characterization of the toxins and the tolerable daily intake (TDI) levels are determined by the dose-response considerations and evaluation of relevance of the endpoints observed in experimental systems for humans. A TDI is an estimate of the amount of a substance in air, food or drinking water that can be taken in daily over a lifetime without appreciable health risk. TDIs are calculated on the basis of laboratory toxicity data to which uncertainty factors are applied. TDIs are used for substances that do not have a reason to be found in food (as opposed to substances that do), such as additives, pesticide residues or veterinary drugs in foods. A dose which gives no-observed-adverse-effect-level (NOAEL) or lowest-observed-adverse-effect-level (LOAEL) for the pivotal effects is identified and thus the tolerable intake limit is determined for each toxin (Larsen et al, 2004).

TDI = (NOAEL or LOAEL) / UF (UF = Uncertainty factor)

The TDI limits of trichothecenes proposed for humans by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and Scientific Committee on Food (SCF) is summarized in Table 4.3.

Table 4.3. TDI levels for Major Trichothecene Toxins

(Adapted from Larsen et al, 2004)

Compound	LOAEL/ NOAEL mg/kg, bw/day	Uncertainty/ Safety factor	t-TDI µg/kg, bw/day
DON	0.1 (NOAEL)	100	1
NIV	0.7 (LOAEL)	1000	0.7
T-2	0.03 (LOAEL)	500	0.06
HT-2	0.03 (LOAEL)	500	0.06

The SCOOP (Scientific Co-Operation on Questions Relating to Food) task 3.2.10 has analyzed the occurrence of *Fusarium* toxins in food and the rate of exposure and the level of intake by the population of EU member states. Among cereals, corn was contaminated with trichothecenes to a higher level than what was observed for other cereals. Most of the occurrence data have proved DON as the frequent contaminant in wheat (Table 1.7) and the consumption data also reported DON as the major trichothecenes toxin in wheat, followed by NIV, T-2 and HT-2 (Schothorst and van Egmond, 2004). Barley, rye and oats have also been shown to contain high levels of DON (Table 1.7). With the exception of a few samples, DON levels reported in majority of cereal products or the raw materials from the EU states have never exceeded the proposed limits of 750 and 500 µg/kg, respectively (Schothorst and van Egmond 2004). The intake level of DON for most of the population groups exists far below the TDI level of 1 µg/kg bw whereas that of the infants is very close to or even exceed the TDI levels (Schothorst and van Egmond 2004). For T-2 and HT-2 the t-TDI level of 0.06 µg/kg bw is exceeded in most of the cases. NIV is the safest among the trichothecenes with mean intake level of 0.05-0.09 µg/kg bw which is far below the t-TDI of 0.7 µg/kg bw (Schothorst and van Egmond 2004).

In this chapter we sought to detect both fungi capable of secreting trichothecenes toxins as well as the toxin levels from various Indian foods directly. This seemed necessary because scanty information was available on occurrence of trichothecenes in Indian food. The development of a rapid and sensitive method for predicting the nature of the fungal contaminant seemed necessary.

4. 2. Materials and Methods

4.2.1. PCR Detection of Trichothecegenic *Fusaria* from Food

The autoclaved maize (10 g) provided with 30-40 % moisture was inoculated with a trichothecene producing *Fusarium* isolate, ICR-PQ-12 (identified as *F. nelsonii*, a DON producer, section 3.3.5) and was incubated at 28 ºC for 3 days. Maize was mixed properly and this was used as the pre-inoculum to infect 10 g of fresh maize. Potato dextrose broth (10 ml) in 50 ml flasks were inoculated with the infected maize, varying in amounts from 0.001-0.8. The flasks were incubated at 28 ºC for a period of 0-72 h. Infected maize from each flask was harvested by filtration. The modified method of Lee et al (2001) was followed for isolation of DNA from equal amounts of the harvested material. PCR was carried out with T5GF2-T5GR2 primers following the procedure described in section 2.3.3.

The *Fusarial* load in each set was determined simultaneously. 100 µl of the broth culture were plated on to PDA plates starting from the increasing dilutions and incubated at 28 ºC for a period of up to 7 days for mould growth. The colony counts from the different dilutions were read using a colony counter and were recorded.

4.2.2. Collection of Food Materials

Food samples were procured from Mysore and Hyderabad city markets and feed samples from a poultry farm in Mysore. 40 raw food ingredients were screened of which 23 materials were cereals and millets such as sorghum (14), wheat (2), barley (1), maize (2) and rice (4); seven were spice samples such as whole chilli (2), turmeric (1), coriander (1), ginger (1), chilli powder (1) and pepper (1); 5 were raw materials for feed preparation such as oil cakes of sunflower (2), safflower (1), groundnut (1) and rice bran (1) and 5 were feed materials such as poultry feed (2), pet food (1) and cattle feed (2).

4.2.3. *Fusarium* Isolation and Maintenance

The food commodities were powdered to pass through 20 BSM sieve. Conventional dilution plating technique (as described in section 2.1.1) on Plate Count Agar [(PCA), HiMedia Mumbai, India] and PDA were employed to assess the total bacteria and yeast and mould count of the samples. 100 µl of the suspensions from successive dilutions were plated on to PCA and PDA plates starting from the increasing

dilutions. The plates were incubated at 37 ºC overnight and at 28 ºC for a period of 7 days for bacterial and yeast and mould growth, respectively. The colony counts from the different dilutions were read using a colony counter and recorded. The purified *Fusarium* isolates were maintained on PDA.

4.2.4. PCR Detection of Trichothecenes in Market Samples

10 g of the sample in aseptic conditions was provided with 35 % moisture (sterile water) and incubated at 30 ºC $\pm$ 2 ºC for 24-48 h based on the microbial load prior to isolation of DNA. The enriched food material was ground properly before extracting DNA. The remaining steps were carried out following the procedure of Lee et al (2001) as described in section 2.3.1. The primers T5GF2 and T5GR2 (the nested primers used for screening of *Fusarium*, refer section 3.2.2.1. Design of Oligonucleotide Primers for PCR Amplification of *tri 5* and Table 3.1) were used for the PCR amplification of *tri 5* following the procedure as described in section 2.3.3. A 10 µl aliquot of the amplicon was separated by 1.5 % (w/v) agarose gel electrophoresis as described earlier (section 2.3.2). Size of the PCR amplicon was obtained by comparing the band size with a 100 bp DNA ladder (Bangalore Genei, India).

4.2.5. Screening of Food and Feed Samples for Trichothecenes

4.2.5.1. Primary Screening by TLC

100 g of each sample was dried at 45 ºC for 48 h and then ground to powder to pass through 20 BSM sieve The toxins were extracted and analyzed for trichothecene toxins following the method of Scott et al (1970) as described earlier (section 2.2.4).

4.2.5.2. Extraction of Trichothecenes for HPLC

HPLC was carried out for the identification and characterization of type A trichothecenes following the procedure of Mateo et al (2002) as described in section 2.2.7. Quantification of trichothecene mycotoxin in food samples were carried out by comparing the peak area of the sample with that of the standard of known concentration.

4.3. Results and Discussion

4.3.1. Sensitivity of PCR for the Detection of Toxigenic *Fusaria* from Food

In this study, maize inoculated with trichothecegenic *Fusarium* (0.001-0.8 g) was added to PDB and incubated for different time intervals (0-72 h) for the enhancement of fungal growth. The colony forming units varied from 1.7 x 10^5 (0.001 infected sample/10 g of uninfected maize) to 150 x 10^8 (0.8 g of infected sample/10 g of uninfected maize) after 48 h enrichment in PDB. PCR analysis of the infected maize with *tri 5* specific primers employing DNA extracts of equal amounts of maize revealed that 7 x 10^6 cfus obtained from 0.004 g of infected sample/10 g of maize were successfully detected in the assay. Mayer et al (2003) has also noticed a positive correlation between the copy number of aflatoxin synthesis gene *nor-1* and the viable *A. flavus* in foods. Positive correlation has been observed between DNA content of *Fusarium* and deoxynivalenol concentration in wheat samples by Schnerr et al (2002). Manonmani et al (2005) have detected 46 x 10^2 and 9 x 10^2 cfu of *A. flavus* in maize and groundnut, respectively using aflatoxin biosynthesis gene (*aflR*) specific primers. They have also observed positive amplification of *aflR* gene from pure cultures of *Aspergillus* with a detection limit of 26 x 10^2 and 32 x 10^2 cfu/ml of PDB. PCR amplification of *aflR* gene has been reported from maize inoculated with *A. flavus* and *A. parasiticus*, after 12 h enrichment of the infected material [(with a spore count of 10^1 and 10^4, respectively) (Somshekar et al, 2004)]. No amplification was observed when samples incubated for 0-6 hours were subjected for PCR. According to this study incubation up to 6 hours of samples with higher fungal loads in PDB was sufficient to allow successful amplification of the *tri 5* fragments (Table 4.4).

Table 4.4. Detection of *tri 5* from Maize Infected with Trichothecegenic *Fusarium*

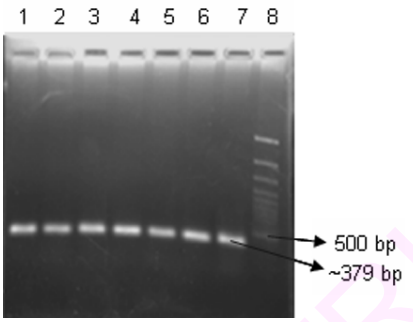
Pre-inoculum (g)/10 ml PDB	PCR after different time period of enrichment (Infected maize as pre-inoculum)						cfu at 48 h enrichment
	0 hr	6 hr	12 hr	24 hr	48 hr	72 hr	
0.001	-	-	-	-	-	-	17 x 10^5
0.002	-	-	-	-	-	+	27 x 10^5
0.004	-	-	-	-	+	+	70 x 10^5
0.006	-	-	-	-	+	+	231 x 10^5
0.008	-	-	-	+	+	+	256 x 10^5
0.01	-	-	-	+	+	+	462 x 10^5
0.02	-	-	-	+	+	+	609 x 10^5
0.04	-	-	-	+	+	+	719 x 10^5
0.06	-	+	+	+	+	+	847 x 10^5
0.08	-	+	+	+	+	+	900 x 10^5
0.1	-	+	+	+	+	+	104 x 10^6
0.2	-	+	+	+	+	+	420 x 10^7
0.4	-	+	+	+	+	+	764 x 10^7
0.6	-	+	+	+	+	+	116 x 10^8
0.8	-	+	+	+	+	+	150 x 10^8

4.3.2. Screening of Food and Feed for the Presence of Trichothecenes

In the present study, an attempt was made to determine the natural occurrence of trichothecenes and trichothecegenic *Fusaria* in food and feed commodities collected from Mysore and Hyderabad using HPLC and PCR. The microbial content of the commodities were analyzed in order to gather primary information on the quality of the materials. Bacterial load as low as zero to fairly high levels of 4 x 10^5 was obtained whereas the viable spore count of fungi ranged from 0 to 7.6 x 10^4 colony forming units in the various samples (Table 4.5). PCR was used for the screening of market samples along with other conventional methods. DNA extracted from seven samples (six sorghum and one poultry feed) supported the amplification of ~379 bp fragment of *tri 5* gene (Fig.4.1). Inhibition of PCR caused by the complex food matrix resulting in false negative results was not observed with any of the toxin positive food material. Positive amplifications were also observed in *Fusarium* strains isolated from the toxin containing samples.

Fig. 4.1. Amplification of 379 bp Fragment of *tri 5* from Food and Feed

Lanes 1: Poultry feed 1, 2: Sorghum C, 3: Sorghum D, 4: Sorghum E, 5: Sorghum J, 6: Sorghum 3, 7: Sorghum 4, 8: 3 kb DNA ladder



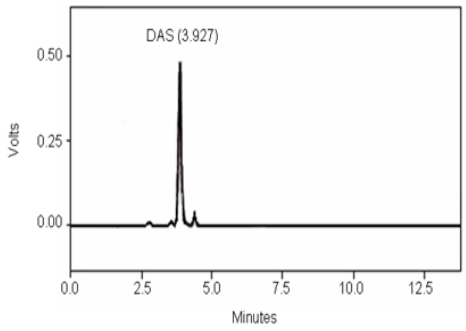
TLC of the different food or feed materials revealed contamination of six sorghum samples with T-2 and DAS. The sample of poultry feed which showed positive reaction in PCR was contaminated with T-2 toxin. None of the type B trichothecenes were detected in any of the samples in the initial screening (Table 4.5.). This was confirmed by HPLC in which T-2 and DAS were detected in sorghum and T-2 toxin alone in poultry feed. HPLC chromatograms obtained for the different samples are given in Fig. 4.2. DAS and T-2 toxins were detected at retention times of 3.8 ($\pm$ 0.1) and 7.02 ($\pm$ 0.1) min, respectively.

Though trichothecene contamination rates second only to aflatoxins, cereal grains, particularly wheat, are the major source of DON worldwide (Larsen et al, 2004). Other toxins like HT-2, NIV and ZEA have also been reported in wheat (Table 1.7). Corn and barley have also been reported to contain DON in quantities lower than that reported from wheat (Placinta et al, 1999; Schothorst and van Egmond, 2004). Reports on contamination of sorghum with trichothecenes are limited (Table 1.7). The few studies of Rukmini and Bhat (1978) and Bhavanishankar and Shantha (1987) have reported the occurrence of T-2 toxin in sorghum in India. Incidence of higher levels of T-2 toxin has also been reported in stored grains of sorghum, rice and groundnuts by Patkar (1993) and Usha (1994). DAS, usually occurring as co-contaminant with T-2 or HT-2 toxin, has been

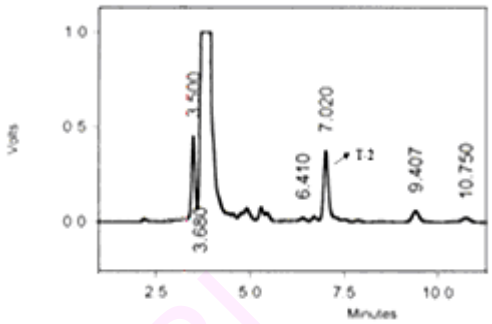
reported from colder European localities following epidemics of *F. sporotrichioides* and *F. poae* (Bottalico and Perrone, 2002). Contamination of cereals and grains with DAS either singly or together with other trichothecenes have been reported from Brazil, Canada, South Africa, Poland and Czech Republic [(Perkowski et al, 1990; Stratton et al, 1993; Furlong et al, 1995; Placinta et al, 1999; Bottalico and Perrone, 2002) (see also Table 1.7)].

Fig. 4.2. Liquid Chromatogram of T-2 and DAS Detected in Food and Feed

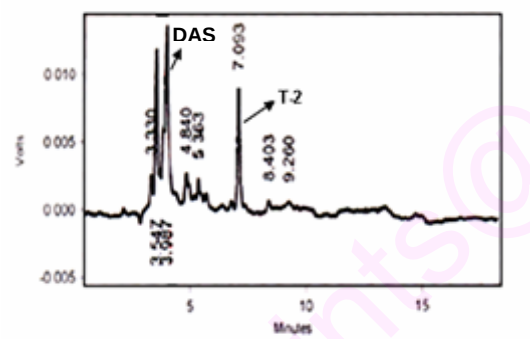
A. DAS standard



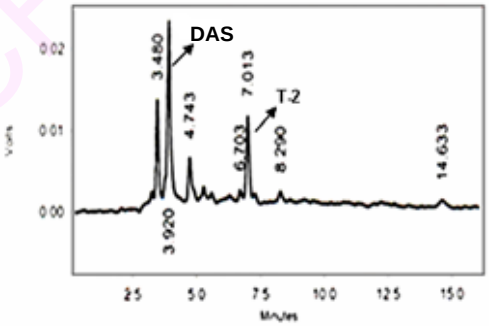
B. T-2 standard



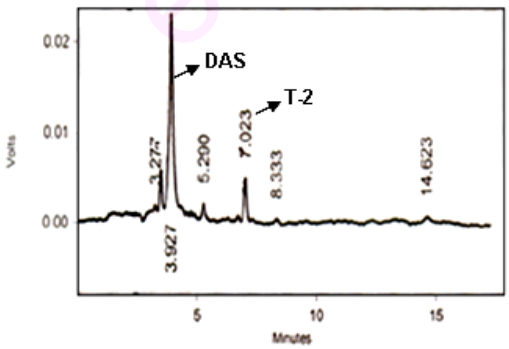
C. Sorghum 3



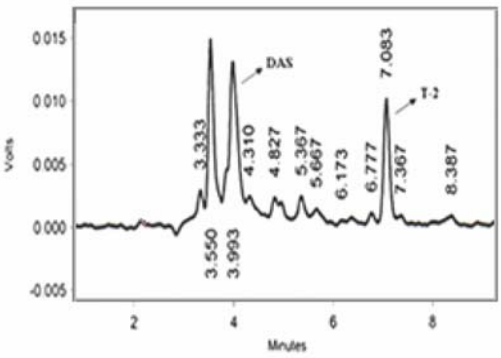
D. Sorghum 4



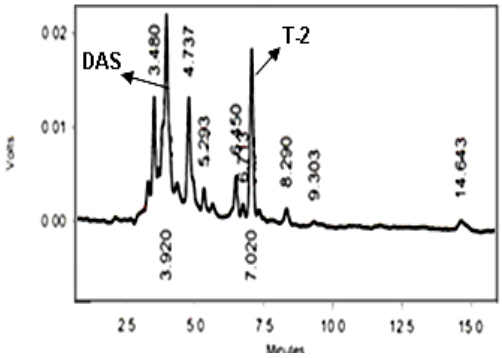
E. Sorghum C



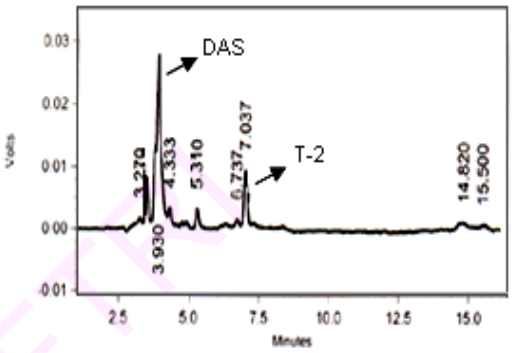
F. Sorghum D



G. Sorghum E



H. Sorghum J



I. Poultry feed 1

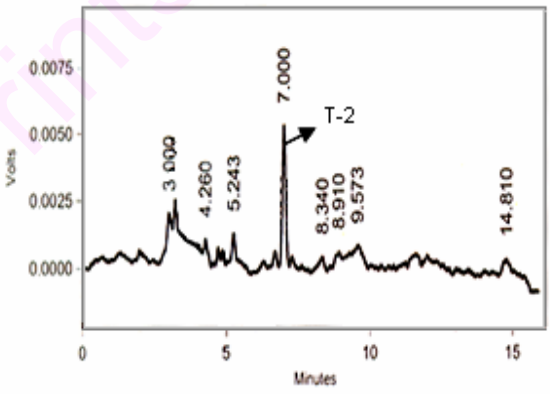


Table 4.5. List of Food and Feed Materials Used and the Type of Trichothecenes Detected

Sl. No.	Sample Name	PCA (cfu/g)	PDA (cfu/g)	*Fusarium* toxin detected			PCR amplification of *tri 5*
				DAS	T-2	DON	
1.	Sorghum-A	0.093(10^2)	46.4(10^2)	-	-	-	-
2.	Sorghum-B	0.011(10^2)	80(10^2)	-	-	-	-
3.	Sorghum-C	0.023(10^2)	8.2(10^2)	+	+	-	+
4.	Sorghum-D	0.24(10^2)	4.3(10^2)	+	+	-	+
5.	Sorghum-E	0.034(10^2)	240(10^2)	+	+	-	+
6.	Sorghum-F	0.14(10^2)	220(10^2)	-	-	-	-
7.	Sorghum-G	0.075(10^2)	17.5(10^2)	-	-	-	-
8.	Sorghum-H	0.32(10^2)	110.4(10^2)	-	-	-	-
9.	Sorghum-I	0.005(10^2)	331.2(10^2)	-	-	-	-
10.	Sorghum-J	0.21(10^2)	102.4(10^2)	+	+	-	+
11.	Cattle feed-CF1	0.14(10^2)	1.6(10^2)	-	-	-	-
12.	Cattle feed-CF2	0.32(10^2)	0.7(10^2)	-	-	-	-
13.	Wheat-W1	0.007(10^2)	0.2(10^2)	-	-	-	-
14.	Wheat-W2	0.005(10^2)	0.8(10^2)	-	-	-	-
15.	Sorghum-S1	0.001(10^2)	0.1(10^2)	-	-	-	-
16.	Sorghum-S2	0.004(10^2)	0.8(10^2)	-	-	-	-
17.	Sorghum-S3	0.24(10^2)	0.1(10^2)	+	+	-	+
18.	Sorghum-S4	0.008(10^2)	0.9(10^2)	+	+	-	+
19.	Barley	0.004(10^2)	1(10^2)	-	-	-	-
20.	Maize 1	0	0.8(10^2)	-	-	-	-
21.	Oil cake-CF3	0.43(10^2)	2.8(10^2)	-	-	-	-
22.	Oil cake-CF4	0.69(10^2)	0.2(10^2)	-	-	-	-
23.	Oil cake-CF5	0.74(10^2)	22.4(10^2)	-	-	-	-
24.	Oil cake-CF6	0.59(10^2)	6.5(10^2)	-	-	-	-
25.	Chilli (Santhepet)	0.345(10^2)	219.2(10^2)	-	-	-	-
26.	Rice-1(Shi)	1(10^2)	760(10^2)	-	-	-	-
27.	Rice-2(Shi)	23(10^2)	49(10^2)	-	-	-	-
28.	Rice-3(Shi)	Floody	106(10^2)	-	-	-	-
29.	Rice-4(Shi)	51(10^2)	19(10^2)	-	-	-	-
30.	Rice Bran	4000(10^2)	12(10^2)	-	-	-	-
31.	Poultry feed 1	105(10^2)	23(10^2)	+	-	-	+
32.	Poultry feed 2	456(10^2)	34(10^2)	-	-	-	-
33.	Whole chilli(sad)	48(10^2)	6(10^2)	-	-	-	-
34.	Turmeric	1(10^2)	1(10^2)	-	-	-	-
35.	Coriander	792(10^2)	64(10^2)	-	-	-	-
36.	Ginger	0(10^2)	2(10^2)	-	-	-	-
37.	Pet food1	72(10^2)	1(10^2)	-	-	-	-
38.	Chilli powder	0(10^2)	0(10^2)	-	-	-	-
39.	Pepper	2656(10^2)	12(10^2)	-	-	-	-
40.	Maize 2	4000(10^2)	640(10^2)	-	-	-	-

4.3.3. Quantification of Trichothecenes

Quantification of type A trichothecenes using HPLC resulted in the detection of T-2 and DAS ranging from 0.012 (± 0.004) to 0.064 (± 0.006) mg/kg and 0.014 (± 0.004) to 0.084 (± 0.004) mg/kg respectively in sorghum samples and 0.13 (± 0.03) mg/kg of T-2 toxin in poultry feed (Table 4.6.). The levels of toxins detected in this study were within the range that has been reported previously from India and other parts of the world (Table 1.7) and was near the lower detection limits of (LOD) of 0.01-0.04 with the exception of poultry feed in which comparatively higher level of T-2 toxin was detected. T-2 levels ranging from 0.006-2.4 mg/kg have been reported in different food materials (Table 1.7). T-2 toxin levels up to 0.8 (wheat) and 40 (maize) mg/kg have been reported respectively from Kashmir and Mysore, India (Bhat et al, 1989; Janardhana et al, 1999). DAS levels of 0.05-3.4 mg/kg of feed have recently been reported from Croatia (Sokolović and Šimpraga 2006). In this study, DAS levels up to 0.084 mg/kg were detected in the sorghum samples. Our analysis failed to detect HT-2 toxin in any of the samples studied. It appears that T-2, HT-2, DAS and other type A toxins are not frequently confronted in the Indian market and if present, they occur only in very low levels.

Table 4.6. Trichothecene Levels Detected in Food and Feed Materials of This Study

Sl. No.	Food sample	Mycotoxin detected (mg/kg)	
		T-2	DAS
1.	Sorghum 3	0.042 (± 0.003)	0.034 (± 0.007)
2.	Sorghum 4	0.012 (± 0.004)	0.084 (± 0.004)
3.	Sorghum C	0.064 (± 0.006)	0.07 (± 0.002)
4.	Sorghum D	0.043 (± 0.0005)	0.058 (± 0.006)
5.	Sorghum E	0.044 (± 0.003)	0.025 (± 0.005)
6.	Sorghum J	0.023 (± 0.006)	0.014 (± 0.004)
7.	Poultry Feed 1	0.13 (± 0.03)	-

Natural infection of cereal crops with toxigenic *Fusarium* results in the accumulation of trichothecenes in cereal kernels beyond permissible levels, the consumption of which will exert deleterious effects on human and animals and resulting finally in mycotoxicosis (section 1.5.5.5.1. Effects of Trichothecenes on Humans; 1.5.5.5.2. Effects of Trichothecenes on Animals). Trichothecenes have phytotoxic activity and affects seed germination and plant growth. Type A toxins such as T-2 and DAS are more toxic to both plants and animals than the type B toxins like DON and NIV. DON is the most water-soluble among the trichothecenes that translocates easily through phloem and serve as an important aggressive factor in Fusarium Head Blight. DON has been found to inhibit wheat coleoptile growth and seedling growth, strongly to fully at 10^{-3} M and 10^{-4} M concentrations respectively (Snijders, 2004). T-2 toxin inhibited the seed germination of *Striga hermonthica* completely at 10^{-5} M concentration and partially (19 %) at 10^{-7} M concentration (Zonno and Vurro, 1999). NIV had inhibitory effect of about 50 % at 10^{-4} M concentration of the toxin.

The microbial loads encountered in the food samples of the present study were very low and did not correspond to the amount of toxin detected. The higher microbial count in the non contaminated materials can be attributed to the constriction of trichothecenes production to a few species of *Fusaria,* mainly belonging to the sections Sporotrichiella and Discolor (Table 1.2). In the case of samples with trichothecene contamination, the toxins may kill other microbial inhabitants of the sample and thus accounts for the less count. Trichothecenes are known to confer aggressiveness and pathogenicity to the producer species so that the flourishing growth of these fungus results in the accumulation of these toxins which may inhibit the protein synthetic mechanism of other fungi that naturally inhabit the food materials (Proctor et al, 1995; Desjardins et al, 1996; Mesterházy et al. 1999). Regulatory limits proposed for trichothecenes is mainly for type B toxins since they are the most frequently listed toxins in majority of the occurrence reports. Regulatory limits for DON in cereals and other ingredients for consumption of humans and animals have been proposed (Tables 4.1 and 4.2). Limits of up to 100 µg/kg of the T-2 toxin in cereals have been fixed, only in Russia. T-2 and DAS levels occurring near the permissible levels (64 and 84 µg/kg,

respectively) were detected in sorghum samples of this study. The levels of these toxins appeared below the statutory limit permitted in food for human consumption. The amount of T-2 toxin detected in the poultry feed sample (0.13 mg/kg) was much lower than what have been proposed for various feed materials across the world (Table 4.2)

4.4. Conclusions

The current study set out to examine 40 different market samples (food and feed) collected from Mysore and Hyderabad (India) market for the presence of trichothecegenic *Fusaria* or the toxins and the levels of these toxins in the respective samples. Microbial load of the samples were analyzed and the bacterial and yeast and mould count varied from 0 to 4 x 10^5 and 0 to 7.6 x 10^4 respectively. PCR was carried out with T5GF1-T5GR2 primers (designed for amplification of *tri 5* gene) using the genomic DNA isolated directly from the food samples. Amplification of ~379 bp fragment of *tri 5* was observed in seven samples (six sorghum and one poultry feed). Primary screening of the samples for trichothecene analysis using TLC resulted in the detection of T-2 toxin and DAS in six sorghum samples and T-2 alone in poultry feed. Quantification of toxins using HPLC resulted in the identification of considerable levels of T-2 in poultry feed whereas low levels were detected in sorghum. The amount of T-2 and DAS varied from 0.012 to 0.064 mg/kg and 0.014 to 0.084 mg/kg respectively in sorghum. T-2 levels of 0.13 mg/kg was detected in the poultry feed. Though there are no fixed limits proposed for T-2 and DAS levels in food for human consumption in India, the amount detected in the present study was lower than the levels that have been permitted in other countries. This study was designed to identify contamination of Indian market samples with the deadly toxins of the trichothecene type. Lack of information on the occurrence and consumption data from India and other countries is a great limitation in assessing the health hazards posed by the toxins or their derivatives. Therefore efforts should be taken to analyze more number of samples with a focus on the detection of a broad range of common contaminants of *Fusarium* species in the food materials consumed by the different strata of population and to assess the levels of toxin that is actually being consumed.

In this chapter the effective use of PCR and HPLC for the detection of trichothecenes in food commodities have been discussed. Another potentially effective method that is widely used in the screening of food materials for mycotoxins is ELISA, where the toxin specific antibodies are generally used for detecting the toxins. To this end we have developed a novel method of ELISA using antibodies raised against trichodiene

synthase (an enzyme involved in the trichothecene biosynthetic pathway) for the detection of trichothecegenic *Fusaria* in pure cultures or food commodities, the subject for the chapter that follows.

Chapter 5.

Antibody to the Tri 5 Protein

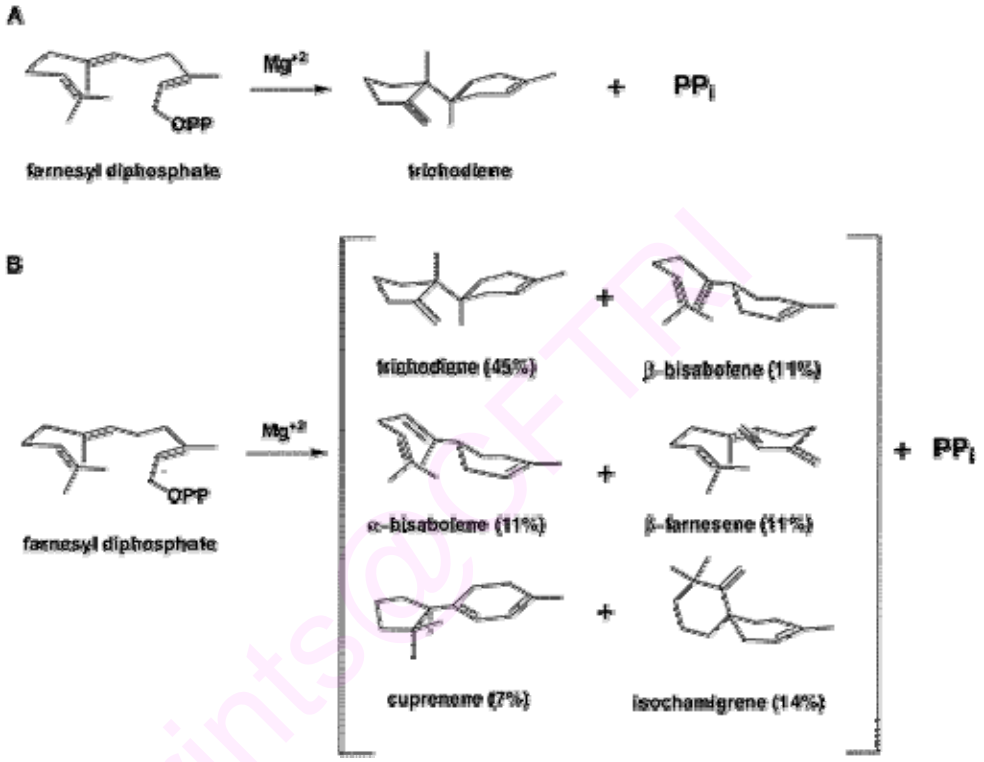
5.1. Introduction

The pathway for the synthesis of trichothecenes has been discussed in section 1.5.5.7 (Biosynthesis and Gene Organization). The synthesis of trichothecenes starts from trans,trans-farnesyl pyrophosphate (PPi/FPP), acted upon by trichodiene synthase to form the natural product trichodiene (Desjardins et al, 1993). Trichodiene has been primarily isolated from *Trichothecium roseum* following studies with tritiated trichodiene (Machida and Nozoe, 1972). Trichodiene is an important component in the pathway of trichothecene biosynthesis and its significance lays in the fact that it is the primary precursor of *Fusarium* trichothecenes. Different trichothecene producers such as *F. sporotrichioides*, *F. culmorum*, and *F. sambucinum* when treated with oxygenation inhibitors have resulted in the accumulation of trichodiene, thereby inhibiting trichothecene production in the initial step itself (Desjardins et al, 1993). Evidences have also been provided by the studies of Beremand (1987) and Plattner et al (1989) using UV mutants of *F. sporotrichioides* that accumulated trichodiene and these exhibited inhibition of the synthesis of T-2 toxin. Trichodiene synthesis is followed by the involvement of an array of minor constituents including several new bicyclic, oxygenated, trichodiene derivatives operating in a metabolic grid of oxygenations, isomerizations, cyclizations and esterifications, leading to the synthesis of different complex trichothecene toxins (Desjardins et al, 1993).

5.1.1. Trichodiene Synthase (Tri 5/Tox 5/TS)

The production of trichothecenes by *Fusarium* occurs through a multi-step pathway in which the enzyme trichodiene synthase catalyze the first unique step of coversion of farnesyl pyrophosphate (FPP) to trichodiene and thereby initiates the cascade of chemical reactions to follow (Fig. 1.14). The enzyme trichodiene synthase is unique in almost all trichothecene producing *Fusarium* (Desjardins et al, 1993). The enzyme is typical of the terpene cyclase types that are involved in the biosynthesis of cyclic terpenoids in both plants and fungi (Desjardins et al, 1993). Cyclization of farnesyl diphosphate to trichodiene on by trichodiene synthase is the first step in the synthesis of trichothecenes (Fig. 5.1).

Fig. 5.1.A. Cyclization of Farnesyl Diphosphate to Trichodiene, B. Sesquiterpene Products Generated by Trichodiene Synthase (Adapted from Rynkiewicz et al, 2001)



(OPP- diphosphate, PPi-inorganic pyrophosphate)

Trichodiene synthase (TS) is a homodimer with a subunit of 45 kDa (Hohn and VanMiddlesworth, 1986). The enzyme requires three Mg^{2+} ions as cofactor for its activity. Two of these ions occupy the metal binding sites in the active site of the enzyme (Rynkiewicz et al, 2001). In presence of pyrophosphate its activity is inhibited (Cane, 1990). The enzyme undergoes substrate induced conformational changes and triggers the cascade of cyclization reactions (Rynkiewicz et al, 2001). The enzyme structure is formed by 17 α-helices, six of which (C, D, G, H, I, and J) define a conical and hydrophobic active site cleft (Rynkiewicz et al, 2001). An aspartate-rich motif DDSKD (starting at residue 100) is located at the C-terminal end of helix D out of which D100 and

D101 are important for catalytic activity of the enzyme. At the C-terminal end of helix J is the "basic motif" DRRYR (residues 302-306). Mutations at R304, Y305, and R306 of this motif results in inhibition of catalytic activity of the enzyme (Rynkiewicz et al, 2001).

Hohn and Beremand (1989b) have observed an increase in TS enzyme activity from undetectable to maximum levels over a period of 3 h and 144 h (early stationary phase of growth), respectively, in the culture filtrates of *F. sporotrichioides* and *G. pulicaris*. They have also monitored changes in the TS polypeptide levels in relation to TS enzyme activity, using antiserum prepared against purified TS from *F. sporotrichioides.* Changes in the TS polypeptide and enzyme activity were corresponding with each other, both in *F. sporotrichioides* and *G. pulicaris* (Hohn and Beremand, 1989b).

5.1.2. Gene for Trichodiene Synthase, *tri 5*/*tox 5*

Trichodiene synthase (Tri 5), encoded by the trichodiene synthase gene *tri 5/ tox 5,* occurs in single copy in *F. sporotrichioides* and *G. pulicaris* (Hohn and Beremand, 1989b; Hohn and Desjardins, 1992). The genes from both the species are highly homogeneous in sequence whereas differences occur near the C terminal end of Tri 5 enzyme in *G. pulicaris*. Studies at the *tri 5* mRNA level in *G. pulicaris* have indicated 47 fold increases within 18-42 h post inoculation whereas the trichodiene synthase enzyme activity increased only 10 fold during the same period (Hohn et al, 1993). Partial control over the *tri 5* gene expression occurs at transcription level in *G. pulicaris* (Hohn et al, 1993).

The gene *tri 5* is 1192 bp in length and have two exons and a single intron almost at its middle. The first exon of length 469 bp is separated from the second exon (658 bp) by an intron varying in length from 50-65 bp in the different trichothecene producers (Fig. 5.2.A). Gene *tri 5* occupies the 26 Kb core trichothecene cluster and is positioned to the immediate downstream of *tri 6*. The expression of *tri 5* is under strict control of the regulatory genes *tri 6* and *tri 10* that are situated upstream and downstream, respectively (Fig. 5.2.B), along with few environmental factors such as nutrients, water activity and

temperature (discussed in section 1.5.7.1. Physico-Chemical Factors Controlling Trichothecene Production).

Fig. 5.2.A. Schematic Diagram of *tri 5* Gene

B. Trichothecene Core Cluster

Hohn and Plattner (1989) and Cane et al (1993) have expressed the coding region of the *tri 5* in *E. coli* resulting in the production of trichodiene. Hohn and Ohlrogge (1991) have observed the presence of trichodiene in leaves from transgenic plants of tobacco that had been transformed with trichodiene synthase gene.

5.1.3. Polyclonal Antibody Production in Chicken

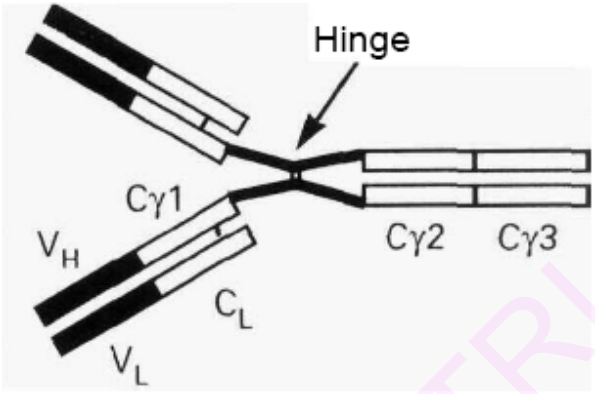
The use of chicken for immunization protocols has resulted in the refinement and limited use of laboratory animals with the greatest advantage that the collection of blood is replaced by antibody extraction from egg yolk. The production of larger quantities of antibodies than in laboratory rodents, and the similar concentrations of specific antibodies in egg yolk and serum promotes the use of chicken in immunization procedures.

5.1.3.1. Avian (Egg Yolk) Immunoglobulin Y: The IgY

Major type of immunoglobulin isolated from chicken is the egg yolk antibodies, immunoglobulin Y (IgY), the 150 kDa homolog of IgG. Other antibodies are also present in egg yolk but occur in negligible amounts (Schade et al, 1996). A normal hen is able to lay 5-7 eggs per week and the amount of IgY antibody contained in one egg yolk amounts to about 50-100 mg (Schade et al, 1996). The average antibody yield will be ~1500-3000 mg which is very high when compared to the antibody yield from any mammal (~200 mg from a 40 ml bleed). The rare advantage that is not available in the case of any other antibody source is that IgYs can be stored without contamination for up to one year at 4 ºC since avian eggs are protected carefully inside the shells. Another advantage is that eggs from immunized chicken provide a continual daily source of polyclonal antibody (Haak-Frendscho, 1994).

The structure of IgY is identical to the major Ig found in serum but is different from the mammalian IgG (Fig.5.3). The fundamental structural differences between IgG and IgY occur in the heavy chain region of these antibodies (Schade et al, 1996). IgG has a heavy γ chain of 50 kDa and consists of four domains: one variable domain (VH) and three constant domains (Cγl-CyS). The Cγl domain is separated from Cγ2 by a hinge region which gives considerable flexibility to the Fab fragments. The heavy chain region, υ, of IgY having a molecular weight of 65 kDa does not have a hinge region and possesses four constant domains (Cυl-Cυ4) in addition to the variable domain. The Cυ2 and Cυ3 domains of IgG are closely related to those of IgY with the exception that the Cυ2 domain is absent in the γ chain, having been replaced by the hinge region. A comparison of the characteristic features of mammalian IgG and yolk IgY is summarized in Table 5.1.

Fig 5.3.A. Structure of Mammalian IgG (Adapted from Schade et al, 1996)



B. Structure of Avian IgY

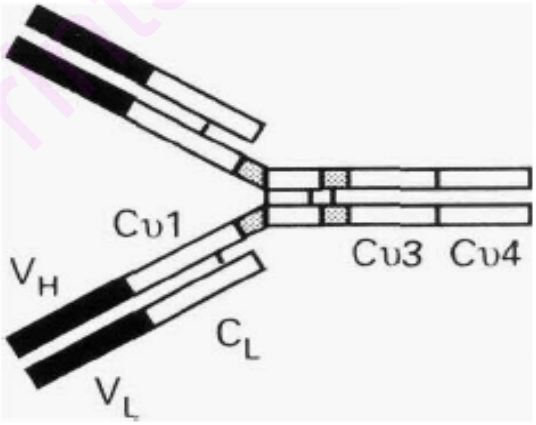


Table 5.1. Comparison of Mammalian IgG and Avian IgY

(Adapted from Schade et al, 1996)

	Mammalian IgG	**Avian IgY**
Antibody sampling	Invasive	Non-invasive
Antibody yield	200 mg IgG per bleed (40 ml blood)	50-100 mg IgY per egg (5-7 eggs per week)
Antibody yield per month	200 mg	~ 1500 mg
Specific antibody yield	5 %	2-10 %
Protein A/protein G binding	Yes	No
Interference with mammaliam IgG	Yes	No
Interference with rheumatoid factor	Yes	No
Activation of mammalian compliment	Yes	No

Chicken used for antibody production are of the SPF chicken i.e., chicken which have been bred free of the specific pathogens or the commercial egg laying ones (Schade et al, 1996). SPF chicken is advantageous in 2 ways; (i) they give high antibody titres and (ii) viral diseases affecting antibody production will be reduced in the case of SPF chicken which may otherwise cause immunomodulatory effects. SPF chicken are specifically used for generating antibodies that are to be used in therapeutic purposes. Commercial egg laying chicken are cheaper, easy to purchase and obtained at the laying stage so that the cost of antibody production will be reduced considerably.

5.1.3.2. Immunization Protocols

Antigen emulsified in Freund's complete or incomplete adjuvant is generally used for immunization of poultry. The antigen concentration to be used depends on the type of antigen and the immune reaction it elicits; in the usual protocols an average antigen concentration of 10 ng-1 mg is used for injection. Chicken those are at least 7 weeks old preferably are immunized with an antigen. An injection volume up to 1 ml is being used for injection at multiple sites, intramuscularly (Schade et al, 1996). Route of immunization depend on the age and type of poultry used for antibody generation. Laying hens, whether kept under field conditions or in the laboratory, can be injected

subcutaneously or intramuscularly at multiple sites in the breast. Chicken can also be vaccinated subcutaneously in the neck. Young animals are preferably injected intramuscularly into the breast muscles due to the difficulty in performing subcutaneous injections (Schade et al, 1996). Chicken can also be used for whole of the laying period (almost 1 year) for vaccination purpose. Vaccination protocols include a primary dose followed by booster doses of the antigen at an interval of 4-6 weeks, with frequent checking of yolk antibody titres. A minimum of two immunizations are required in the process and further boosters depend on the amount of antibody produced. In case of low antibody titres a larger number of booster immunizations should be given during the laying period (Schade et al, 1996). Vaccination protocols for immunization of chicken may be summarized as follows.

Table 5.2. Vaccination Protocols for Immunization of Chicken

(Adapted from Schade et al, 1996)

Adjuvant	Freund's incomplete adjuvant, Specol, lipopeptide (Pam_3-Cys-Ser-$[lys]_4$; 250 μg)
Antigen dose	10 ng-1 mg
Injection site	Intramuscular (field studies; young laboratory chickens) Subcutaneous (older laboratory chickens)
Injection volume	< 1 ml
Injection frequency	2-3 times; boosters during laying period
Vaccination interval	4-8 weeks
Use of chickens	Entire laying period (about 1 year)

The use of antibodies for the specific detection of various trichothecenes has been reported (1.5.8.2. Immunochemical Methods). ELISA and RIA have been developed for the qualitative and quantitative analysis of toxins. Other than the reports of Gan et al (1997), the immunoassays defined for trichothecene analysis make use of antibodies developed against the various toxins or their conjugates which are highly specific that a particular toxin can be detected at a time. The antibodies developed by Gan et al (1997) against exoantigens of *Fusarium* were efficient in detection of cultures of *Fusarium*; but it could not distinguish between toxigenic and non-toxigenic species. In this study development of an ELISA method for the group specific detection of trichothecegenic *Fusarium* have been attempted using antibody raised against trichodiene synthase.

5.2. Materials and Methods

5.2.1. Fungal Cultures

F. asiaticum (NCIM 651) was used for amplification of the *tri 5* fragment. (This fungi obtained as *Gibberella saubinetti* from NCIM, Chandigarh, India, was identified as *F. asiatcum* in this study). Mycelial extracts of isolates of *Fusarium* such as ICR1, ICR50, ICR-PQ-12, ICR-PQ-2, ICR-PQ-13, FM246, Isolate 1, *F. asiaticum* (NCIM 651), *Aspergillus niger* and *Penicillium* were used for characterization of antibody. The isolates ICR-PQ-12 and ICR103 that were previously identified as producers of DON (Table 3.3) and T-2 toxin (Table 3.4), respectively, were used in studying the synthesis of the Tri 5 protein in relation to toxin production.

5.2.2. Cloning of *tri 5* Gene Fragment in pRSETA

A schematic diagram of the strategy used for cloning of *tri 5* gene is given in Fig. 5.4. A fragment of the gene *tri 5* was amplified from the genomic DNA of *F. asiaticum* (*G. saubinetti*) following the procedure as described in section 2.3.3. PCR was carried out using the oligonucleotide primers T5GF3-T5GR1 that specifically amplified a portion of *tri 5* gene outside the intron region. Semi-nested primer T5GR2 that prime within the T5GF3-T5GR1 amplified *tri 5* fragment was used to check the authenticity of PCR product. List of primers used in the study are given in Table 5.3.

Table 5.3. List of Primers used

Sl. No.	Primer name	Sequence
1.	T5GF3	5'-GGGATGCTGGATTGAGCAG-3'
2.	T5GR1	5'-TYACTCCACTAGCTCAATTG-3'
3,	T5GR2	5'-CACACCTCACCCTCCTTCT-3'

PCR conditions

Initial denaturation: 94 °C for 5 min
Denaturation: 94 °C for 1 min
Annealing: 55 °C for 1 min
Extension: 72 °C for 1 min
} 34 cycles
Final extension: 72 °C for 10 min

Cloning of the *tri 5* gene fragment into pTZ57R/T was carried out following the procedure described in section 2.6. The recombinant plasmid designated pTZ-T5 was screened for the insertion of *tri 5* by PCR and insert release using the restriction enzymes *BamHI* and *EcoRI* (as described in sections 2.9.1 and 2.9.2, respectively). Direction of insertion of the gene in pTZ-T5 was ascertained as described in 2.9.3 and 2.9.4. The *tri 5* fragment from pTZ-T5 was sub-cloned into the pRSETA vector as described in section 2.10. The restriction enzymes *BamHI-KpnI* were used to excise the fragment off from pTZ-T5 and the fragment was ligated to *BamHI-KpnI* cut pRSETA vector. The recombinant plasmid named pRA-T5 was screened and checked through PCR and release of the insert using *BamHI-KpnI* restriction enzymes. The plasmid pRA-T5 was finally transformed into the expression host *E. coli* BL21. The construct pTZ-T5 was sequenced at the Bangalore Genei, Bangalore using M13 forward universal primer.

5.2.3. Over-Expression of Recombinant Tri 5 in *E. coli*

The gene *tri 5* was ligated to pRSETA in fusion with 6-His tag such that the recombinant Tri 5 would be a fusion protein having a His-tag at C terminal end of the protein. Over-expression of the recombinant Tri 5 protein was carried out following the procedure as described in section 2.12.

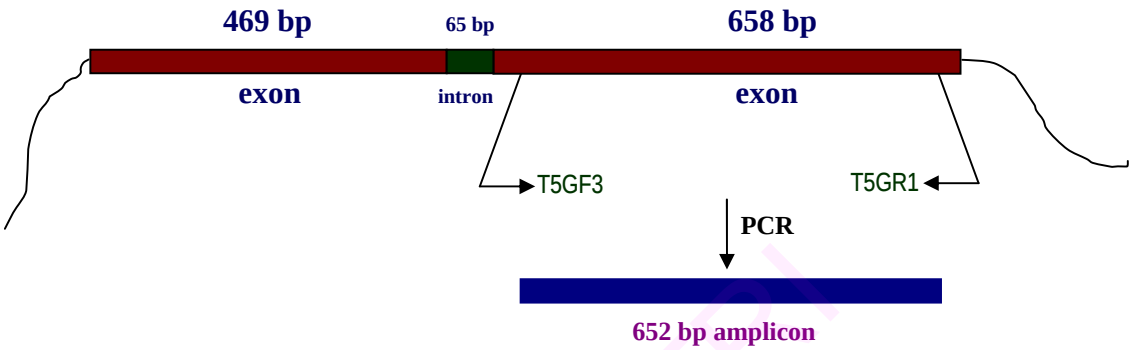
5.2.4. Immunization of poultry

Egg laying poultry (n=2), 20 weeks old, were collected from a poultry farm in Mysore. One was used for immunization and the other was kept as control.

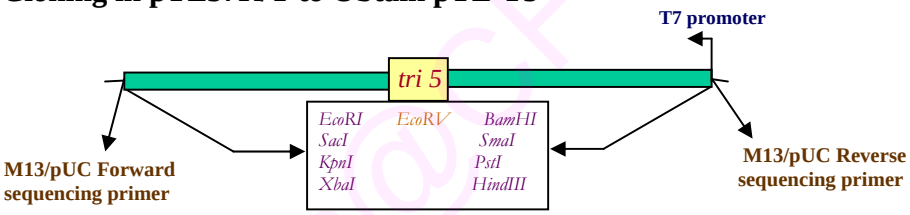
Immunization of poultry with purified Tri 5 was carried out following the procedure as described in section 2.15.

Fig. 5.4. Schematic Diagram of Cloning Strategy

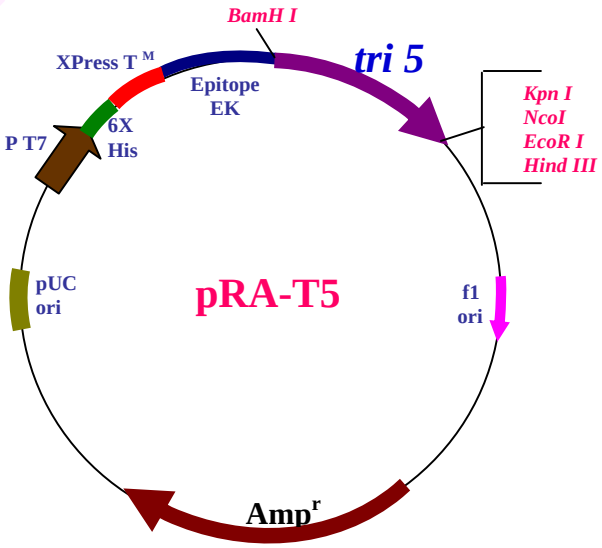
1. PCR



2. Cloning in pTZ57R/T to Obtain pTZ-T5



3. Cloning in pRSETA to Obtain pRA-T5



5.2.5. Isolation of IgY

IgYs were isolated from both immunized and non-immunized poultry following the procedure as described in section 2.16.

5.2.6. Characterization of Antibody

IgYs produced against recombinant Tri 5 was isolated and characterized using Western Blot (section 2.17) and ELISA (section 2.18).

5.2.7. Estimation of Protein

5.2.7.1. Total Protein in Recombinant *E. coli*

The cells of recombinant *E. coli* were grown in 50 ml of LB broth and induced with IPTG after attaining OD_{600} of 0.8-1. IPTG concentration ranging from 0.1-1 mM were added to the cultures and incubated at 37 °C for 6-15 h. Cells from each set was harvested by centrifugation at 4000-6000 rpm for 10 min. The cells were washed in 1 ml sterile water to remove the media components. Cell pellet obtained after centrifugation at 4000-6000 rpm for 10 min were resuspended in lysis buffer (100 mM EDTA, 100 mM β-mercapto ethanol and 10 mM PMSF). The cells were lysed by sonication. Supernatant was collected after centrifugation of the cell lysate at 10,000 rpm for 10 min. 10 µl of the supernatant was diluted with 100 µl deionized water. The amount of protein in 10 µl of the diluted sample was determined by Bradford's method (section 2.14).

5.2.7.2. Total Protein in IgY

Protein in the IgYs isolated from eggs collected from the immunized and non-immunized hens was estimated. 10 µl of the IgY isolate was diluted with 100 µl deionized water. The amount of protein in 10 µl of the diluted sample was determined by Bradford's method (section 2.14).

5.2.7.3. Relative Band Intensity of 28 kDa Protein

Cell lysate of *E. coli* bearing pRA-T5 was prepared following the procedure as described earlier (section 5.2.7.1). The supernatant was separated using SDS-PAGE following the procedure described in section 2.11. The gels were stained with Coomassie Brilliant Blue R-250 and the relative band intensity of the over-expressed protein across the different the gel lanes were determined using the facility available in the gel documentation unit (Chemidoc, BIO-RAD Laboratories, Italy).

5.2.8. Preparation of Antigen

5.2.8.1. *E. coli* Cell Extract

For characterization of antibody, cell lysates from *E. coli* BL21 bearing pRA-T5 and pRSETA, the host *E. coli* BL21 (prepared as described in section 5.2.7.1) and the purified Tri 5 fusion protein were used as source of antigen. The supernatant obtained after *E. coli* cell lysis was dialyzed against deionized water, lyophilized and finally suspended in minimum volume of PBS, which were further used as antigen in western blot and ELISA. The purified Tri 5 fusion protein was also suspended in PBS.

5.2.8.2. Extract of Fungal Mycelia

The specificity of IgY against Tri 5 was determined by the immunoblotting of mycelial extracts of trichothecene producers of *Fusarium*. Fungal strains listed in section 5.2.1 were inoculated into 50 ml GYEP media (Glucose 5 %, yeast extract 0.1 %, peptone 0.1 %) in 250 ml flasks and were incubated at 28 °C for 1 week. The mycelia were harvested by filtration through Whatman No. 1 filter paper, washed three times using sterile deionized water and freeze dried. The freeze dried mycelia were powdered using sterile mortar and pestle. Equal amount (1 g each) of powdered mycelia was weighed out to which 1 ml of extraction buffer (100 mM EDTA, 100 mM β-mercapto ethanol and 10 mM PMSF) was added and mixed well by vortexing for 10 min. The mixture was heated in a boiling water bath for 5-10 min after which the extract was collected by centrifugation at 8000 rpm for 10 min. The extract, after dialyzing against deionized water, was used as antigen in Western Blots and ELISA.

5.2.8.3. Buffer Extracts of Rice

10 g of previously autoclaved rice samples provided with 30-40 % moisture were inoculated with toxigenic ICR-PQ-12 and non-toxigenic *Fusarium* [ICR82 (2), a trichothecene negative isolate of this study]. These rice samples were incubated for one week at 28 °C after which they were ground to fine powder. 1 g each of the powdered rice was extracted with PBS buffer/ lysis buffer (100 mM EDTA, 100 mM β-mercapto ethanol and 10 mM PMSF)/ water (1 ml/g). The suspension was filtered out and diluted in PBS (1:1, 1:2, 1:3, 1:4, 1:5) to be ready for the assay. Extracts from autoclaved plain

rice and rice inoculated with trichothecene non-producing strains of *Fusarium* were used as negative controls.

5.2.9. Antibody Dilutions and Titrations

IgY isolated from immunized and non-immunized poultry were used for Western blot. Antibodies diluted to an appropriate concentration (1:1000, 1:2000, 1:3000, 1:4000, 1:5000) were prepared 1 h before they were added.

Antibody concentrations to be used for ELISA was optimized using serial dilutions of the antibody from immunized hen (1:20-1:600, in multiples of 20) prepared in PBS buffer.

5.2.10. Influence of Nutrients on Synthesis of Trichodiene Synthase (Tri 5) and Trichothecene Production (DON and T-2)

5.2.10.1. Different Carbohydrates as Carbon Source

Different carbon sources were tested for their influence on synthesis of trichodiene synthase (Tri 5) and thereby trichothecene production. The *Fusarium* isolates ICR-PQ-12 (DON producer) and ICR 103 (T-2 producer) were selected for the study. Liquid GYEP (section 2.2.1), the specific media for toxin production, was used for culturing the fungi. Carbohydrates such as fructose, maltose, lactose, mannose, glucose, sucrose and xylose (SRL, Mumbai, India) were used to replace glucose individually. All carbon sources were used at a concentration of 5 % w/v. Inoculations were made by transferring 100 µl spore suspension from one week old cultures to 100 ml conical flasks containing 20 ml of the media that was incorporated with the respective carbohydrates. The flasks were incubated for a period of 7 days at 28 °C.

5.2.10.2. Varying Concentration of Glucose and Peptone

For studying the effect of varying amounts of carbon and nitrogen sources on Tri 5 and trichothecenes, glucose (SRL, Mumbai, India) and peptone (HiMedia, Mumbai, India) at concentrations ranging from 1.25-5 % and 0.025-2 %, respectively, were incorporated in GYEP media (section 2.2.1). 20 ml of the media in 100 ml conical flasks were inoculated and incubated as described in section 5.2.10.1 for a period of one week.

5.2.10.3. Analysis of Tri 5

The cultures of fungi listed in section 5.2.1 were prepared as described in 5.2.8.2. Mycelia collected by filtration were washed with sterile deionized water and freeze dried. The dried mycelia were ground in a sterile mortar and pestle to obtain a fine powder. 0.1 mg each of the powdered mycelia from different experimental sets were suspended in 100 µl lysis buffer (100 mM EDTA, 100 mM β-mercapto ethanol and 10 mM PMSF) and was vortexed for 1 min. The mycelial lysates were collected by centrifugation and the supernatant was checked for Tri 5 using ELISA, following the procedure as described in section 2.18. The lysate at a concentration of 1:1 dilution was used for the assay.

5.2.10.4. Trichothecene Detection

The different sets of culture filtrates were extracted with chloroform and heat concentrated to dryness. The samples were further processed and the detection and identification of DON and T-2 by HPLC and GC, respectively, were undertaken (sections 2.2.6 and 2.2.5)

5.2.11. Time Course Production of Tri 5 and Trichothecenes (DON)

20 ml of liquid GYEP media (section 2.2.1) in 100 ml flasks was inoculated with 100 µl spore suspension from one week old culture of ICR-PQ-12. The cultures were incubated at 28 °C in a shaker incubator at 150 rpm. Mycelia were harvested at regular intervals from individual flasks at two days interval, starting from 3 days of incubation, for a period of 19 days. The culture filtrates were analyzed for DON by HPLC method following the procedure described in section 2.2.6. Amount of Tri 5 in the mycelial lysate was assayed by ELISA (section 2.18).

5.3. Results and Discussion

5.3.1. Cloning and Expression of Recombinant Trichodiene Synthase (Tri 5)

In this study, PCR fragment of the expected size of 652 bp was amplified from the genomic DNA of *Fusarium* and was cloned initially into pTZ57R/T to obtain pTZ-T5 (Fig. 5.5.A). Amplification of the 652 bp PCR fragment was obtained only from pTZ-T5 (Fig. 5.5.B). Restriction digestion of pTZ-T5 using *BamHI-EcoRI* enzymes also resulted in the release of 652 bp fragment, only from pTZ-T5.

Fig. 5.5.A. Ligation of *tri 5* in pTZ57R/T

Lanes 1: Control vector, 2: Recombinant, 3: Non-recombinant

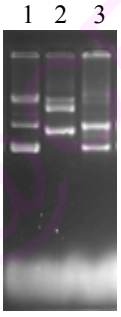
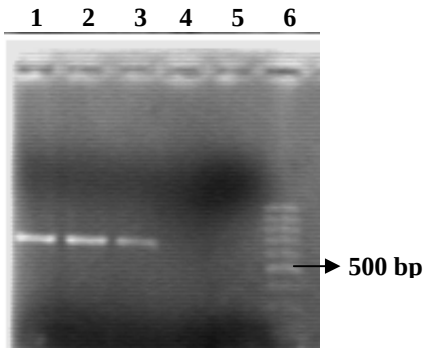


Fig. 5.5.B. Amplification of *tri 5* from pTZ-T5

Lanes 1 and 2: Amplification from pTZ-T5; 3: Amplification from genomic DNA of *Fusarium*; 4 and 5: No amplification from control pTZ57R; 6: 100 bp DNA ladder



The *tri 5* fragment in pTZ-T5 was sequenced. The sequence was compared with the *tri 5* gene sequences of known trichothecene producing *Fusarium* along with the trichothecene producers belonging to other genera of fungi such as *Trichotheceum*, *Myrotheceium* and *Stachybotrys*-all of which are macrocyclic trichothecene producers (Fig. 3.4.A). The sequence was found to be closest to that of *F. asiaticum* [(AY102604) 100 % similarity] and less similar to that of *Stachybotrys chartarum* (75 %) when BLAST was used. Sequence of the EF-1α gene and molecular phylogeny analysis also resulted in the identification of the culture as *F. asiaticum* (3.3.5).

The fragment of *tri 5* was subcloned into pRSETA such that the resultant plasmid pRA-T5 has the fragment in frame with start codon (ATG), 6-histidine, XpressT^{M} and epitope enterokinase cleavage site [(EK) (Fig. 5.6.)]. The *tri 5* fragment, chimaeric with some vector sequences, from the start ATG to the stop TGA coded for a protein with 257 amino acids. The protein sequence that was deduced from the gene sequence of the cloned fragment was identical to sequences of trichodiene synthase from different *Fusarium* species present in the NCBI data base (Fig. 5.7). Similarly as observed in the nucleotide sequence comparison (Fig. 3.4.B), the protein sequence also showed more homology to that of *F. asiaticum*.

Fig. 5.6. Translated Sequence of the *tri 5* Fragment in pRA-T5

```
1   atgcggggtt ctcatcatca tcatcatcat ggtatggcta gcatgactgg tggacagcaa atgggtcggg atctgtacga cgatgacgat
    m  r  g  s  h  h  h  h  h  h  g  m  a  s  m  t  g  g  q  q  m  g  r  d  l  y  d  d  d  d

91  aaggacgatg acgataagct aggcctaaag ggatgcttga ttgagcagta taactttggt ggattcccag gatctgatga ctaccctcaa
    k  d  d  d  d  k  l  g  l  k  g  c  l  i  e  q  y  n  f  g  g  f  p  g  s  d  d  y  p  q

181 ttccttcgac gcatgaatgg cctgggtcat tgtgttgggg cttctctatg gcccaaggag ctgttcgacg agcgaaagaa tttcctcgaa
    f  l  r  r  m  n  g  l  g  h  c  v  g  a  s  l  w  p  k  e  l  f  d  e  r  k  n  f  l  e

271 atcacgacag ccgttgccca gatggagaac tggatggttt gggtcaacga tctcatgtca ttctacaagg aattcgacga tgagcgtgac
    i  t  t  a  v  a  q  m  e  n  w  m  v  w  v  n  d  l  m  s  f  y  k  e  f  d  d  e  r  d

361 cagatcagtc tggtcaagaa ctttgtcacc tgccatgaga tcactctcga tgaagccttg gagaagctca cgcaggaaac tctgcactcg
    q  i  s  l  v  k  n  f  v  t  c  h  e  i  t  l  d  e  a  l  e  k  l  t  q  e  t  l  h  s

451 tccaagcaga tggttgctgt cttcgcggac aaggaccctc aggtgatgga cacgattgag tgtttcatgc acggctacgt tacgtggcat
    s  k  q  m  v  a  v  f  a  d  k  d  p  q  v  m  d  t  i  e  c  f  m  h  g  y  v  t  w  h

541 ttatgcgacg ccagataccg cctccatgag atttatgaaa aggtcaagga tcaggataca gaggacgcta agaagttctg caagttcttt
    l  c  d  a  r  y  r  l  h  e  i  y  e  k  v  k  d  q  d  t  e  d  a  k  k  f  c  k  f  f

631 gagcaggcag ccaatgtcgg cgccgttgca ccctcggagt gggcttatcc acaagttgca cagctggcaa ccaatgtcgg cgccgttgcg
    e  q  a  a  n  v  g  a  v  a  p  s  e  w  a  y  p  q  v  a  q  l  a  t  n  v  g  a  v  a

721 ccctcggagt gggcttaccc accagttgca cagctggcaa tagtggagtg atagatctac gtaagcgctc catggatgga attcgaagct
    p  s  e  w  a  y  p  p  v  a  q  l  a  i  v  e  -  -  i  y  v  s  a  p  w  m  e  f  e  a
```


(Start codon and stop codon depicted in red, 6-histidine tag in blue, *tri 5* reading frame in pink and vector part in green)

Fig. 5.7. Alignment of the Sequence of Tri 5 Protein from NCIM 651 (*F. asiaticum*) With that from Other Species.

The amino acids highlighted in yellow indicate the difference in sequence of the protein fragment used in the current study with that of the known Tri 5 protein

F. pseudogram: *F. psuedograminearum; F. sporotrich*: *F. sporotrichioides*; *F. langseth: F. langsethiae*; *F. astroamer*: F. *astroamericanum*; *F. mesoamer*: *F. mesoamericaum*

Protein expression was monitored, both in the presence and absence of IPTG. IPTG at a concentration of 0.1-1mM IPTG was added after the *E. coli* culture had obtained sufficient growth, both in the recombinant and controls. The cultures were incubated further at 37 ºC for different time intervals of 6-15 h. Protein from cell lysates of *E. coli* BL21 bearing pRA-T5, pRSETA vector and host *E. coli* BL21 with and without IPTG induction, were separated using 15 % (w/v) SDS-PAGE. Higher levels of total protein were observed in the cell lysate of recombinant *E. coli* [(bearing pRA-T5) (Fig. 5.8)]. The protein was maximally expressed in cells induced with 0.4 mM IPTG concentration over an incubation period of 12 h (Fig. 5.9). Recombinant protein fragment of size ~28 kDa size was over-expressed only in recombinant *E. coli* bearing pRA-T5 and not in any of the controls [(pRSETA in *E. coli* BL21 and *E. coli* BL21 host) (Fig. 5.10)]. The protein fragment of specific size was finally purified using gel elution (Fig. 5.11) which was used to immunize poultry.

Fig. 5.8. Total Protein Content at Different IPTG Concentrations for Different Incubation Periods

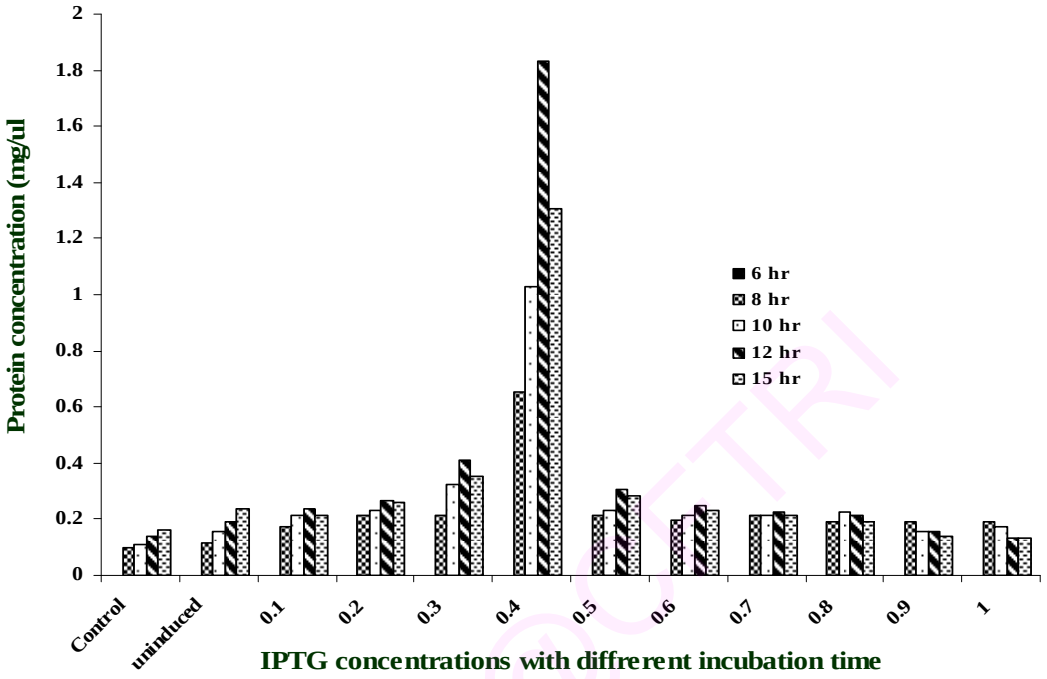


Fig. 5.9. Relative Band Intensity of the 28 kDa Protein at Different IPTG Concentrations for Different Incubation Periods

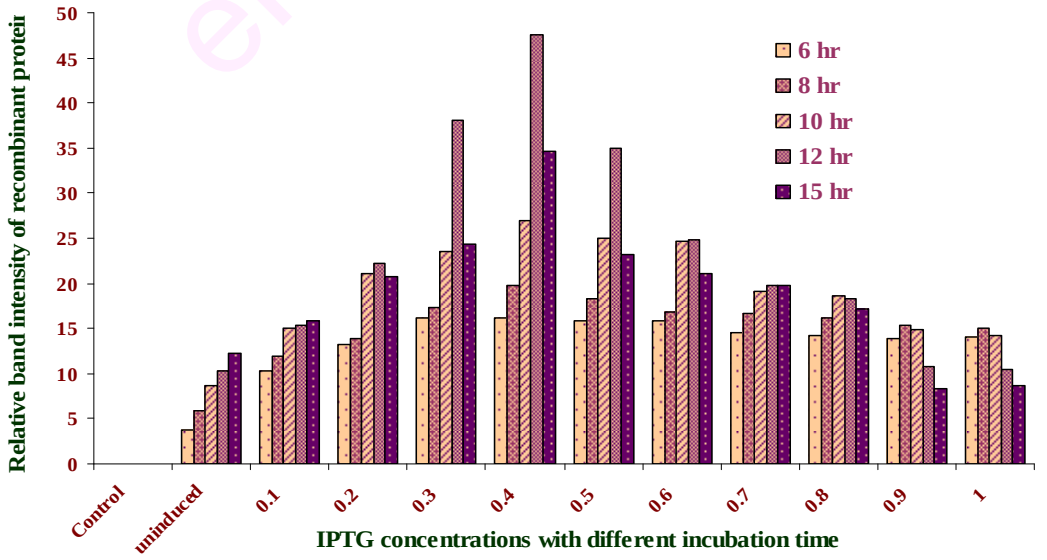


Fig. 5.10. SDS PAGE showing Over-Expression of *tri 5*

Lanes 1 and 6: Protein size marker, 2: Cell lysate of *E. coli* BL21, 3: Cell lysate of *E. coli* BL21 bearing pRSETA, 4: Cell lysate of *E. coli* BL21 bearing pRA-T5

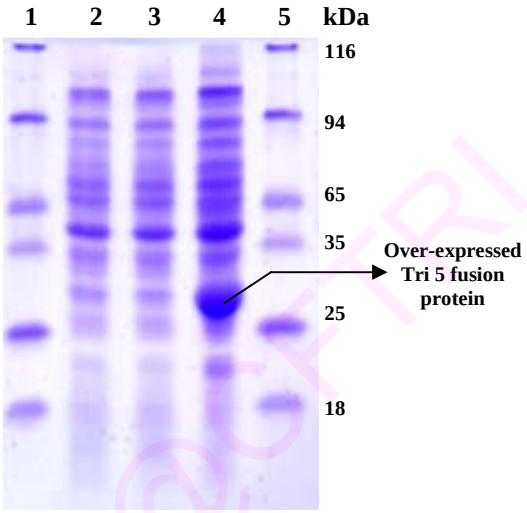
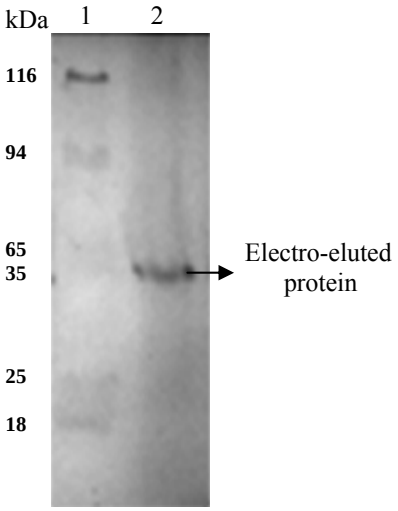


Fig. 5.11. SDS PAGE of the Electro-Eluted Tri 5 Protein

Lane 1: Protein size marker; Lane 2: Purified protein



5.3.2. Generation of Antibodies against Recombinant Tri 5

In this study, antibody against Tri 5 fusion protein was raised in poultry. The antigen was injected at multiple sites into the pectoral muscle of poultry. Booster doses were scheduled at 4 weeks interval after the first dose and eggs were collected daily after the first injection, for a period of 17 weeks.

IgY antibody titre was monitored regularly throughout the immunization period for both the immunized and the control hen. The protein content in yolk which has been an indicator of the levels of IgY antibody that was likely to be present in the egg was estimated. The protein content in the IgY isolated from immunized poultry increased at a relatively constant level until seventh week of immunization. The protein content decreased after the seventh week, i.e., three weeks after second booster; the titre reduced relatively and attained a steady level. The titre of IgY from control hen remained the same throughout the period of experiment. Protein content of the IgYs estimated at weekly intervals is presented in the Fig. 5.12. The titre of IgY (0.048) isolated from eggs collected on the 7^{th} week was highest and was therefore chosen for further work (Fig. 5.13).

Fig. 5.12. IgY Protein Content

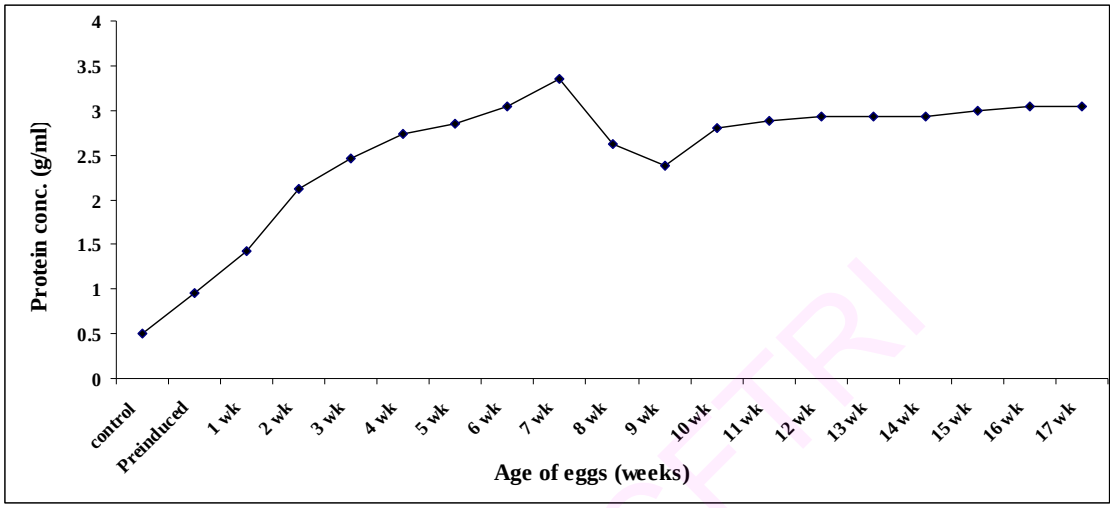
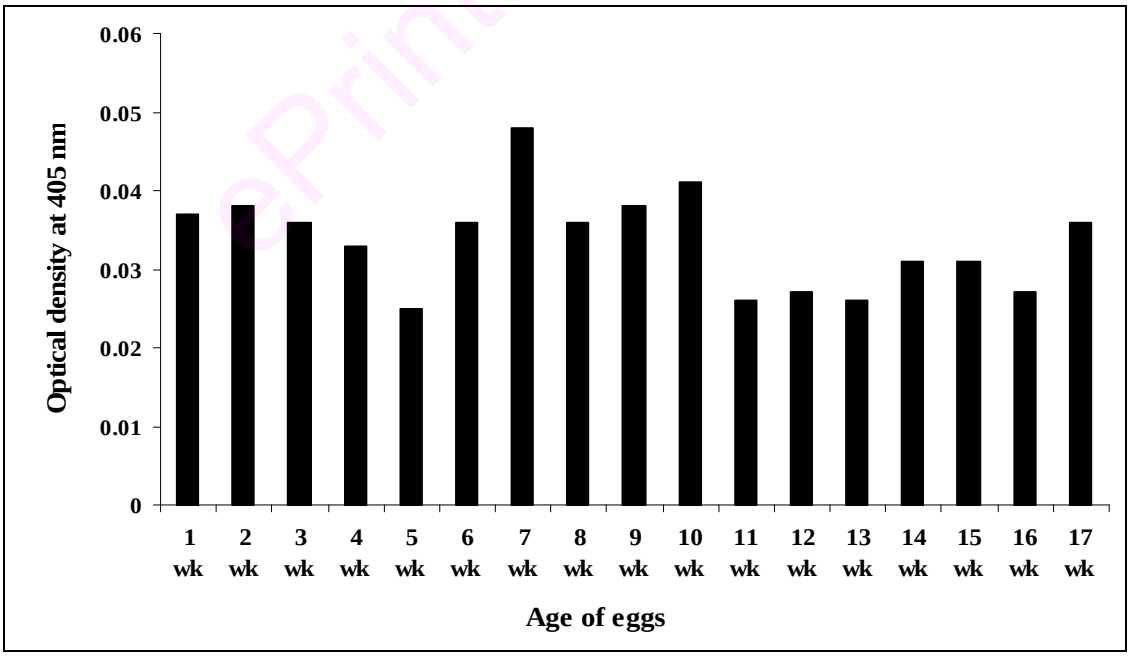


Fig. 5.13. IgY Antibody Titre



5.3.3. Characterization of IgY

5.3.3.1. Cross Reactivity of Anti-Tri 5 IgY

The specificity of IgY raised against *tri 5* was tested using antigens from other fungi such as *Penicillium* and *Aspergillus* and from both trichothecegenic and non-trichothecegenic *Fusaria*. Reaction with the anti-Tri 5 IgY was considerably less while using the extracts of ICR82(2), the trichothecene non-producer *Fusarium* and with that of *Penicillium* (discussed in detail in the following sections)

5.3.3.2. Immunoblotting or Western Blot

Specificity of the antibody against the recombinant protein in *E. coli* was determined through immunoblotting. The major antigens that reacted with the antibody were a ~28 and 40 kDa proteins (Fig. 5.14 and 5.15) contained in the cell extracts of *E. coli*: pRA-T5 and trichothecene producing *Fusarium*, respectively (at primary and secondary antibody dilutions of 1:2000 and 1:3000 respectively). However, no significant bands were observed in the immunoblots of cell extracts of *E. coli*, *E. coli* transformed with the vector pRSETA and from mycelial extracts of non-trichothecene producers. (Fig. 5.14 and 5.15). Immunoblotting of SDS-PAGE gels containing mycelial extracts of trichothecene producing *Fusaria* resulted in the identification of a protein fragment whose molecular weight was slightly higher than that of the Tri 5 fusion protein. Immunoblots using IgY against Tri 5 from mycelial lysates of trichothecegenic *Fusaria* is provided in Fig. 5.15. Specific reaction to anti *tri 5* IgY was observed only in trichothecene producers and not from mycelial extracts of trichothecene non-producers or fungi of other genera.

Fig. 5.14. Western Blot using Cell Extracts of *E. coli* BL21 bearing pRA-T5

Lanes 1: Protein size marker, 2: Cell lysate of *E. coli* BL21 bearing pRA-T5 (SDS-PAGE), 3 and 5: Cell lysate of *E. coli* BL21 bearing pRSETA, 4 and 6: Cell lysate of *E. coli* BL21 bearing pRA-T5

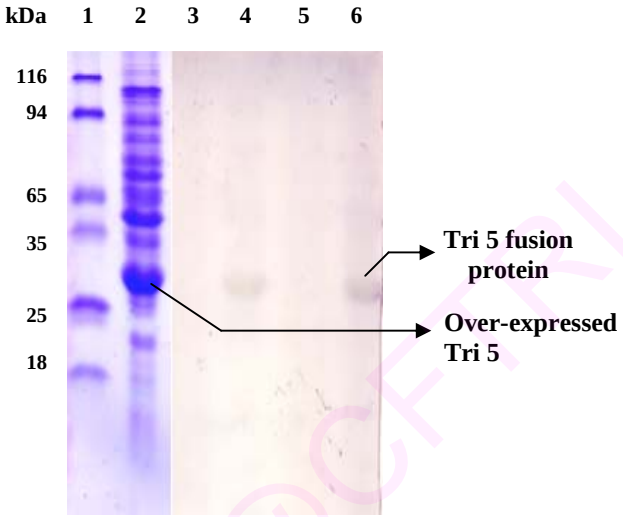
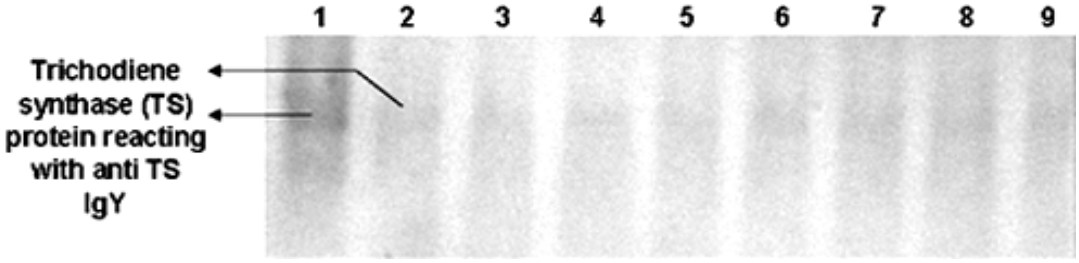


Fig. 5.15. Immuno-Detection of the Tri 5 Protein in Mycelial Extracts of Trichothecegenic *Fusarium*

Lanes 1: FM246, 2: ICR-PQ-2, 3: ICR50, 4: FM246, 5: ICR-PQ-12, 6: ICR-PQ-13, 7: ICR1, 8: Isolate 1, 9: Standard strain NCIM 651



5.3.3.3. ELISA

The optical density (OD) of the product from the reaction of different dilutions of antibody and antigen was recorded to determine the concentration of each that resulted in the highest titre in ELISA. Antigen dilutions of 1:2 for *E. coli*: pRA-T5 and 1:3 for mycelial extracts of trichothecegenic *Fusarium* was identified suitable. Antibody dilutions that reacted effectively with the antigen were of the order of 1:400-1:500. Secondary antibody was used at a dilution of 1:1000. Higher OD values were obtained in ELISA when cell lysates of *E. coli*:pRA-T5 and mycelial lysates of trichothecegenic *Fusarium* were used as antigen, both of which contained a copy of *tri 5* (Fig. 5.16). No significant reaction was obtained while using extracts from the negative controls such as *E. coli*:pRSETA, *E. coli* BL21 and non-trichothecene producing fungi, since they all lacked a *tri 5* gene.

Titres for rice extracts prepared using lysis buffer (section 5.2.8.3. Buffer Extracts of Rice), PBS and water was monitored. PBS was identified as a better extraction buffer since the titres of the extracts of rice inoculated with trichothecene positive *Fusarium* was significantly higher than that of control rice or rice inoculated with negative culture (Table 5.4). No significant difference in titre was observed between the rice samples when water was used for extraction.

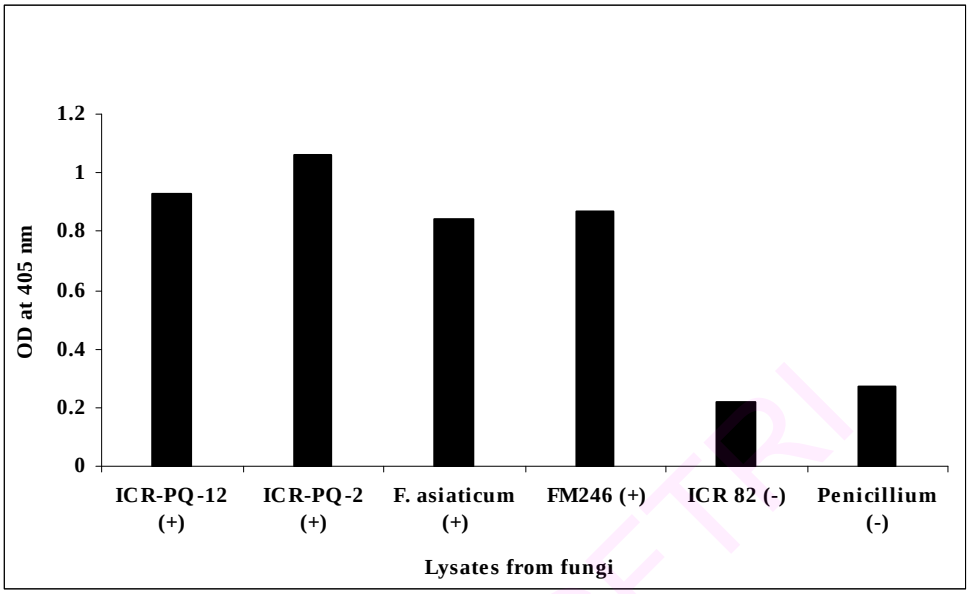
ELISA for the detection of contamination in food with trichothecegenic *Fusaria* was standardized using rice that was artificially inoculated with trichothecegenic and non-trichothecegenic *Fusaria.* A positive correlation between trichothecene contamination in rice and ELISA was observed. Higher optical densities were obtained in ELISA with extracts from rice samples inoculated with trichothecegenic *Fusaria* than from those inoculated with non-trichothecegenic *Fusarium* [ICR82(2)] or from the extract of rice (Fig. 5.17).

Table 5.4. ELISA of Trichothecegenic *Fusarium* Infected Rice using Different Extraction Buffers

Sl. No.	Extraction buffer	Rice inoculated with +ve culture	Rice inoculated with -ve culture	Control rice
1.	PBS	1.37	0.765	0.142
2.	Protease cocktail buffer	1.344	0.868	0.657
3.	Deionized water	0.214	0.184	0.140

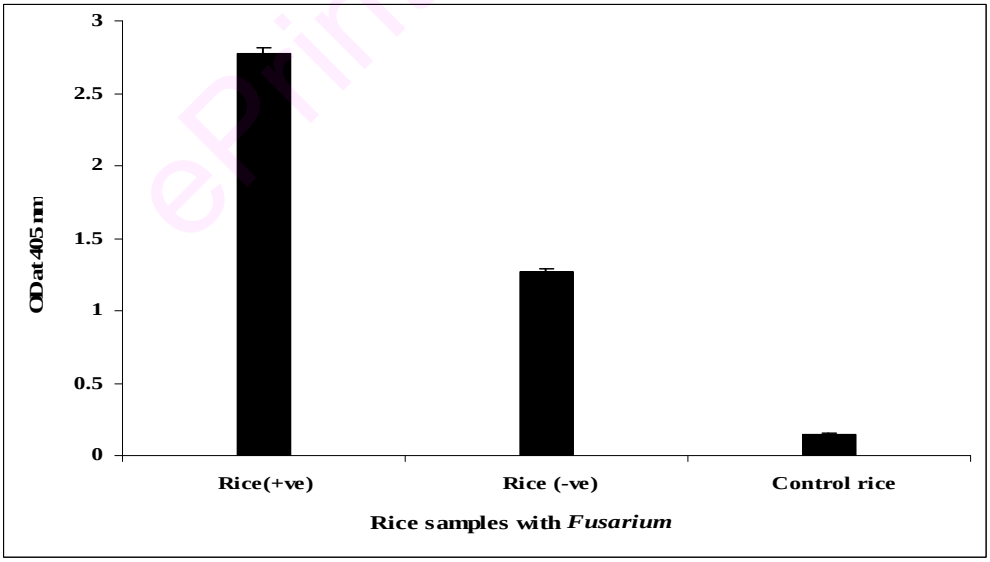
ELISA for the detection of trichothecene contaminated food was standardized using rice that was artificially inoculated with trichothecegenic and non-trichothecegenic *Fusaria.* The results revealed a positive correlation between trichothecene contamination in rice and ELISA. Higher optical densities were obtained in ELISA with extracts from rice samples inoculated with trichothecegenic *Fusaria* than from those inoculated with non-trichothecegenic *Fusarium* or from the extract of rice (Fig. 5.17).

Fig. 5.16. ELISA using Mycelial Lysates as Antigen Source



+ and – sign indicates cultures positive and negative for trichothecene production, respectively.

Fig. 5.17. ELISA of Rice Samples Inoculated with Trichothecene Producing and Non-Producing *Fusarium*



+ and – sign indicates positive and negative for trichothecene producing *Fusaria*, respectively

5.3.4. Synthesis of Tri 5 in Relation to Trichothecene Production

Synthesis of trichodiene synthase (Tri 5) as estimated through ELISA by the *Fusarium* isolate ICR103 was highest in the presence of mannose, sucrose and glucose and least in the presence of maltose and xylose. Sucrose, mannose and fructose supported higher levels of T-2 production as assayed by GC than did xylose, lactose and maltose (Fig. 5.18). Ueno et al (1975) have reported of high production of T-2 toxin by *F. solani* in presence of 1 % glucose, sucrose and galactose

The amount of T-2 toxin secreted and the level of Tri 5 produced by the isolate ICR103 when grown in different amounts of glucose was inverse of each other. Higher levels of T-2 were secreted at the high glucose levels while the least amount of the enzyme was synthesized then (Fig. 5.19). Similar picture have been obtained from the studies of both Ueno et al (1975) and Cullen et al (1982) for T-2 production by *F. solani* and *F. tricinctum* in different culture media. It is probable that more trichothecene is secreted from the fungi at higher glucose levels than at lower levels.

Fungi grown in varying concentration of peptone secreted similar amounts of the T-2 toxin while the levels of Tri 5 in the mycelial extract of ICR103 decreased with increasing concentration of peptone (Fig. 5.20). Increasing amounts of peptone have been reported not to support T-2 toxin production in VN media by *F. tricinctum* (Cullen et al, 1982). High toxin levels have been reported in media containing no peptone. Ueno et al (1975) also have noticed a decrease in T-2 toxin production by *F. solani* in presence of higher amounts of peptone.

Fig. 5.18. Effect of Different Carbohydrates on Production of T-2 and Tri 5

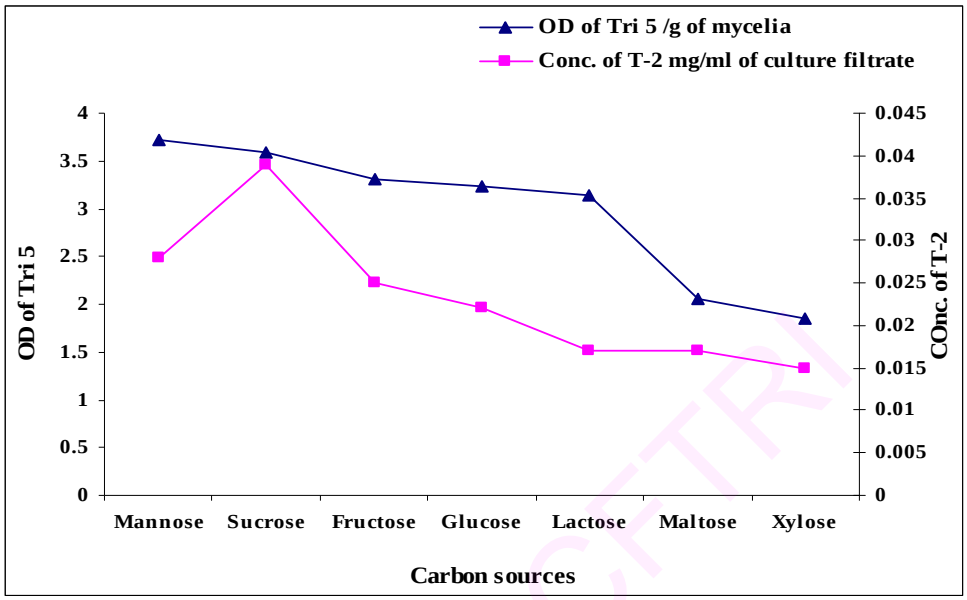


Fig. 5.19. Effect of Glucose on Production of T-2 and Tri 5

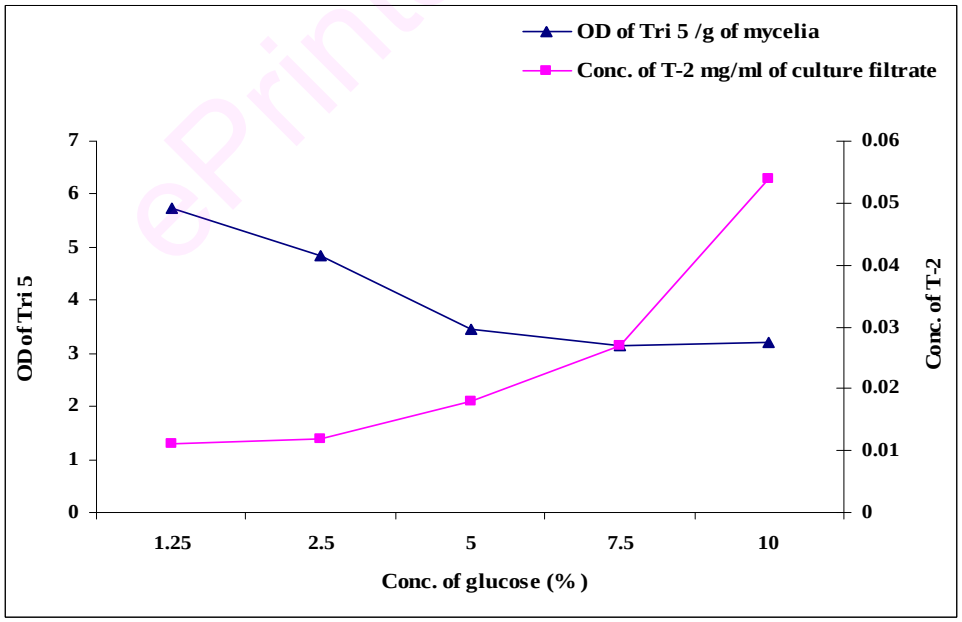
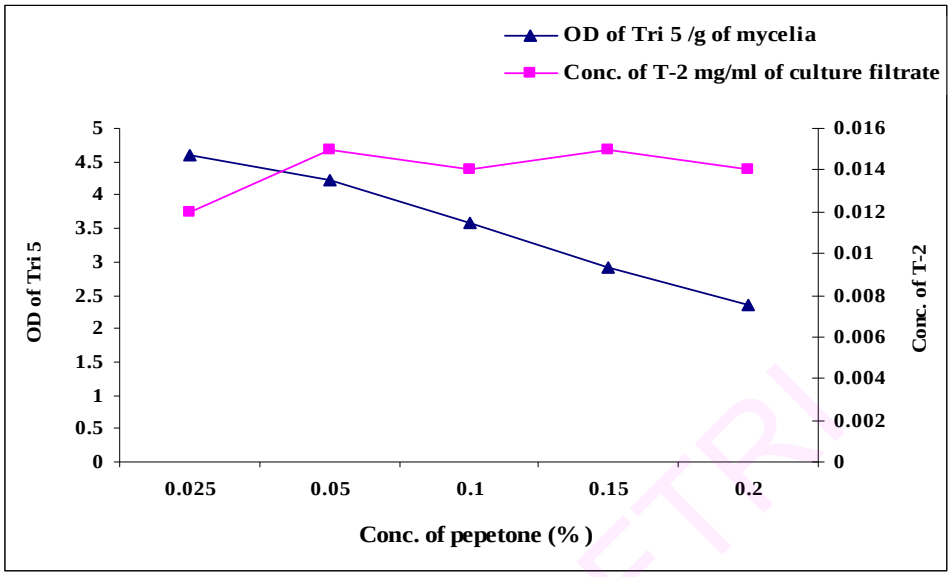


Fig. 5.20. Effect of Peptone on Production of T-2 and Tri 5



The amount of DON secreted by the *Fusarial* isolate ICR-PQ-12 and the level of the Tri 5 synthesized during differing periods of growth were estimated. Highest levels of DON were observed at 3-7 days post inoculation after which there was a reduction in toxin content. The amount of trichodiene synthase (Tri 5) peaked after 3-5 days of incubation followed by a gradual decrease upto 13 days and attaining finally a steady state (Fig.5.21). T-2 toxin was produced maximally by *F. solani* in GYEP media containing 1 % glucose after 5 days of incubation (Ueno et al 1975). Hohn and Beremand (1989b) observed maximal expression of Tri 5 polypeptide and TS activity after 144 h of *Fusarium* growth.

The influence of different carbohydrates on accumulation of DON by *Fusarium* isolate ICR-PQ-12 was studied. Higher levels of DON was secreted by the fungus in glucose containing media (0.133 mg/ml) followed by that containing sucrose (0.079 mg/ml) or maltose (0.069 mg/ml). Concordant results were obtained when mycelial lysate of the isolate was assayed for the levels of Tri 5 protein (Fig.5.22). Higher titres for Tri 5 were obtained from the mycelia of fungi grown in the presence of sucrose and glucose.

Fig. 5.21. Time Course Production of DON and Tri 5

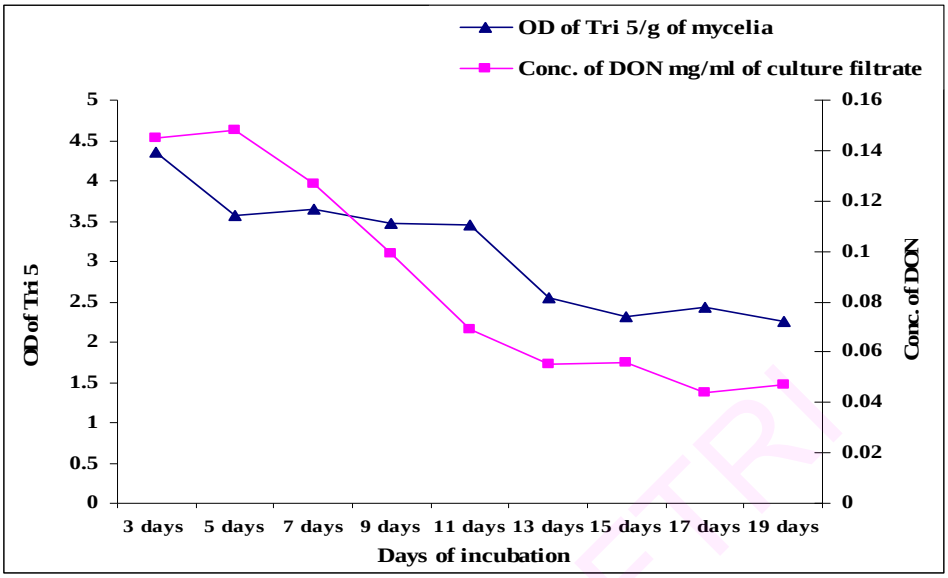
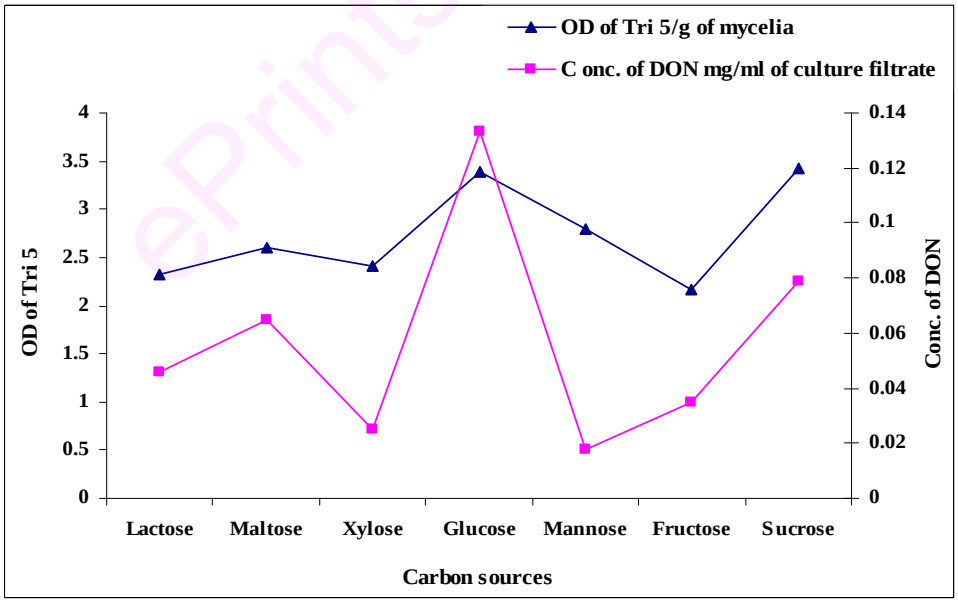


Fig. 5.22. Effect of Different Carbohydrates on Production of DON and Tri 5



The amount of DON secreted as assayed through HPLC and the level of Tri 5 produced as estimated through ELISA by the isolate ICR-PQ-12, when grown in different amounts of glucose, were inverse of each other. Higher levels of DON were secreted at the high glucose levels while least amount of the enzyme was then synthesized (Fig. 5.23). The pattern is very similar to that seen with the isolate ICR103 with respect to the relation between the amount of trichodiene synthase synthesized and secretion of T-2 toxin. Lower levels of DON, produced by *F. graminearum*, with increase in amount of glucose have been reported by Miller et al (1983). Hohn and Beremand (1989b) on the other hand have reported of an increase in trichothecene concentration with decrease in glucose concentration from 5 % to 3 %. Just as the case with T-2 production and Tri 5 synthesis in the isolate ICR103, increasing peptone levels in the media decreased production of DON as well as the level of the Tri 5 protein in ICR-PQ-12 (Fig. 5.24).

Fig. 5.23. Effect of Glucose on Production of DON and Tri 5

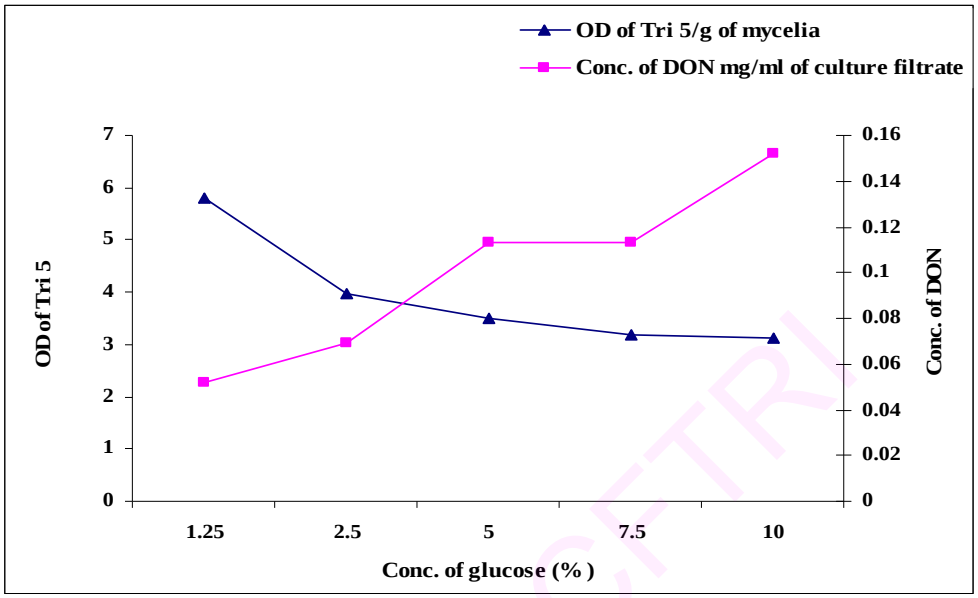
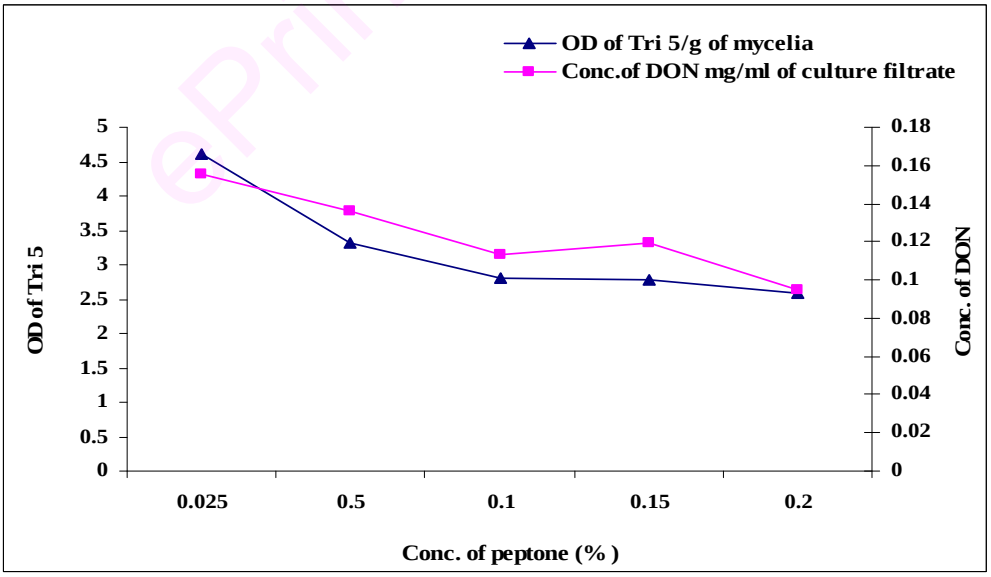


Fig. 5.24. Effect of Peptone on Production of DON and Tri 5



The differential production of trichothecenes with varying glucose and peptone concentrations or different carbon sources may be due to the regulatory effect of glucose or other nutrients on trichothecene synthetic pathway i.e, by affecting synthesis of metabolites in the trichothecene core cluster or by blocking the primary metabolites that are necessarily involved in toxin production. Tag et al (2001) have also suggested the existence of control factors other than the *tri* genes and their product enzymes that play a pivotal role in trichothecene synthesis (Fig. 1.16). It may be hypothesized that different carbohydrates influences the level of different transcription factors which in turn influences synthesis of Tri 5 and eventually secretion of the toxin. The role of promoters of genes involved in trichothecene biosynthesis is the subject of the next chapter.

5.4. Conclusions

In conclusion, the antibody raised against Tri 5 fusion protein exhibited high specificity for trichothecegenic *Fusarium*. It was shown that the antibody raised against trichodiene synthase (Tri 5) have efficiently detected the presence of trichothecegenic *Fusaria*. This could serve as a versatile and less tedious substitute for other detection methods in place today. Use of the antibody to estimate levels of Tri 5 protein revealed a complex control on its synthesis by different sugars or even by different levels of glucose in the media.

Chapter 6.

Analysis of Promoters of Genes Involved in Trichothecene Biosynthesis

6.1. Introduction

6.1.1. What is Promoter?

Promoter is the region of non-coding nucleotide sequences at the transcriptional start region of an operon, characterized by the presence of a number of conserved sequences. A promoter is the regulatory DNA fragment and in most cases situated upstream (towards the 5') of a gene (Promoter, 2008). Promoter is the region where RNA polymerase (RNAP) binds and signals the start point of RNA synthesis. There are proteins called transcription factors which can recognize specific sequences in the promoter region and regulate the synthesis of RNAs complimentary to the coding region of the genes. It provides a control point for regulated gene expression by working in concert with other regulatory elements such as enhancers, silencers, boundary elements, insulators etc and directs the level of gene expression (Promoter, 2008).

6.1.2. Basic Elements of a Promoter

The basic elements of a promoter are (i) core promoter, (ii) proximal promoter and (iii) distal promoter (Promoter, 2008).

6.1.2.1. Core Promoter

This is the minimal region of promoter sequence required for proper initiation of gene transcription (Komarnytsky and Borisjuk, 2003; Promoter, 2008). The basic features necessarily required in a DNA fragment upstream to the gene start codon to make it the core promoter include

a. Transcription Start Site (TSS)
b. Approximately -34
c. A binding site for RNAP
d. General transcription factor binding sites

6.1.2.2. Proximal Promoter

These are the proximal sequences situated upstream of the genes that tend to contain primary regulatory elements (Promoter, 2008). The elements that constitute a proximal promoter include

a. Approximately -250
b. Specific transcription factor binding sites/upstream cis acting elements

6.1.2.3. Distal Promoter

These are the distal sequences situated upstream of the genes that may contain additional regulatory elements (Promoter, 2008). Distal promoters often have a weaker influence than the proximal promoters. The basic elements of a distal promoter include

a. Anything further upstream (but not an enhancer or other regulatory region whose influence is positional/orientation independent)
b. Specific transcription factor binding sites

6.1.3. Prokaryotic Promoters

The core promoter of prokaryotes necessarily consists of two short sequences at -10 and -35 positions upstream from the transcription start site (Promoter, 2008). The sequence at -10 called the 'Pribnow box' (also known as Pribnow-Schaller box or the -10 element) usually consists of the six nucleotides TATAAT (Pribnow, 1975; Schaller et al, 1975). The Pribnow box is absolutely essential to start transcription in prokaryotes. Other sequence at -35 (or the -35 element) usually consists of six nucleotides TTGACA whose presence is responsible for high transcription rate of a gene. The optimal spacing between -10 element and -35 elements suggested is 17 bp. Some promoters contain so-called "extended -10 element" with a consensus sequence of 5'-TGNTATAAT-3'. Prokaryotic promoters consist of specific elements called sigma factors which help in enhancing RNAP binding to the promoter and also targets RNAP to the genes to be transcribed (Promoter, 2008).

6.1.4. Eukaryotic Promoters

Eukaryotic core promoters are generally characterized by the presence of a 'TATA box' (similar to Pribnow box in prokaryotes) with the consensus sequence TATAAA, which in turn binds a TATA binding protein and assists in the formation of the RNA polymerase transcriptional complex (Smale and Kadonaga, 2003). The TATA box typically lies very close to the transcriptional start site (often within 50 bases). In eukaryotes, regulatory elements are placed several kilobases away from the transcriptional start site in certain cases which bind the transcription factors to form the transcriptional complex. The placement of regulatory sequences far from the actual site

of transcription results in the bending of DNA on itself bringing the transcriptional complexes to their proximity.

Hohn et al (1999) have studied the promoter region of genes involved in trichothecene synthesis for the presence of core binding sites for Tri 6 [(section 1.5.7.2.1. Tri 6]. A 401 nucleotide sequence upstream from the *Tox5* start ATG of *G. pulicaris* has been identified that efficiently regulates the expression of a β-galactosidase reporter gene (Hohn et al, 1993). The existence of two alleles *Tox5-1* and *Tox5-2* were identified depending on the presence or absence of a 42 nucleotide tandem repeat sequence, located 280 bp upstream from start ATG. *G. pulicaris* strains containing the *Tox5-1* allele produced higher levels of trichothecenes when compared to those bearing *Tox5-2*, which produced lower or undetectable levels of trichothecenes. Presence or absence of duplication of the 42 nucleotide repeat sequence was identified not responsible for higher trichothecene production (Hohn et al, 1993). Chen et al (2000) have observed increased production of trichothecenes following integration of the *tri 5* promoter at the genomic *tri 5* site of *F. graminearum*. There is a great shortage of studies addressing the characterization of promoters for the *tri* genes. Much remains to be learnt about promoter region of *tri* genes, motifs that are recognized by the transcription factors or even the transcription factors itself. Part of the task was taken in the current study where the promoter sequences of *tri 5*, *tri 6* and *tri 10* were analyzed using promoter analysis softwares for the prediction of motifs involved in nutrient specific response.

6.1.5. Reporter Gene Assays for Promoter Studies

A reporter gene is a gene that confers certain characteristics on organisms that can be easily identified and measured. Reporter genes are generally used to determine whether the gene of interest has been taken up by or expressed in the cell or organism. It is important to use a reporter gene that is not natively expressed in the cell or organism under study since the expression of the reporter is being used as a marker for successful uptake of the gene of interest. Commonly used reporter genes that induce visually identifiable characteristics in cell culture, animals or plants include (i) the gene that encodes jelly fish (*Aequorea victoria*) green fluorescent protein (GFP) which causes cells that express it to glow green under UV light (Misteli and Spector, 1997; Spellig et al,

1996), (ii) the enzyme luciferase which catalyzes a reaction with luciferin to produce light (Ow et al, 1986), (iii) the gene *uidA* that encodes β-glucuronidase (also known as GUS) whose reaction with X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) generates a blue colour in the transformants (Jefferson et al, 1987), (iv) the *lacZ* gene which encodes the enzyme β-galactosidase that gives a blue colour when grown on a medium containing X-gal in presence of IPTG (Fire et al, 1990), (v) chloramphenicol acetyl transferase (CAT) gene which confers resistance to the antibiotic chloramphenicol (Potrykus and Wegrzyn, 2001) etc.

Reporter gene expressions under the control of promoters that are activated under specific conditions provide a faithful representation of a gene's expression pattern in the micro-environment of the fungus during its growth in the plant. Introduction of reporter genes to trichothecegenic *Fusarium* allow visualization, subcellular localization and quantification of fungi in the infected tissue. In the present study, expression of the gene *tri 5* was studied by assaying the expression of GUS under the control of *tri 5* promoter.

6.2. Materials and Methods

6.2.1. Cloning of Promoter Fragment of *tri 5* in pCAMBIA1304

6.2.1.1. Bacterial Strains and Plasmids

E. coli strains DH5α and *Agrobacterium tumefaciens* EHA 105 were used as cloning and co-cultivation hosts, respectively. *E. coli* cultures were maintained at 4 °C on Luria Bertani (LB) agar medium. *Agrobacterium* cultures were grown on LB agar plates containing rifampicin (10 µg/ml) and incubated at 28 °C for 2-3days. The cultures were maintained at 4 °C.

The plasmid pTZ57R/T (MBI Fermentas InsT/A Cloning kit) was used as the primary cloning vector. The vectors pRSETB and pCAMBIA1304 were used as intermediate and reporter gene expression hosts, respectively.

6.2.1.2. Oligonuleotide Primers

Oligonucleotide primers T5PF2-T5PR2 for cloning of *tri 5* promoter region were designed outside the intron region, based on the *tri 5* sequences available in the data bank. Two primers 5PF1 and 5PF2 were designed within the priming region of T5PF2-T5PR2 primers for cloning as well as for use as nested primer. The sequences of the primers used in the study are given in Table 6.1.

Table 6.1. List of Primers Used

Sl. No.	Primer name	Sequence
1.	T5PF2	5'-AACACCCCTACCRBGAACAC -3'
2.	T5PR2	5'-GGCAGCYTTGTTGTAAGCAT -3'
3.	5PF1	5'- CAGTTGCAKTGCATTCGGG-3'
4.	5PF2	5'-GTACCTTTGCAGGGAATGAG -3'
5.	*hptII*F	5'-CGGAAGTGCTTGACATTGG-3'
6.	*hptII*R	5'-AGAAGAAGATGTTGGCGA-3'

6.2.1.3. Cloning of Promoter fragments for *tri 5* in pCAMBIA1304

Promoter fragment of the gene *tri 5* (1000 bp upstream from the start site) was amplified from the genomic DNA of the isolate ICR-PQ-12 using specific primers (Table 6.1), following the procedure described in section 2.3.3.

PCR conditions

Initial denaturation: 94 °C for 5 min

Denaturation: 94 °C for 1 min
Annealing*: 55 °C for 1 min } 34 cycles
Extension: 72 °C for 1min

Final extension: 72 °C for 10 min

*55 °C for *tri 5* primers and 52 °C for *hptII* primers

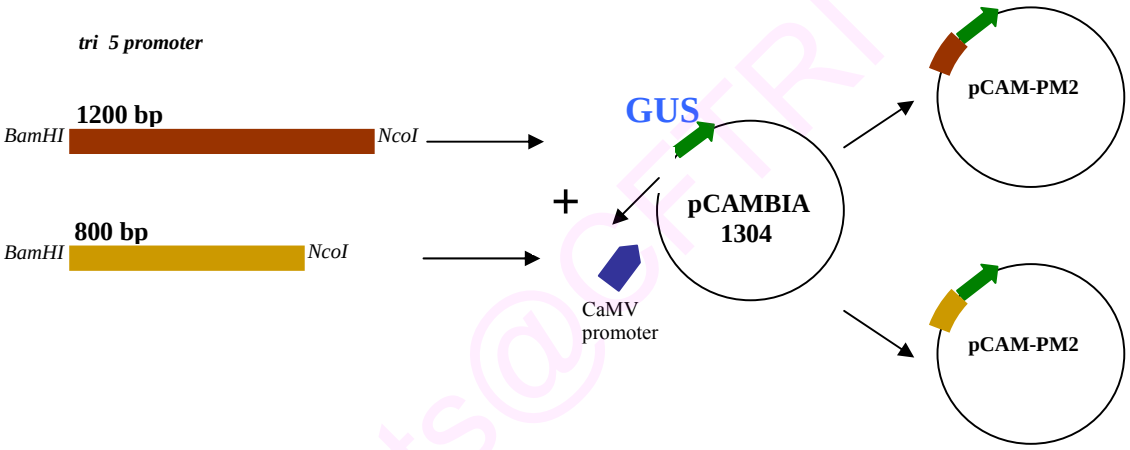
PCR amplified DNA fragments of size 1200 and 800 bp were purified and cloned in T-tailed vector pTZ57R/T following the procedure described earlier (sections 2.6, 2.7, 2.8 and 2.9). The constructs were designated as pT-PM1 and pT-PM2, respectively. The promoter fragment inserted in pT-PM1 was sequenced using M13 forward primer following the procedure described in section 2.5. The promoter fragment was excised off from pT-PM1 and pT-PM2 constructs using *HindIII-EcoRI* restriction enzymes and was ligated to the same sites of pRSETB which was used as the intermediate vector (procedure described in section 2.10). The resulting constructs were named pRB-PM1 and pRB-PM2. Promoter fragments were cut off using *BamHI-NcoI* restriction enzymes and the insert was further mobilized into *BamHI-NcoI* cut pCAMBIA1304 (procedure described in section 2.10). The final constructs were named pCAM-PM1 and pCAM-PM2 (Fig. 6.1), respectively, that contained the gene for GUS under the control of *tri 5* promoter.

6.2.2. Transformation of *Fusarium*

The concentration of hygromycin required for killing of *Fusarium* and cefotoxime and augmentin for that of *Agrobacterium* cells were determined prior to *Fusarium* transformation. The constructs pCAM-PM1 and pCAM-PM2 were transformed into competent *Agrobacterium c*ells as described in section 2.19.1. Positive clones which

grew on kanamycin plates were picked up by colony PCR, using primers specific for *tri 5* promoter sequence and the hygromycin phosphotransferase gene *hptII* (procedure described in sections 6.2.3 and 2.3.3). The transformed cells were co-cultured with *Fusarium* putative spores as described in section 2.19.2 to attain transformation of *Fusarium*. The transformants were transferred to progressively higher hygromycin concentrations: 50, 100, 150, 200, 250, 300 µg/ml.

Fig. 6.1. Schematic Representation of Cloning Strategy



6.2.3 Colony PCR

Single colony of *Agrobacterium* was picked up with a sterile micro-tip and inoculated into 2 ml LB broth. The tubes were incubated for 1 h in a shaker incubator at 250 rpm. The cultures were transferred to sterile micro-centrifuge tubes and the cells were harvested by centrifugation at 4000 rpm for 10 min. The cells were washed in sterile deionized water and lysed by incubating in a boiling water bath for 5-10 min. The cell debris was removed by centrifugation at 8000 rpm for 10 min. 1 µl of the supernatant was used as template in the PCR reaction mixture.

6.2.4. GUS Activity

Histochemical staining for GUS was carried out following the modified method of Jefferson et al (1987). The putative transformants bearing pCAM-PM1 and pCAM-PM2 and the wild type *Fusaria* were grown in GYEP media (containing 300 µg of hygromycin) at 28 °C for 2 days. Mycelia were harvested by centrifugation at 8000 rpm for 10 min and

washed in sterile deionized water. Mycelia were treated with 4 % cellulose in 0.1 M sodium acetate (pH 5). After incubation at 37 °C overnight, the mycelia were washed in sterile deionized water and immersed in 200 µl of X-Gluc [(5-bromo-4-chloro-3-indolyl glucuronide) (Sigma, St. Louis, USA)] staining solution [(2 mM X-Gluc, 50 mM phosphate buffer (pH 7), 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1 % triton X-100). Mycelia were incubated at 37 °C overnight for colour development. The mycelia were observed under a microscope (OLYMPUS CKX41) and photos were digitally captured at a primary magnification of 400 X.

6.2.5. *In Silico* Identification of *cis* Elements in Promoter of *tri 5, tri 6* and *tri 10*

6.2.5.1. Promoter Sequences

In this chapter, DNA sequences 1000 bp upstream from the gene start site was called a promoter. Sequence of promoters of *tri 5*, *tri 6* and *tri 10* of common trichothecene producers such as *F. culmorum, F. cerealis, F. sporotrichioides and F. graminearum* were retrieved from the database. The sequences were searched for *cis* acting elements using various computer programs.

6.2.5.2. Softwares Used

Pairwise alignment of the sequences was carried out using Dialign 2 (Morgenstern, 1999). TRANSFAC® and its module TRANSCompel® (Matys et al, 2006) were used to analyze the promoter sequences for location of basic elements required in a promoter. PATCH (Pattern search for transcription factor binding sites), a program included in TRANSFAC®, in conjunction with TRED [(Transcriptional Regulatory Element Database) (Zhao et al, 2005)] was used to make predictions of specific motifs in the promoter regions of *tri* genes. The promoter sequences of *tri 5*, *tri 6* and *tri 10* were queried using these programs and an attempt was made to predict and possibly locate the *cis* acting elements or motifs related to nutrient specific response in trichothecene production.

6.3. Results and Discussion

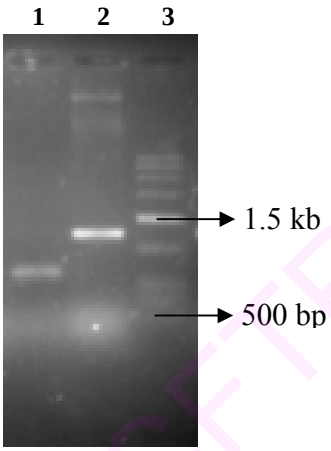
6.3.1. Cloning of *tri 5*Promoter

PCR fragments of size ~800 and 1200 bp were amplified from the genomic DNA of ICR-PQ-12, a DON producer (section 3.3.5), using primers T5PF2-T5PR2. Amplification of 800 and 400 bp nested fragments using the PF1 and PF2 primers authenticated specificity of 1200 and 800 bp fragments, respectively. The promoter fragments were cloned into pTZ57R/T to obtain pT-PM1 and pT-PM2 constructs Amplification of the promoter fragments with specific and nested primers and release of the inserted fragment using *EcoRI-BamHI* enzymes were obtained from the pT-PM1 and pT-PM2 constructs. Sequencing of the *tri 5* promoter fragment in pT-PM1 using M13 forward primer was carried out for checking the authenticity of the constructs and also for checking the orientation of the insert. A 700 bp sequence upstream of the *tri 5* start site was obtained after sequencing and was homologous in sequence to that of *F. asiaticum* (AY102604). This is in concordance with the results obtained after sequencing of the EF-1α gene fragment and molecular phylogenetic analysis of ICR-PQ-12 (section 3.3.5; Fig. 3.8.).

The promoter fragments released from pT-PM1 and pT-PM2 were inserted into *HindIII-EcoRI* cut pRSETB vector so as to introduce a *NcoI* site at the 3' end of the promoter fragment. The promoter fragment was finally ligated into pCAMBIA1304 in such a way that the CaMV promoter was excised off from the vector which was replaced with the *tri 5* promoter (Fig. 6.1). The promoter-GUS fusion constructs were designated as pCAM-PM1 and pCAM-PM2. Release of the inserted fragment using *BamHI-NcoI* from pCAM-PM1 and pCAM-PM2 constructs is shown in Fig. 6.2.

Fig. 6.2. Release of insert from pCAM-PM1 and pCAM-PM2

Lanes 1: 800 bp promoter fragment released from pCAM-PM2, 2: 1200 bp promoter fragment released from pCAM-PM1, 3: 3 kb DNA ladder



6.3.2. Transformation of *Fusarium*

Varying concentrations of hygromycin, cefotoxime and augmentin were assayed to identify their amounts that were required for killing of the wild-type fungi and *Agrobacterium*, respectively. Presence of hygromycin (300 µg/ml) in the media completely inhibited *Fusarium* growth. *Agrobacterium* cells were killed in presence of 300 µg/ml each of cefotoxime and augmention. The putative transformants of ICR-PQ-12 bearing the constructs pCAM-PM1 and pCAM-PM2 after infecting with *Agrobacterium* cells were observed as pin point colonies that later spread over the entire agar surface and were capable of growing in up to 300 µg/ml of hygromycin.

6.3.3. Histochemical GUS staining

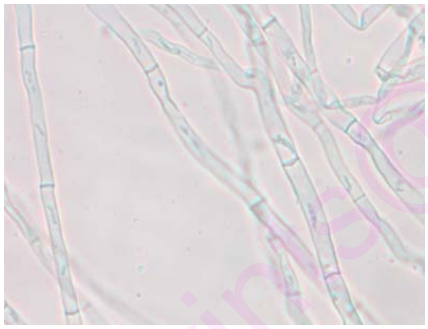
The transformants were subjected to qualitative GUS assay after growing in the trichothecene production media. GUS activity was observed in the *Fusarium* isolate ICR-PQ-12 that was transformed with the constructs pCAM-PM1 and pCAM-PM2 (Fig. 6.3.C and 6.3.D). This is consistent with the observation of Hohn et al (1993) who demonstrated that GUS expression occurred under the control of *tri 5* promoter fragment

of length 401 nucleotides upstream from the *tri 5* start site. GUS activity was not detected in the wild type *Fusaria*, which served as a negative control (Fig 6.3.A). *Fusarium* that was transformed with pCAM-PM2 produced more intense colour than those bearing the bigger fragment of 1200 bp. It appears as though the truncated promoter is more active than the full length promoter. This may be because of the presence of a repressor in the full promoter which suggests that there is a strict control over trichodiene synthase during trichothecene production in *Fusarium*.

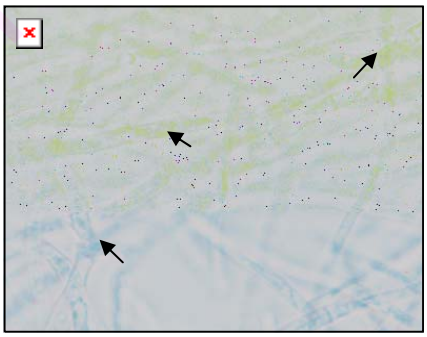
Fig. 6.3. Histochemical Staining for GUS in Transformed *Fusarium*

(Arrows indicate staining)

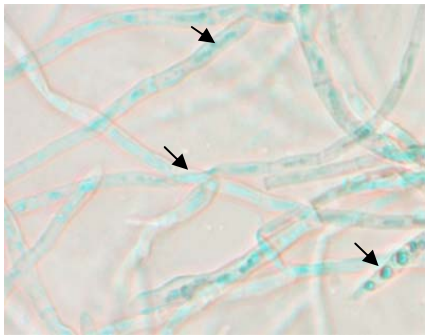
A. Wild type untransformed



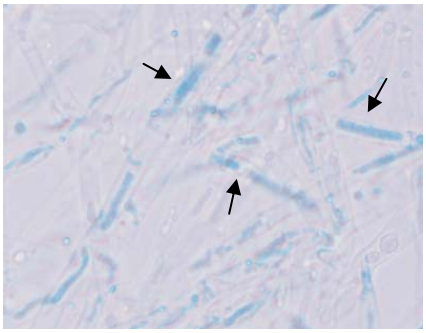
B. *Fusarium* bearing pCAMBIA1304



C. *Fusarium* bearing pCAM-PM1



D. *Fusarium* bearing pCAM-PM2



The genes involved in trichothecene biosynthetic pathway has been investigated, however limited number of studies are available for the promoter regions of these genes.

6.3.4.1. *In Silico* Analysis of the *tri 5* Promoter Sequence

Multiple alignments of the promoter sequences of the *tri 5* gene of common trichothecene producing *Fusaria* are given in Fig 6.4. The promoter sequences revealed considerable variations across the different trichothecene producing *Fusaria*. These variations in the promoter sequences could be species specific which in turn may serve as a fingerprint in the identification of a particular species. The results of analysis of promoter sequences from different strains of the same *Fusarium* species namely *F. graminearum* also exhibited variations among the different strains (Fig. 6.5). These inter and intra specific variations at the promoter region of the gene among different strains may be responsible for their differential response to toxin production in varied growth conditions.

Fig. 6.4. Multiple Alignment of *tri 5* Promoter Sequences

```
ICR-PQ-12              1   ---------- ---------- ---------- ---------- ----------    1   ---------- ---------- ---------- ---------- ----------
F. graminearum         8   ACATGGGTTG ATTGAAAACG GGATTCGGGA TTCTGTACtg tACTCTGTAC   58   AGAGTACAGA GTACTTCGTC GACATGGGGG AATAACCACC GTTTCCCGCC
F.culmorum            15   ACATGGGTTA ATTGAAAACG GGATTCGGGA TTCTGT---- -ACTCTGTAC   60   AGAGTACAGA GTACTTCGTC GACATGGGGG AGTAACCGCC GTTTCCCGCC
F.cerealis            47   ACAAGGGTTA ATCAAGAACA GGATTCATGA TTCCGTACTC T---------   88   ---------- GTACTACGTC AACATGGGGG AATAATCACT GTTCTTAGCC
F.sporotrichioides    94   ACGAGTacga ctaaggatg- -------GGA TTCGGAATTC C---------  127   ---------- GTACTGCGTC GACAACGGAG AATAATCACC ATTTAGCGCa

ICR-PQ-12              1   ---------- ---------- ---------- ---------- ----------    1   ---------- ---------- ---------- ---------- ----------
F. graminearum       108   CCTTGTTAAG CTAAGCGTTT TTAATATGGA AAACGGAGTT CATATACAGT  158   AGAGTCAACA AGATCTGCAA CCTATCAGTG CTTA--AATG CAATTCCAGC
F.culmorum           110   CCTTGTTAAG CTAAGCGTTT TTAATATGGA AAACGGAGTT CATCTACAGT  160   AGAGTCGACA AGATCTGCAA TCTATCAGTG CTTA--AATG CAGTTCCAGC
F.cerealis           128   CCTTGTTAAG CTAAGTATTT CAgcatgt-- AAATGGAGTT CATCCACGGT  176   AGAGTTGACA AGATCTGCAC tatcatcggt atttaatgtc --GTTCTAGC
F.sporotrichioides   167   a---GTTAAG TTATCTATGT CAaatac-GA AAACAAGATT TATCTACGGC  213   ACAGCCGAGG ACATCTGTgt tctagttcgt gtatagAACA CAATGCTAGA

ICR-PQ-12              1   ---------- ---------- ---------- ---------- ----------    1   ---------- ---------- ---------- ---------- -------AGC
F. graminearum       206   TACGCGACTG TCAGGCACCG GCAATGGAGA TCTTGGCTCA GATTCTAGTC  256   TAACTAACAC ACAGGAAAGG GGTTGTAGAT CGTACAGTTA AATTC-GAGC
F.culmorum           208   TACGCGACTT TCAGGCACCG GCAATGGAGA TCTTGGCTCA GATTCTAGTC  258   TAACTAACAC ATAGGAAAGG GGTTGTAGAT CGTACAGCTA AATTCAGAGC
F.cerealis           224   TACGCGACCT TCAGGTACCG GTAATGGAGA CCTTGGCCAA TATTCC----  270   -----AACAC ACAGGAAATG GGTTGTAGAT CATACAGCTA AATTCAGAGC
F.sporotrichioides   263   TACGCGg--T TTAGACACAG GTGATGGAGA TCTTAGTACA GATTCC----  307   -----CGCAC AAAGGAAGGG ttcaag-GAT AGTACAGCTA AATTCAGAGC

ICR-PQ-12              1   ---------- ---------- ---------- ---------- ----------    1   ---------- ---------- ---------- ---------- -------AGC
F. graminearum       206   TACGCGACTG TCAGGCACCG GCAATGGAGA TCTTGGCTCA GATTCTAGTC  256   TAACTAACAC ACAGGAAAGG GGTTGTAGAT CGTACAGTTA AATTC-GAGC
F.culmorum           208   TACGCGACTT TCAGGCACCG GCAATGGAGA TCTTGGCTCA GATTCTAGTC  258   TAACTAACAC ATAGGAAAGG GGTTGTAGAT CGTACAGCTA AATTCAGAGC
F.cerealis           224   TACGCGACCT TCAGGTACCG GTAATGGAGA CCTTGGCCAA TATTCC----  270   -----AACAC ACAGGAAATG GGTTGTAGAT CATACAGCTA AATTCAGAGC
F.sporotrichioides   263   TACGCGg--T TTAGACACAG GTGATGGAGA TCTTAGTACA GATTCC----  307   -----CGCAC AAAGGAAGGG ttcaag-GAT AGTACAGCTA AATTCAGAGC

ICR-PQ-12              4   CTCCTGCTAA ACCAGAAAGG GCTAAGTTGC CCAAA-CTTA TTCAAC---G   50   CAGTTGCAGT GCATTCGGGA GTCAACTCCG CGGGATATAT GTGATGGCCG
F. graminearum       305   CTCCTGCTAA ACCAAAAAGG GCTAAGTTGC CCAAACCTTA TTCAAC---G  352   CAGTTGCAGT GCATTCGGGA GCCAACTCCG CGGGACATAT GTGATGGCCG
F.culmorum           308   CTCCTGCTAA ACCTAAAAGG GCTAAGTTGC CCAAACTTTA TTCAAC---G  355   CAGTTGCAGT GCATTCGGGA GCCAACTCCG CGGGATATAT GTGATGGCCG
F.cerealis           315   CGCTTGCTAA ACCCAAAATG GATAAGTTGC TTGAACCTTA TTCAAC---G  362   CAGTTGCATT GCATTCGGGA GCCAACTCCG CAGGATATAT GTAACGGCCG
F.sporotrichioides   351   CTCCCGCTAA ct-AAACAAG GCTAAGTTGg -TGAACCTTA TTCAAagatG  399   CGGTTGCAGT ACATCCGGGA ACGAACggtt ttGAATATGT GTAACAGCCG
```

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ICR-PQ-12           100   GCAGTT-CGT AGTGCTGATC ATAAAAGTGG TCATATTTAA GGCCTGTCGC   149   CCAGGTAGAC TTTTGCCAGG GCACAA---- ---TATAACA CCGCGGTTAC
F. graminearum      402   GCAGTTTCGT AGTGCTGATC ATAAAAGTGG TCATATTTAA GGCCTGTCGC   452   CCAGGTAGAC TTTTGCCAGG GCACAA---- ---TATAACA CCGCGGCTAC
F.culmorum          405   GCAGTTTCGT AGTGCTGATC ATAAAAGTGG TCATATTTAA GGCCTGTCGC   455   CCAGGTAGAC TTTTGCCAGG GCACAA---- ---TATAACA CCGCGGCTAC
F.cerealis          412   GCAGTTTCGT GGTGTTGATG ATAAAAGTGG TCATCCTTAA GGCCTGTCAC   462   CCAGGTAAGC TTTTGCCAGG GCAGAA---- ---AATAACT CCGTGGCTGC
F.sporotrichioides  449   ACGGGTTTGC AGGGTatATC ATAAAAGTGG TTACACTTAA GGCCTTTCGC   499   CttatTAAGC TTTTGACAGG GCACAA---- ---AATAACT CCGTGGCTGC

ICR-PQ-12           192   CTAGGTAAGT GAGGCTTTCT TCTGTGTTGA TAGACTGTCG TTTCACTAGT   242   CCAAACATAG ACCACACGGA CAATGACCGA ACTCAATATC CCGATCCAAG
F. graminearum      495   CTGGGTAAGT GAGGCTTTCT TCTGTGTTGA TAGACGGTCG TTTCACTAGT   545   CCAAACACAA ACCACGCGGA CAATGACCGA ACTCAATATC CCGATCCAAG
F.culmorum          498   CTAGGTAAGT GAGGCTTTCT TCTGTGTTGA TAGACGGTCG TTTCACTAGT   548   CCAAACACAG ACC--ACGGA CAATGACCGA ACTCAATATC CCGATCCAAG
F.cerealis          505   CTAGTTAAGT GAGGCTTTCT TCTGTGTTGA TAGACGGCTG TTTCACCAGT   555   CCAAACACAG CCC--ACGGA CAACAACCGA ACCCAACATG CCAACTCAAG
F.sporotrichioides  542   TTGGACAAGT GAGGCTTCCT CCCCTATCGA CAGAAGGCTA TTCCACTAGT   592   TCAAACTCAG tt---ACGGA CAACAACCGA ACTCAA---- ----------

ICR-PQ-12           292   GATTGGTCCC TAGATTTAGG CCTACTCCCA GCCCTTTGAT ACTAGCATCT   342   GGCACCAATC GCTTGTGTAG GTCTACCAAG TGTG--TCGA GCTAAAGACA
F. graminearum      595   GATTGGTCCC TAGATTTAGG CCTACTCCCG GCCCTTTGAT ACTAGCATCT   645   GGCACCAATC GCTTGTGTAG GTCTACCAAG TGTG--TCGA GCTAAAGAAA
F.culmorum          596   GATTGATCCC TAGATTTAGG CCTACTCCCG GCCCTTTGAT ACTAGCATCT   646   AGCACCAATC GCTTGTGTAA GTCTACCAAG TGTG--TCGA GCTAAAGACA
F.cerealis          603   GATTGATCCC TAGATTTAGG CCTACTCCCT GTCCTTTGAC ACTAGCATCT   653   AGCACCAATC GCTTGTGTAG GTCTACCAAG TGTA--TTGA GCTAAAGACA
F.sporotrichioides  625   ---------- -AGCTTCAGG CCTACTCCTC GTCCATTGAC ACCAGGATCC   664   AGGACCAATC GCTTGCGTAG CTAAACCAAA TGctctTCGA GCTAAAGACA

ICR-PQ-12           390   -AAATGAACC AAGAGTTTGC TCCAAGAGCC GGATGTTTTT CTGATACCTG   439   TAGCCTTGCA GGGAACGAGA GAGCATGTCC ATACATCATG GTCTCTCTTC
F. graminearum      693   -AAATGAACT AAGCGTTTGC TCCAAGAGCC GGATGTTTTT CTGATACCTG   742   TAGCCTTGCA GGGAACGAGA GAGCATGTCC ATACATCATG GTCTCTCTTC
F.culmorum          694   -AAATGAACC AAGAGTTTGC TCCAAGAGCC GGAT-GTTTT CTGATATCTG   742   TAGCCTTGCA GGGAATGAGA GAGCACGTCC ATACGTCAAG GTCTCTCTCC
F.cerealis          701   aAAATGAACC AAGAGTTTGC TCCAAGAGCC GGAT-GTTTT CTAATATCTG   750   TAGCCTTGCA GGGAATGAGA AAGCACGTCC ATACGGCACG GTCTtcaaga
F.sporotrichioides  714   -AACCGGACT AATCCTTcct tctggGAGCC GAAG-TCTTT CTCAACTTCG   762   TAGTTTTGCA TGAGACGAGG GGGATCAAGC CTACGTCACG GTCTATGT-C

ICR-PQ-12           489   ACAACCGTCT GGTTGGGGAC GCTATTCGCA TTGACTTTGG ATCAGTCTTA   539   AGGCCTAACA ATACAATCTT GACTAATAAA TGTGTATGGG TCGAGATGTT
F. graminearum      792   ACAACCGTCT GGTTGGGGAC GCTATTCGCA TTGACTTTGG ATCAGTCTTA   842   AGGCCTAACA ATACAATCTT GACTAATAAA TGTGTATGGG TCGAGATGTT
F.culmorum          792   ACGACCGTCT GGTTGGGGAC GCTATTCGCA TTGACTTTGG ATCAGTCTTT   842   AGGCCTAACA ATACAATCTT GACTAATAgC TGTATACGGG TCCAGATGTT
F.cerealis          800   gaGACCATCT GGTTGGGGAC GCTATTCGCA TTGGCTTTaG ATCAGTCTTT   850   AGGCCTAACA AGACAATATT GACTAACAAC TGTGTACGGG TCGAGATGTT
F.sporotrichioides  811   ACAGGTAGCC GGTTGGGGAG GCTGTTGGCA TCGATaTTGG CTCAGTCTTT   861   AGGCCTAACA AGAAAATGTC AACTAGCAAt aGTGTACGGT TCGAGATGGT
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Fig. 6.5. Multiple Alignment of the *tri 5* Promoter Sequence from Five Strains of *F. graminearum*

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F. gram (AF336365)  34   GCCCCTTGTT AAGCTAAGTA TTTCgGCAT- -GTAAACGGA GTTCATCCAC   82   GGTAGAGTTG ACAAGATCTG CACTCTCATC GGTACTTAAT GTCGTTCTAG
F. gram (AB060689)   1   ------TGTT AAGCTAAGCG TTTTTAATAT GGAAAACGGA GTTCATATAC   45   AGTAGAGTCA ACAAGATCTG CAACCTATCA GTGCTTAAAT GCAATTCCAG
F. gram (AY102587)  50   GCCCCTTGTT AAGCTAAGTA CTCCaGCAT- -GTAAACGGA GTTCATCCAC   98   GGTAGAGTTG ACAAGATCTG CACTCTCATC GGTATTTGAT GTAGTTCTAG
F. gram (AF359361)  15   GCCCCTTGTT GAGCTAAGCG TTTTTAATAT GGAAAACGGA GTTCATCTAC   65   AGTAGAGTCG ACAAGATCTG CAATCTATCA GTGCTTAAAT GCAGTTCCAG
F. gram (AY102605)  21   GCCCCTTGTT GAGCTAAGCG TTTTTAATAT GGAAAACGGA GTTCATCTAC   71   AGTAGAGTCG ACAAGATCTG CAATCTATCA GTGCTTAAAT GCAGTTCCAG

F. gram (AF336365) 132   CTACGCGACC TTCAGGTACT GGTAATGGAG ATCTTGGCCA ATATTCCA--  180   ---------- ------ACAC ACAGGAAATG GGTTGTAGAT CATACAGCTA
F. gram (AB060689)  95   CTACGCGACT gTCAGGCACC GGCAATGGAG ATCTTGGCTC AGATTCTAgt  145   ctaactA--- ------ACAC ACAGGAAAGG GGTTGTAGAT CGTACAGTTA
F. gram (AY102587) 148   CTACGCGACC TTCAGGTACC GGTAATGGAG ATCTTGGCCA ATATTCC---  195   ------A--- ------ACAC ACAGGAAATG GGTTGTAGAT CTTACAGCTA
F. gram (AF359361) 115   CTACGCGACT TTCAGGTACC GGCAATGGAG ATCTTGGCTC AGATTCT---  162   ------AGTC TAACTAACAC ACAGGAAAGG GGTTGTAGAT CGTACAGCTA
F. gram (AY102605) 121   CTACGCGACT TTCAGGTACC GGCAATGGAG ATCTTGGCTC AGATTCT---  168   ------AGTC TAACTAACAC ACAGGAAAGG GGTTGTAGAT CGTACAGCTA

F. gram (AF336365) 214   AATTCAGAAC CGCTTGCTAA ACCCAAAATG GATAAGTAAC TTGAACCTTA  264   TTCAACGCAG TTGCATTGCA TTCGGGAGCC AACTCCGCGG GATATATGTA
F. gram (AB060689) 186   AATTCAGAGC CTCCTGCTAA ACCAAAAAGG GCTAAGTTGC CCAAACCTTA  236   TTCAACGCAG TTGCAGTGCA TTCGGGAGCC AACTCCGCGG GACATATGTG
F. gram (AY102587) 230   AATTCAGAGC CGCTTGCTAA ACCCAAAATG GATAACTTGC TTGAACCTTG  280   TTCAACGCAG TTGCATTGCA TTCGGGAGCC AACTCCGCGG GATATATGTA
F. gram (AF359361) 206   AATTCAGAGC CTCCTGCTAA ACCTAAAAGG GCTAAGTTGC CCAAACCTTA  256   TTCAACGCAG TTGCAGTGCA TTCGGGAGCC AGCTCCGCGG GATATATGTG
F. gram (AY102605) 212   AATTCAGAGC CTCCTGCTAA ACCTAAAAGG GCTAAGTTGC CCAAACCTTA  262   TTCAACGCAG TTGCAGTGCA TTCGGGAGCC AGCTCCGCGG GATATATGTG

F. gram (AF336365) 314   ACGGCCGGCA GTTTCGTGGT GTTGATGATA AAAGTGGTCA TCCTTAAGGC  464   CCAAACACAG CCCACG--GA CAACAACCGA ACCCAACATG CCAACTCAAG
F. gram (AB060689) 286   ATGGCCGGCA GTTTCGTAGT GCTGATCATA AAAGTGGTCA TATTTAAGGC  436   CCAAACACAA ACCACGcgGA CAATGACCGA ACTCAATATC CCGATCCAAG
F. gram (AY102587) 330   ACGGCCGGCA GTTTCGTGGT GTTGATGATA AAAGTGGCCA TCCTTAAGGC  480   CCAAACACAG CCCACG--GA CAACAACCGA ACTCAACATC CTGACCCAAG
F. gram (AF359361) 306   ATGGCCGGCA GTTTCGTAGT GCTGATCATA AAAGTGGTCA TATTTAAGGC  456   CCAAACACAG ACCACG--GA CAACGACCGA ACTCAATATC CCGATCCAAG
F. gram (AY102605) 312   ATGGCCGGCA GTTTCGTAGT GCTGATCATA AAAGTGGTCA TATTTAAGGC  462   CCAAACACAG ACCACG--GA CAACGACCGA ACTCAATATC CCGATCCAAG

F. gram (AF336365) 562   AGCACCAATC GCTTGTGTAG GTCTACCAAG TGTATTGAGC TAAAGACAAA  612   AATGAACCAA GAGTTTGCTC CAAGAGCCGG ATG-TTTTCT AATATCTGTA
F. gram (AB060689) 536   GGCACCAATC GCTTGTGTAG GTCTACCAAG TGTGTCGAGC TAAAGA-AAA  585   AATGAACTAA GCGTTTGCTC CAAGAGCCGG ATGTTTTTCT GATACCTGTA
F. gram (AY102587) 578   AGTACCAATC GCTTGTGTAG GTCTACCAAA TGTATTGAGC TAAAGACAAA  628   AATGAACCAA GAGGTTGCTC CAGGAGCCGG ATGTTTT-CT GATGTCTATA
F. gram (AF359361) 554   AGCACCAATC GCTTGTGTAA GTCTACCAAG CGTGTCGAGC TAAAGA-CAA  603   AATGAACCAA GAGTTTGTTC CAAGAGCCGG ATGTTTT-CT GATATCTGTA
F. gram (AY102605) 560   AGCACCAATC GCTTGTGTAA GTCTACCAAG CGTGTCGAGC TAAAGA-CAA  609   AATGAACCAA GAGTTTGTTC CAAGAGCCGG ATGTTTT-CT GATATCTGTA
```

Matrix scan analysis of the promoter sequences using TRED (Transcription regulatory element database) identified the TATA-box to be located at -92, relative to the start ATG the *Fusarium* species. The location of TATA-box is represented in Fig. 6.6.

Tri 6, one of the regulatory elements for trichothecene synthesis, brings about its effect by binding to the promoter sequences of the genes involved in trichothecene synthesis (discussed in section 1.5.7.2. Molecular Factors Regulating Trichothecene Biosynthesis). The core binding sequence required for Tri 6 binding has been identified as 'TNAGGCCT' (Hohn et al, 1999). Promoter sequences of the *tri 5* gene from commonly encountered trichothecegenic *Fusaria* were searched for the presence of Tri 6 core binding sequence. Three motifs each was observed in the upstream region of *tri 5* start ATG, as also observed by Hohn et al (1999). The distance between the first two motifs was approximately 162-164 bp for all *Fusarium* species with the exception of *F. sporotrichioides* whose motifs were 136 bp apart. The second and third Tri 6 binding motifs were 222 bp apart for all the species studied. The *tri 5* promoter regions contained either of the three sequences TTAGGCCT, TAAGGCCT and TCAGGCCT (i.e, three forms of the basic TNAGGCCT sequence where N represented by T, A and C) specific for Tri 6 binding (Fig. 6.6).

In the previous chapter we have discussed about the synthesis of Tri 5 protein in relation to trichothecene production in presence of varying carbon and nitrogen sources. In this chapter attempts were made to locate binding sites of transcription factors involved in the sensing of nutrient levels. Motif searches of the *tri 5* promoter region using PATCH revealed the AGGGG motif specific for MSN4 binding. The protein MSN4 (Transfac No. T01258 from *S. cerevisiae*) is involved in carbon utilization (Estruch and Carlson, 1993). Proteins involved in the glucose repression system plays significant role in the utilization of other carbohydrates also (Carmona et al, 2002). MIG1 (Transfac No. T00509 from *S. cerevisiae*) is a protein involved in glucose repression in *S. cerevisiae* and has binding sites in the promoters of glucose repressed genes such as GAL1, GAL4, SUC2, and MAL (Carmona et al, 2002). In this study, analysis of *tri 5* promoter sequences of different species revealed the presence of motifs specific for

Fig. 6.6. Transcription Factor Binding Sites in the Promoter Sequence of *tri 5* (match highlighted)

>AY134892 *F. culmorum*

```
ACTTTCAGGCACCGGCAATGGAGATCTTGGCTCAGATTCTAGTCTAACTA     2150
ACACATAGGAAAGGGGGTTGTAGATCGTACAGCTAAATTCAGAGCCTCCTG    2200
CTAAACCTAAAAGGGCTAAGTTGCCCAAACTTTATTCAACGCAGTTGCAG     2250
TGCATTCGGGAGCCAACTCCGCGGGATATATGTGATGGCCGGCAGTTTCG     2300
TAGTGCTGATCATAAAAGTGGTCATATTTAAGGCCTGTCGCCCAGGTAGA     2350
TTCTTCTGTGTTGATAGACGGTCGTTTCACTAGTCCAAACACAGACCACG     2450
GACAATGACCGAACTCAATATCCCGATCCAAGGATTGATCCCTAGATTTA     2500
GGCCTACTCCCGGCCCTTTGATACTAGCATCTAGCACCAATCGCTTGTGT     2550
AAGTCTACCAAGTGTGTCGAGCTAAAGACAAAATGAACCAAGAGTTTGCT     2600
CCAAGAGCCGGATGTTTTCTGATATCTGTAGCCTTGCAGGGAATGAGAGA     2650
GCACGTCCATACGTCAAGGTCTCTCTCCACGACCGTCTGGTTGGGGACGC     2700
TATTCGCATTGACTTTGGATCAGTCTTTAGGCCTAACAATACAATCTTGA     2750
CTAATAGCTGTATACGGGTCCAGATGTTTGCCATGTAATGATATATAGTT     2800
AATAGCAACAGCACTTTGTAATAAGAGAATGACAAGGACTTGACTTGTTA     2850
ATTTATTGAATAACTGTTACCAGTACAACCTTGCCATC                 2888
```

>AY102574 *F. cerealis*

```
GTAGAGTTGACAAGAACTGCACTCTCATCGGTACTTAATGTAGTACTAGC     2150
TACGCGACCTTCAGGTACCGGTAATGGAGATCTTGGCCAATATTCCAACA     2200
CACAGGAAATGGGTTGTAGATCGTACAGCTAAATTCAGAGCCGCTTGCTA     2250
AACCCAAAATGGATACGTTGCTTGAACCTTATTCAACGCAGTTGCATTGC     2300
ATTCGGGAGCCAACTCCGCAGGATATATGTAACGGCCGGCAGTTTCGTGG     2350
TGTTGATGATAAAAGTGGTCACCCTTAAGGCCTGTCACCCAGGTAAGCTT     2400
TTGCCAGGGCAGAATATAACACCGCGGCTACCTAGTTAAGTGAGGCTTTC     2450
TTCTGTGTTGATAGACGGCTGTTTCACCAGTCCAAACACAGCCCACGGAC     2500
AACAACCGAACCCAACATGCCAACTCAAGGATTGATCCCTAGATTTAGGC     2550
CTACTCCCTGTCCTTTGACACTAGCATCTAGCACCAATCGCTTGTGTAGG     2600
TCTACCAAGTGTATTGAGCTAAAGACAAAAATGAACCAAGAGTTTGCTCC     2650
AAGAGCCGGATGTTTTCTAATATCTGTAGCCTTGCAGGGAATGAGAAAGC     2700
ACGTCCATACGGCACGGTCTTGAAGAGAGACCATCTGGTTGGGGACGCTA     2750
TTCGCATTGGCTTTAGATCAGTCTTTAGGCCTAACAAGACAATATTGACT     2800
AACAACTGTGTACGGGTCGAGATGTTTGTCATCTAATGATATATAGTTAA     2850
TAGCAGCAGCACTTTGTAACAATAGAATAAAAACGACTTGACTTGTTGAT     2900
TCATTGAATAACTGTTACCAATACACCTTTGCCATC                   2936
```

TATATA: TATA box, TNAGGCCT: Core binding site for Tri 6

>AY359360 *F. sporotrichioides*

```
AGCGCAAGTTAAGTTATCTATGTCAAATACGAAAACAAGATTTATCTACG     1500
CTAAACAAGGCTAAGTTGGTGAACCTTATTCAAAGATGCGGTTGCAGTAC    1700
ATCCGGGAACGAACGGTTTTGAATATGTGTAACAGCCGACGGGTTTGCAG     1750
GGTATATCATAAAAGTGGTTACACTTAAGGCCTTTCGCCTTATTAAGCTT     1800
TTGACAGGGCACAAAATAACTCCGTGGCTGCTTGGACAAGTGAGGCTTCC     1850
TCCCCTATCGACAGAAGGCTATTCCACTAGTTCAAACTCAGTTACGGACA     1900
ACAACCGAACTCAAAGCTTCAGGCCTACTCCTCGTCCATTGACACCAGGA     1950
TCCAGGACCAATCGCTTGCGTAGCTAAACCAAATGCTCTTCGAGCTAAAG     2000
ACAAACCGGACTAATCCTTCCTTCTGGGAGCCGAAGTCTTTCTCAACTTC     2050
GTAGTTTTGCATGAGACGAGGGGGATCAAGCCTACGTCACGGTCTATGTC     2100
ACAGGTAGCCGGTTGGGGAGGCTGTTGGCATCGATATTGGCTCAGTCTTT     2150
AGGCCTAACAAGAAAATGTCAACTAGCAATAGTGTACGGTTCGAGATGGT     2200
TGCCATCTAATGATATATAGTTGGTAGCAACGGCACTTTGTTATAAGACA     2250
ATGATAGCGATCTAGTTTGTTGATTCATCAAGAATTGTTACCAATACACC     2300
TTGGCCAATA                                             2310
```

>*AB060689. F. graminearum*

```
AAATGCAATTCCAGCTACGCGACTGTCAGGCACCGGCAATGGAGATCTTG     2150
GCTCAGATTCTAGTCTAACTAACACACAGGAAAGGGGTTGTAGATCGTAC     2200
AGTTAAATTCAGAGCCTCCTGCTAAACCAAAAAGGGCTAAGTTGCCCAAA     2250
CCTTATTCAACGCAGTTGCAGTGCATTCGGGAGCCAACTCCGCGGGACAT     2300
ATGTGATGGCCGGCAGTTTCGTAGTGCTGATCATAAAAGTGGTCATATTT     2350
AAGGCCTGTCGCCCAGGTAGACTTTTGCCAGGGCACAATATAACACCGCG     2400
GCTACCTGGGTAAGTGAGGCTTTCTTCTGTGTTGATAGACGGTCGTTTCA     2450
CTAGTCCAAACACAAACCACGCGGACAATGACCGAACTCAATATCCCGAT     2500
CCAAGGATTGGTCCCTAGATTTAGGCCTACTCCCGGCCCTTTGATACTAG     2550
CATCTGGCACCAATCGCTTGTGTAGGTCTACCAAGTGTGTCGAGCTAAAG     2600
AAAAAATGAACTAAGCGTTTGCTCCAAGAGCCGGATGTTTTTCTGATACC     2650
TGTAGCCTTGCAGGGAACGAGAGAGCATGTCCATACATCATGGTCTCTCT     2700
TCACAACCGTCTGGTTGGGGACGCTATTCGCATTGACTTTGGATCAGTCT     2750
TAAGGCCTAACAATACAATCTTGACTAATAAATGTGTATGGGTCGAGATG     2800
TTTGCCATCTATTGATATATAGTTAATAGCAACAGCACTTCGTAACAAGA     2850
GAATGACAACGACTTAACTTTTTGATTTATTGAATCATTTCTACCAGTAC     2900
AACCTTGCCATC                                           2912
```

ATTTT/TTTTA: MIG1 binding, AGGGG: MSN4 binding,

TATCTM: NIT2 binding

MIG1 binding (Fig. 6.6). NIT2, a protein specific for activation of nitrogen mediated genes was also identified from the promoter region of *tri 5* (Fig. 6.6). NIT2 (Transfac No. T00627) is a positive acting novel regulatory gene in *Neurospora crassa*. It is hypothesized that there may be specific motifs in the *tri 5* promoter which plays crucial role in the differential expression of *tri 5* and the production of varying amounts of trichothecenes under varied nutrient conditions.

6.3.4.2. *In Silico* Analysis of the *tri 6* Promoter Sequence

The sequences between the *tri 4* stop and the *tri 6* start sequences were used in the analysis. Variations in the sequences for this region from different species of *Fusarium* were revealed in the multiple sequence alignment (Fig. 6.7). It was inferred that the differential regulation of trichothecene production by the various *Fusarium* species may be mediated through the variant promoter. None of the four variants of the Tri 6 core binding sequence (TNAGGCCT) such as TGAGGCCT, TAAGGCCT, TCAGGCCT, TTAGGCCT were observed in the sequences of the *tri 6* promoter. It may be presumed that Tri 6 protein does not therefore regulate its own synthesis. The motifs for binding of MSN4 (AGGGG) and MIG1 were observed in the promoter region of different trichothecene producers such as *F. graminearum*, *F. culmorum* and *F. cerealis* (Fig. 6.7). The *tri 6* promoter of *F. sporotrichioides* contained motifs for MIG1 binding whereas the MSN4 binding motif was not observed (Fig. 6.8). The promoter sequence also contained the motif TATCAT specific for NIT2 binding (Fig. 6.8). Tag et al (2001) have predicted the possibility of a regulatory loop involved in the synthesis of trichothecenes. It was suggested that the physico-chemical factors might exert their effect either directly on the *tri* genes or via an alternate pathway, under the control of *tri 6* and *tri 10*. In the previous chapter we had noticed the differential production of Tri 5 and trichothecene toxin production in response to different carbohydrates and also to varying concentrations of glucose and peptone (section 5.3.4. Synthesis of Tri 5 in Relation to Trichothecene Production**)**. The presence of MSN4, MIG1 and NIT2 specific motifs in the promoter region of *tri 5* and *tri 6* indicates that *tri* genes do play a role in the varied trichothecene production in response to differential nutrient make up in the media

Fig. 6.7. Multiple Alignment of *tri 6* Promoter Sequences

```
F.culmorum          186   CGTAAACGCT -CACAACTCT GAAGCTGTCC TCAGTATCGC CATGTCAGAT   640   TGGTCA-AAA GTATGTACAT GGATGGTCTT GCACAGAAGA ------CAGC
F.graminearum       248   CGTAAACGCT -CACAACTTT GAAGCTATCC TCAGTATCGC TATGTCAAAT   707   TGGTCAGAAA GTATGTACAT GGATGGTCTT GCACAGGAGC ------CAGC
F.cerealis          250   CGCAAAGGCT -CACAACTCT GAAGCTGTCC TCAGTATCAC CATGTCCGAT   709   TGGTCAGAAA GTATGTACAT GGATGGTCTT GCACAGAAGC ------CAGC
F.sporotrichioides  248   CGTGAAAGCT tCACAGCTAT GAGGCTGTAC TCAGTAACGC CATGCTAGAT   712   CGGTCA-AGG ATATGTAC-- --ATGGTCTT GTACAAAgta cagataCAGC

F.culmorum          235   AAGCTTCGCA AGAGCGTCCG ---------- GTCGGAAACT CACCAATCAA   683   CTCGAGTGTT ATGCAGACTG TCACGGCTGC AGTAAGTTG- ----------
F.graminearum       297   AAGCTTCGCA AGAGTGTCTG ---------- GCCGGAAACT CACCAATCAA   751   CTCAAGTGTT GTGCAAACTG TCAAGGCTGC AGTAAGTTGG C------ACA
F.cerealis          299   GAGGTTCGCA GGAGTGTCCG ---------- GTCGAAAACT CACCAATCAA   753   CTCAAGTGTT GTGCAAACTG TCAAGGCTGC AGTAAGTTGG C------ACA
F.sporotrichioides  298   AGGCTTTGCC AAA-TGTCCt tacttagtaa GTCGAACACT AACCAATCAA   757   CTCAGCTATT ATGCAAACTG CCAAGGCTGC AGCCAGTGGt taccgttACA

F.culmorum          275   CTCGACAGGA CGAACAAGGG GTCTGAAAGG CCTGGCAGGC CTGACAGGAG   722   ----CCACAG ACTTGAATCG ATTATCATTG ACCGTTCGGA AGCG--CTCT
F.graminearum       337   CTCAACAGGA CGAACAAGGG GTCTGAAAGG CCTGGCAGGC CTGACAGGAG   795   CTC-CCACAG ACTAGAATCG ATTATCATTG ACCGTCCGAA AGCCTTCTCT
F.cerealis          339   CTCAACAGGA CGAACAAGGG GTCTGAAAGG CCTGGCAGGC CTGACAGGAG   797   CTC-CCACAG ACTTGAATCG ATTATCATTG ACCGTCCGGA AGCGTTCTCT
F.sporotrichioides  347   TCCACCAAGG TGAACAt-GG GTCTGAAAGG CCTGGCTGGC CTGACAGGAG   807   CTtgCCGCAT TCTTAAATGT GTTATCATTG AACGTTAGAG AACG--CTTC

F.culmorum          325   CGATAAAATG TGAGAAGAGA TATGCCGATA CAACCGTGTA ACTTGTGAAA   766   GTTAGGAATC TTTCTAGACC ACAACTAC-C ACTTTGGCAT CTGCATACTA
F.graminearum       387   CGATAAAATG TGAGGAGAGA TATGCCGACA CAACCGTGTA ACTTGTGAAA   844   GTTTGGAATC TTTCTAGACC ACAACTAC-C ACTTTAGCAT CTGCATGCCA
F.cerealis          389   CGATAAAAGG TGAGAGGAGA TATGCCGATA CAACCGTGTA ACTTGTGAAA   846   GTTTGGAATC TTTCTAGACC ACAACTAC-C ACTTTAGCAT CTGCATGCCA
F.sporotrichioides  396   CAATAGATTG AGAGAAGGGA TATGCCGGTA CAACCGGGTA ACTTcgGAAA   855   ATTTGGAATC CTGTCAGCTT ACAGCTTCtC TTTTCAGTAT CTGTATGCCA

F.culmorum          375   CGGGGCATGG AATCCCATGG CAAGTTCTG- ---------- ----------   815   ACACTAGTAG -CCACATAGT AAACCTTCAA CTGCCGCCGC ATCAAACTGT
F.graminearum       437   CGGGGCATGG AATCCCATGG TAAGTTCTG- ---------- ----------   893   GCACTAGTAG -CCACATAGT GAACCTTCAA CTGCCGCCGC ATCAAACTGT
F.cerealis          439   CGGGGCATGG AATCCCATGG TAGGTTCTG- ---------- ----------   895   GCACTAGTAG -CCACATAGT GAACCTTCAA CTGCCGCCGC ATCAAACTGT
F.sporotrichioides  446   CGGGGTATGC AGCCGCCAAG AGAGTTtcta cgatcggtgg attgtcaacg   905   GCAGTGATAG tCCGCATACG GAAGCTACAA CTGGCGTAG- --CAAACTGT

F.culmorum          404   ------GGGT CAGCAGCAAC TGA---ATTG CCTACGACTC AAGAAGTGCA   864   AAACAGGTAC CGGCCGACGC GTCTCGGATA AGaATAC--- CTTTTAAACT
F.graminearum       466   ------GGGT TAGCAGCAAC CGA---ATTG CCCACGACTC AAGAAGTGCA   942   AAATAGGTAC CGGCCGACGC GTCTCGGATA AG-ATAC--- CTTTTAGACT
F.cerealis          468   ------GGGT TAGCAGCAAC TGA---ATTG CCCACGACTC AAGAGGTGCA   944   AAATAGGTAC CGGCCGACGC GTCTCGGATA AG-ATAC--- CTTTTAAACT
F.sporotrichioides  496   atcggcGGAT TGTCAACGAT CGgtggATTG TCAACAGTTG AAGATGTGAA   952   AAATAtt--- -----GACGT GACTCGGAGT AT-ATcaata CCGTTGAACT

F.culmorum          445   TCCTTTCACC GGCGGCTTAT CCGAAGTTGC TGCCGATCAG ATGCAGACAC   911   GCCGTAGCAA ACTGTAATTG TCGGTACTTC T--CGGACAA TATTTTTATG
F.graminearum       507   TTCTTTCACC GGCGGATTAT CCGAAGTTGC TGCCGATCAG ATGCAGACAC   988   GCCGTAGCAA ACTGTAATTG TCGGTACTTC T--CGGACAA TATTCTTGTG
F.cerealis          509   TTCTTTCACC GGCGGGTTAT CCGAAGTTGC TGCCGATCAG ATGCAGACAC   990   GCCGTAGCAA ACTGTAATTG TCGGTACTTC T--CGGACAA TATTTTGTG
F.sporotrichioides  546   CCCTCTCGTC CGCGGA---- ---------- ---------- ---CAGACAT   993   GCCGTAGTAA ACTGTAAATG TCGGCACTTg ctgCGGGCAC TAa-CTTGAG

F.culmorum          495   ACATGCAGAG GGGTACGACT GCGCGGAAGA ATAAGAATCA TCAGTGCGCC  1009    TATCAATCGT GTCCCATCCC ATcaaggcTC AAGCCATCTT TTTTTttttt
F.graminearum       557   ACATGCAGAG TGGTACGACT GCACAGAAGA ATAAGAAGCA TCAGTGCGCC  1086    TACCAATCGT GTCCCCTCTC AT-------C AAGCCATCTT TTTTCCCT--
F.cerealis          559   ACATGCAGAA TGGTACGACT GCACAGAAGA ATAAGAAGCA TCAGTGCGCC  1088    TACCAATCGT GTCCCCTCCC AT-------C AAGCCATCTT TTTTTCCT--
F.sporotrichioides  569   GCATGCAGAG TGGTACGATT CTACGGAACA ATAa-AAGGA CCAATGCGCC  1092    TACCAATCGT cctg------ --------TT AAGCCAACTT TTT-------

F.culmorum          595   CCAGGGT--- --CTTGTCTC GAAATATCTT TGTCTACCGA GACCCATGCA  1059    ttttgCATCA CCAACCAATA TATTGAACAT CTATT-TTGA CTACCCTCGA
F.graminearum       657   CCAGGGTAGG GTCTTGTCTC AAAATATCTT TGTCTCCCGA GACCCATGCA  1127    -----CATCA CCAGCCGATA CATTGAACGT CTATT-TTAA CTACATTCGA
F.cerealis          659   CCAGGGTAGG GTCTTGTCTC AAAATATCTT TGTCTCCAGA GACCCATGCA  1129    -----CATCA CCAACCGATA CATTGAACGT CTATT-TTAA CTACACTCGA
F.sporotrichioides  668   a------AGG ATCTTGTCTC AAACTATCTT ccagTGCCGG GACCGATGCA  1121    ---------A CTCATCAATA CATTGAACGT ATATccTTAT ACTCTCACTA
```

Fig. 6.8. Transcription Factor Binding Sites in the Promoter Sequence of *tri 6* (match highlighted)

>AY134892 *F. culmorum*

```
CTCTGAAGCTGTCCTCAGTATCGCCATGTCAGATAAGCTTCGCAAGAGCG     250
TCCGGTCGGAAACTCACCAATCAACTCGACAGGACGAACAAGGGGTCTGA     300
AAGGCCTGGCAGGCCTGACAGGAGCGATAAAATGTGAGAAGAGATATGCC     350
GATACAACCGTGTAACTTGTGAAACGGGGCATGGAATCCCATGGCAAGTT     400
CTGGGGTCAGCAGCAACTGAATTGCCTACGACTCAAGAAGTGCATCCTTT     450
CACCGGCGGCTTATCCGAAGTTGCTGCCGATCAGATGCAGACACACATGC     500
AGAGGGGTACGACTGCGCGGAAGAATAAGAATCATCAGTGCGCCGCAATG     550
TTAAAAACTGATGTGCGGAAGCAACATTAAGCTTTGGAGGCATGCCAGGG     600
TCTTGTCTCGAAATATCTTTGTCTACCGAGACCCATGCATGGTCAAAAGT     650
ATGTACATGGATGGTCTTGCACAGAAGACAGCCTCGAGTGTTATGCAGAC     700
TGTCACGGCTGCAGTAAGTTGCCACAGACTTGAATCGATTATCATTGACC     750
GTTCGGAAGCGCTCTGTTAGGAATCTTTCTAGACCACAACTACCACTTTG     800
GCATCTGCATACTAACACTAGTAGCCACATAGTAAACCTTCAACTGCCGC     850
CGCATCAAACTGTAAACAGGTACCGGCCGACGCGTCTCGGATAAGAATAC     900
CTTTTAAACTGCCGTAGCAAACTGTAATTGTCGGTACTTCTCGGACAATA     950
TTTTTATGGCTTCCCGAAGCTTTCACTTTTAATAAAACTTGATCCGAATA    1000
AGAAACTTTATCAATCGTGTCCCATCCCATCAAGGCTCAAGCCATCTTTT    1050
TTTTTTTTTTTTGCATCACCAACCAATATATTGAACATCTATTTTGACTA    1100
CCCTCGAAAT                                            1110
```

>AY102574 *F. cerealis*

```
GCAAAGGCTCACAACTCTGAAGCTGTCCTCAGTATCACCATGTCCGATGA     300
GGTTCGCAGGAGTGTCCGGTCGAAAACTCACCAATCAACTCAACAGGACG     350
AACAAGGGGTCTGAAAGGCCTGGCAGGCCTGACAGGAGCGATAAAAGGTG     400
AGAGGAGATATGCCGATACAACCGTGTAACTTGTGAAACGGGGCATGGAA     450
TCCCATGGTAGGTTCTGGGGTTAGCAGCAACTGAATTGCCCACGACTCAA     500
GAGGTGCATTCTTTCACCGGCGGGTTATCCGAAGTTGCTGCCGATCAGAT     550
GCAGACACACATGCAGAATGGTACGACTGCACAGAAGAATAAGAAGCATC     600
AGTGCGCCGCAATGTTAAAAACTGATGTGCGGAAGCAACATTAAGCTTTG     650
GAGACATGCCAGGGTAGGGTCTTGTCTCAAAATATCTTTGTCTCCAGAGA     700
CCCATGCATGGTCAGAAAGTATGTACATGGATGGTCTTGCACAGAAGCCA     750
GCCTCAAGTGTTGTGCAAACTGTCAAGGCTGCAGTAAGTTGGCACACTCC     800
CACAGACTTGAATCGATTATCATTGACCGTCCGGAAGCGTTCTCTGTTTG     850
GAATCTTTCTAGACCACAACTACCACTTTAGCATCTGCATGCCAGCACTA     900
GTAGCCACATAGTGAACCTTCAACTGCCGCCGCATCAAACTGTAAATAGG     950
TACCGGCCGACGCGTCTCGGATAAGATACCTTTTAAACTGCCGTAGCAAA    1000
CTGTAATTGTCGGTACTTCTCGGACAATATTTTTGTGACTTTGAGAAGCT    1050
TTTACTCTTAATAAAACTTCATCTGAATAAGAAACTTTACCAATCGTGTC    1100
CCCTCCCATCAAGCCATCTTTTTTTCCTCATCACCAACCGATACATTGAA    1150
CGTCTATTTTAACTACACTCGAGATGATTTACATGGAGGACGAATCTCAC    1200
TACGAATCTT                                            1210
```

TTTAAA/TAAATA: TATA-Box, ATTTT/TTTTA: MIG1 binding

>*AB060689. F. graminearum*

```
AAACGCTCACAACTTTGAAGCTATCCTCAGTATCGCTATGTCAAATAAGC     300
TTCGCAAGAGTGTCTGGCCGGAAACTCACCAATCAACTCAACAGGACGAA     350
CAAGGGGTCTGAAAGGCCTGGCAGGCCTGACAGGAGCGATAAAATGTGAG     400
GAGAGATATGCCGACACAACCGTGTAACTTGTGAAACGGGGCATGGAATC     450
CCATGGTAAGTTCTGGGGTTAGCAGCAACCGAATTGCCCACGACTCAAGA     500
AGTGCATTCTTTCACCGGCGGATTATCCGAAGTTGCTGCCGATCAGATGC     550
AGACACACATGCAGAGTGGTACGACTGCACAGAAGAATAAGAAGCATCAG     600
TGCGCCGCAAGGTTAAAAACTGATGTGCGGAAACAACATTAAGCTTTGGA     650
GACATGCCAGGGTAGGGTCTTGTCTCAAAATATCTTTGTCTCCCGAGACC     700
CATGCATGGTCAGAAAGTATGTACATGGATGGTCTTGCACAGGAGCCAGC     750
CTCAAGTGTTGTGCAAACTGTCAAGGCTGCAGTAAGTTGGCACACTCCCA     800
CAGACTAGAATCGATTATCATTGACCGTCCGAAAGCCTTCTCTGTTTGGA     850
ATCTTTCTAGACCACAACTACCACTTTAGCATCTGCATGCCAGCACTAGT     900
AGCCACATAGTGAACCTTCAACTGCCGCCGCATCAAACTGTAAATAGGTA     950
CCGGCCGACGCGTCTCGGATAAGATACCTTTTAGACTGCCGTAGCAAACT    1000
GTAATTGTCGGTACTTCTCGGACAATATTCTTGTGACTTTGAGAAGCTTT    1050
CGCTCTTAATAAAACTTCATCTGAATAAGAAACTTTACCAATCGTGTCCC    1100
CTCTCATCAAGCCATCTTTTTTCCCTCATCACCAGCCGATACATTGAACG    1150
TCTATTTTAACTACATTCGAGATGATTTAC                         1180
```

AY359360 *F. sporotrichioides*

```
GAAAGCTTCACAGCTATGAGGCTGTACTCAGTAACGCCATGCTAGATAGG     300
CTTTGCCAAATGTCCTTACTTAGTAAGTCGAACACTAACCAATCAATCCA     350
CCAAGGTGAACAfTGGGTCTGAAAGGCCTGGCTGGCCTGACAGGAGCAATA    400
GATTGAGAGAAGGGATATGCCGGTACAACCGGGTAACTTCGGAAACGGGG     450
TATGCAGCCGCCAAGAGAGTTTCTACGATCGGTGGATTGTCAACGATCGG     500
CGGATTGTCAACGATCGGTGGATTGTCAACAGTTGAAGATGTGAACCCTC     550
TCGTCCGCGGACAGACATGCATGCAGAGTGGTACGATTCTACGGAACAAT     600
AAAAGGACCAATGCGCCGCAATGTTAAGAGCTAATGCACGGAAGCAACAT     650
TAGGCTTTGGAGGCATCAAGGATCTTGTCTCAAACTATCTTCCAGTGCCG     700
GGACCGATGCACGGTCAAGGATATGTACATGGTCTTGTACAAAGTACAGA     750
TACAGCCTCAGCTATTATGCAAACTGCCAAGGCTGCAGCCAGTGGTTACC     800
GTTACACTTGCCGCATTCTTAAATGTGTTATCATTGAACGTTAGAGAACG     850
CTTCATTTGGAATCCTGTCAGCTTACAGCTTCTCTTTTCAGTATCTGTAT     900
GCCAGCAGTGATAGTCCGCATACGGAAGCTACAACTGGCGTAGCAAACTG     950
TAAATATTGACGTGACTCGGAGTATATCAATACCGTTGAACTGCCGTAGT    1000
AAACTGTAAATGTCGGCACTTGCTGCGGGCACTAACTTGAGGCTGTTAGA    1050
GACATTCACTCTTAATAAAACGTCGTCTGAATAAGAGACTTTACCAATCG    1100
TCCTGTTAAGCCAACTTTTTACTCATCAATACATTGAACGTATATCCTTA    1150
TACTCTCACTAAATGATTTA                                  1170
```

AGGGG: MSN4 binding, TATCAT: NIT2 binding

6.3.4.3. *In Silico* Analysis of the *tri 5* Promoter Sequence

The distance of the presumed promoter region for *tri 10* for the *Fusarium* species varied from 584-662 bp. Variations in the sequences for this region from different species of *Fusarium* were revealed in multiple sequence alignment (Fig. 6.9). The sequences were devoid of the *tri 6* core binding site, as also reported by Tag et al (2001). The promoter sequence for *tri 10* was characterized for the presence of NIT2 (motif TATCTA) and MIG1 (motif AATTG) binding sites (Fig. 6.10). 1-3 motifs were observed for the binding of MIG1 and one motif each were observed for binding of NIT2 with the exception of *F. cerealis* which contained only MIG1 binding motif. Unlike those of *tri 5* and *tri 6*, the promoter sequence of the gene *tri 10* did not contain MSN4 binding site.

6.3.4.4. Identification of sequences homologous to MIG1, MSN4 and NIT2 sequences in the *Fusarium graminearum* Genome Database (FGDB)

The MIPS [(Munich Information Center for Protein Sequences) (MIPS-GSF, Neuherberg, Germany)] *Fusarium graminearum* genome database (FGDB; on http://mips.gsf.de/genre/proj/fusarium/) established by Güldener et al (2006) provides information on more than 14,000 genes of *F. graminearum.* The GenRE (Genome Research Environment) database provides information on the gene structure of one third of the ~ 14, 000 protein coding genes of *F. graminearum*. There is provision for retrieval of information on genes, proteins and contigs in FGDB. Primary analysis of promoters of genes *tri 5*, *tri 6* and *tri 10* revealed the presence of motifs specific for the binding of certain proteins involved in carbon and nitrogen utilization namely MIG1, MSN4 and NIT2. The BLAST service established in FGDB database was used to search for the presence of proteins involved in the utilization of carbon and nitrogen in trichothecegenic *Fusaria*. The protein sequences of MIG1 (accession no. YGL035C) and MSN4 (accession no. YKL062W) from *Saccharomyces cerevisiae* when queried against the database showed some homology to hypothetical carbon repression proteins. The protein sequence of *Neurospora crassa* for the gene NIT2 revealed homogeneity with the nitrogen catabolic enzyme regulatory protein in the *Fusarium graminearum* database.

Fig. 6.9. Multiple Alignment of *tri 10* Promoter Sequences

Fig. 6.10. Transcription Factor Binding Sites in the Promoter Sequence of *tri 10* (match highlighted)

>AY134892 *F. culmorum*

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ACCGAAGGCGAGCTTGGAAGTATGTTTTGCGGGTACGGATACTCGTTTGG      50
AGAATGGTGGTCTGTTATAATGATTACAAATAGTTTGGTCGTGTTTTGTT     100
AGAATGAACAGTTGAACAAGGATAATTACTTCGGAATAGGCAGTTGAAAC     150
TGAATGTCCGTATGTAACCTGAGCCTGTAACCATTTCCCACTCGAGTGCA     200
GGCTTTTGCGTAACCAAGTCTGTACACCCGTCGGTGCGACAGGGCTACCC     250
CCAACCCTGCAACTGCAGCTGCAGCTGGCAGCCTGGTAGACTGGCGCTAC     300
GAACTGTAGTGCGATGCGGGAATCTTGTACCCGCTCGGAGGTTGGAGGGA     350
GCTGGCACATTCTCTAGACCCGCGAATTGATCTTCAAAGCGCTTGCGTTT     400
TGTCCAGGTCAGTGACCATACCCTTGCCTTTCCGCACCACCCAAACGTCC     450
ACTGAACGAGGCGTACAGAAACCACACAAGATAAGGTTTAATGCCTGCTT     500
GAGCACTATGAGGGACACGACACTTCTGTAAAACTCTATCCTTGCATTAT     550
ATTGTAACATCGTTTAACTTCTCCACGCCCATTCCTTCCCGTCTTTCCCC     600
CAACCTCAATTGTATGCCAACCAACAATCATCAAACTATCATTATTATTG     650
TCGTTAGTCATCATGGATTTCCCAAAGCCT                         680
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>AY359360 *F. sporotrichioides*

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GTGACCGAAGGTGGTTTGGAAGTATGTTTTGCGGGTACTCGCTAGGAGAA      50
TACTGGCCATTTATCATGATTACAAATAGCTTGGTTTTGTTTTTTATTAG     100
TCTAGAATGTACGGTTGAACAAGGATAATTACTACAGGATAGGCAGTGTG     150
AACTGGTTTTTGAAACCCGAGCCTGTAAGCATCCCACTTGACTGCAGGCT     200
TTTGCATGGCTGCCTAGGTTATACCTGTTACGGTCTCAGTGCGACAGGGC     250
TATCCCGGCTCTGCGCCGCAGTAGCCGCTCTGTAGTGTGGTGCGGGAATC     300
TTCTACCCGATCGGAGGCTGGGGAACTTGTTTTACACCGAGTTTACGCAT     350
TCCAGCCGTGTCTTCTTCAAATCGCTGACCTAGATCCATGTCTACTTGTT     400
CCATCTAACGTTCATTGAACAAGGCGTACAGAAACCGCACCAAGTAAAGT     450
CTCATGCCCGCTCAACCACCACTGGGTACACGGCACATCTGTTAAACTCT     500
ATCCTTGCATTATATTGTAACATCGCCTAACTTCTCCACGCACTATTCTT     550
TCGTGTTTTTTATCCAACCTCAATTGTATACCACCCAGCAATCATCAGAT     600
TACTATTTCTGTTAGTCATC                                   620
```

AATTG: MIG1 binding, TATCAT: NIT2 binding,

>*AB060689. F. graminearum*

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ACCGAAGGCGAGGTTGGAAGTATGTTTTGCGGGTACTCGTTTGAAGAATA      50
CTGGTCTGATTAGTTATAGAATGATTACAAATAGTTTGGTTGTGGTTTTG     100
TCGTAATATACATACAGTTGAACAAGGATAATTACTAGACTAGATACCTA     150
CGGAATAGGCAGTTGAAACTGAATGGCTGTATGTAACCTGAGCCTGTAAC     200
CATTTCCCACTCGAGTGCAGGCTTTTGCGTAACCAAGTCTGTACACCCGT     250
CGGTGCGACAGGGCTACCCCCAACCCTGCAACTGCATCTGCATCTGCAGC     300
TGGCAGACTGGCGCTACGAATTGTAGTGCGATGCGGGAATCTTGTACCCG     350
CTCGGAAGTTGAAGGTTAGGTCTAGTTTTGTTCCGAGCTGGCACACTTCC     400
TAGTACCGCGAATTGATCTTCAAAGCGCTTGCGTTTTGCCCAGATCAGTG     450
ACCATACCCTTGTCTTTCCGCACCACCCAAACGTCCACTGAACGAGGCGT     500
TCAGAAACCACACAAGATAAGGTTTCATGCCTGCTTGAGCACTATGAGAG     550
ACACGACACTTCTGTAAAACTCTATCCTTGCATTATATTGTAACATCGTT     600
TAACTTCTCCACGCCCATTCCTTCCCGTTTTTCCCCCAACCTCAATTGTA     650
TGCCAACCAACAATCATCAAATTATCATTATTATTGTCGTTAGTCATCAT     700
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>AY102574 *F. cerealis*

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GAGTGACCGAATGCGAGTTTGGAAGTATGATTTGCGGGTACTCGTTACAT      50
AGGAGAAATACTGGTCTGTTATAATGATTACAAATAGCTTGCTTGGTTGT     100
GTTTTTGTTACATAGAATGTACGGTTGAACAAGGATAATTACCACGGAAT     150
AGGCAGCTGAAACTAATTGTCTGTAACCTGAGCCTGTAACCATTTCCCAC     200
TCGAGTGCAGGCTTTTGCGTAACCAAGTTACACCCGTCGGTGCGACAAGG     250
CTACCCCCGAACCCTGCAACTGTAGCTGCAGCTGGCAGAATGGTAGACCG     300
GTAGACTGGCGCTACGAACTGTAGTGCGATGCGGGAATCTTGTACCCGCT     350
CGGAGGTTGGAGGGAGCTGGCACATTCTTTAGACCCGCGAATTGATCTTC     400
AAAGCGCTTGCGTCTTGTCCAGATCAGTGACCACACCCTTGTTTTTTTCG     450
CACCACCCAACGTCCACTGAATGAGGCGTACAGAAACCACACAAGATAAA     500
GTTTAACGCCTGCTTGAGCGCTGTTGACGGACACGACAATCCTGCAAAAC     550
TCTATCCTTGCATTATATTGTAACATCGTTTAACTTCTCCACGCCCATTC     600
CTTCCCGTTTTCCCCCAACCTCAATTGTATGCCGACCAACAATCATCAAA     650
TTATTATTATTGCCGTTAGTCATCATGGAT                         680
```

MSN4 binding site is absent

6.4. Conclusions

Truncated fragments of promoter sequences spanning a distance of 1200 and 800 bp upstream from the *tri 5* start site was ligated in fusion with GUS in pCAMBIA1304 and were introduced into the isolate ICR-PQ-12 (*F. nelsonii*) following *Agrobacterium* mediated transformation. Qualitative GUS assay resulted in the staining of *Fusarial* hyphae with the characteristic blue colour in transformants alone. *In silico* analysis of the promoter sequences of *tri 5*, *tri 6* and *tri 10* from different species of *Fusarium* resulted in the identification of *cis* acting elements namely MSN4, MIG1 and NIT2 that are specifically involved in nutrient utilization. The absence of MSN4 binding motif in promoter for *tri 10* was noted. Further laboratory studies have to be carried out to identify the role of nutrients on the activation of promoters of genes involved in trichothecene production in *Fusarium*.

Chapter 7.

Future Perspectives

Future Perspectives

In the course of this study entitled "Molecular Approaches to the Study of Trichothecene Production in *Fusarium* Species", many observations were made. These observations and a survey of the literature have pointed out directions that future research may take up. Some of these are listed below.

1. A highly specific set of primers were developed for the group specific detection of trichothecegenic *Fusaria*. One third of the 167 *Fusarium* isolates obtained from various food materials were identified to be producers of one or more trichothecenes and to contain the *tri 5* gene. The toxin produced by a few of the isolates was profiled in detail and the species to which they belonged identified. This study may represent the diversity of *Fusarium* in the country to some extent. Molecular taxonomy involving a greater gene number with isolates from different parts of the country would provide enhanced information on the diversity of *Fusarium* as well as possible migration of the fungi throughout the country.

2. The levels of trichothecenes that were encountered in food and feed commodities in the present study were lower than has been proposed as unacceptable in different parts of the world. The scope of the study initiated here would have to be broadened for continuous and larger scale monitoring of the trichothecenes in Indian food and feed and particularly in infant food formulations.

3. An antibody was developed in poultry using the over-expressed Tri 5 as immunogen. The antibody was successful in discriminating trichothecene producers from the non-producers and was further used for assaying Tri 5 protein expression in relation to trichothecene production under varying nutrient conditions. The antibody may be used for the detection of trichothecene producing fungi in food and feed materials. A study of the expression of the Tri 5 protein may help in the development of improved methods for controlling the accumulation of trichothecene.

4. *In silico* analysis on promoters of the genes *tri 5*, *tri 6* and *tri 10* involved in trichothecene synthesis resulted in the identification of *cis* elements specific for nutrient take up which may be responsible for the differential synthesis of trichothecenes in presence of different sugars and nitrogen sources. Promoter fusion constructs have been made during the course of this work. The reason why the truncated promoter drives GUS expression to a higher level than did the full length promoter needs to be investigated. The effect of the process of infestation on the level of expression of GUS in these constructs would yield valuable information on *Fusarium* biology.

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Appendix

Appendix

Fig. A.1.A. Thin Layer Chromatogram of T-2 toxin

Lane 1: T-2 standard, Lane 2: ICR103, Lane 3: FM553, Lane 4: T-2 standard

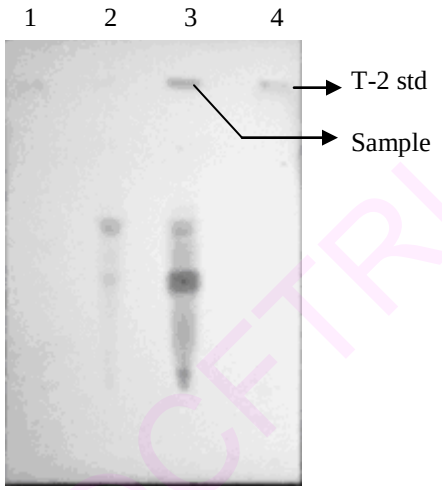


Fig. A.1.B. Thin Layer Chromatogram of DON

Lane 1: FM246, Lane 2: DON standard, Lane 3: ICR61

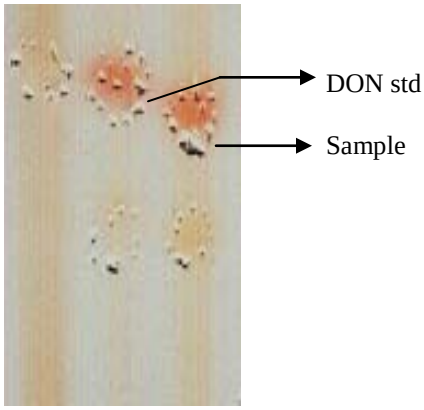
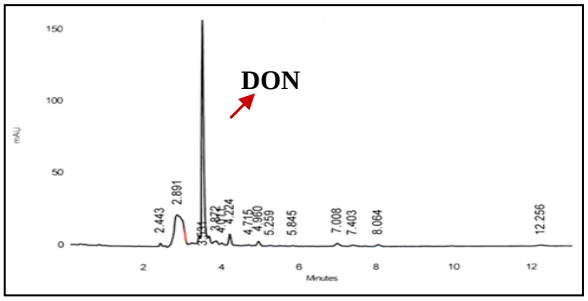
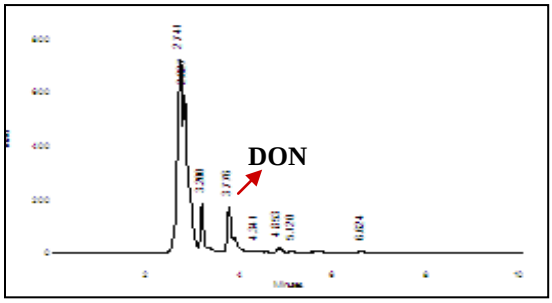


Fig. A.2. HPLC Chromatogram of DON

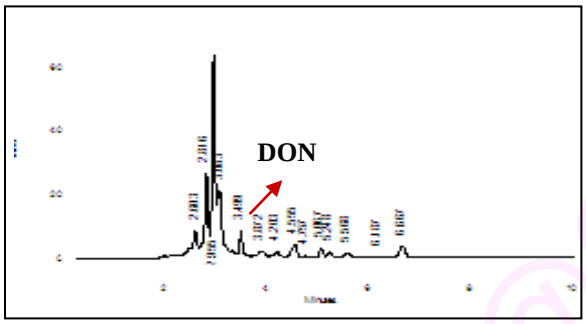
A.2.1. DON Standard



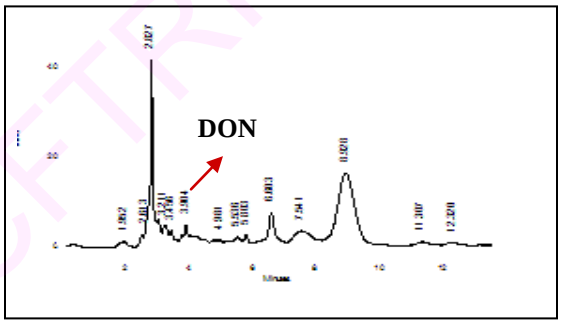
A.2.2. ICR50



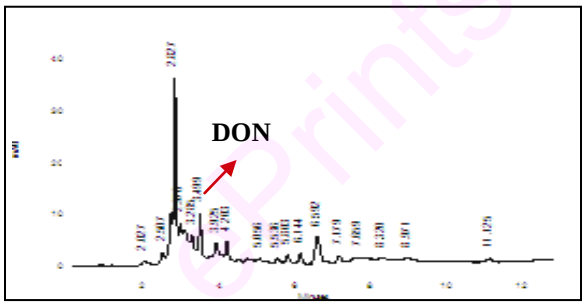
A.2.3. Isolate 1



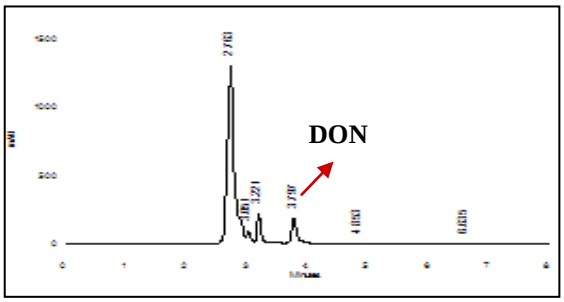
A.2.4. ICR-PQ-13



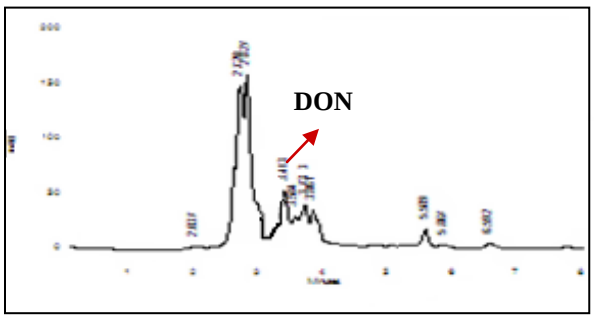
A.2.5. ICR11



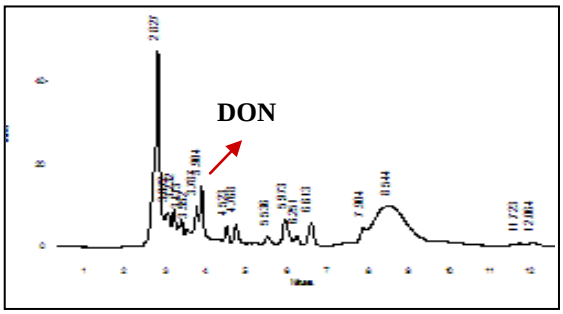
A.2.6. ICR-PQ-9



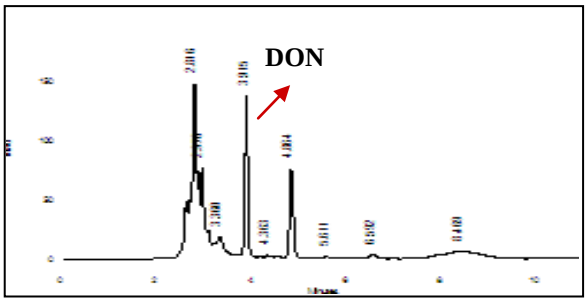
A.2.7. ICR-PQ-2



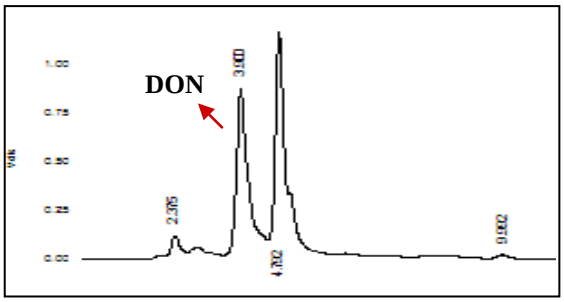
A.2.8. ICR15



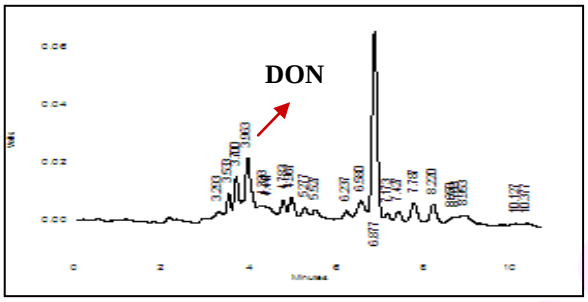
A.2. 9. ICR18



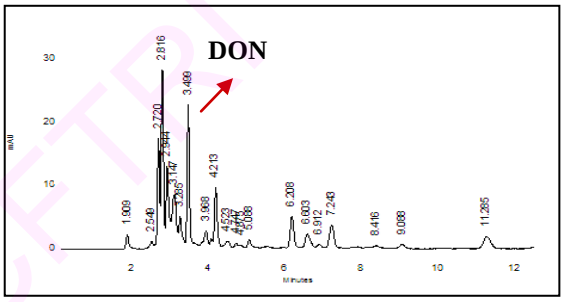
A.2.10. ICR-PQ-4



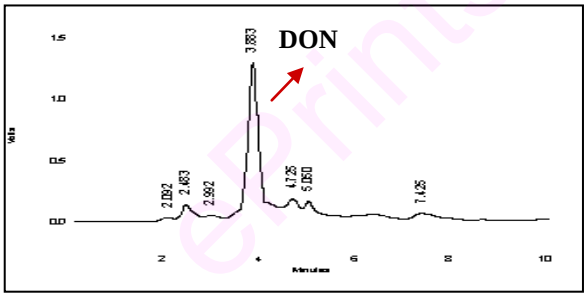
A.2.11. ICR96



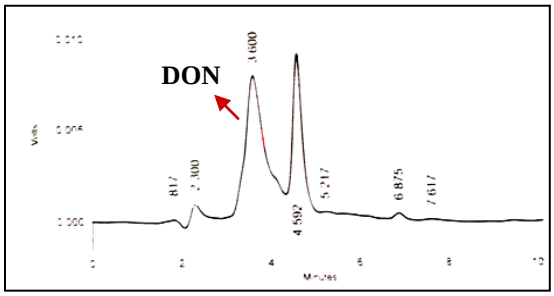
A.2.12. FM302



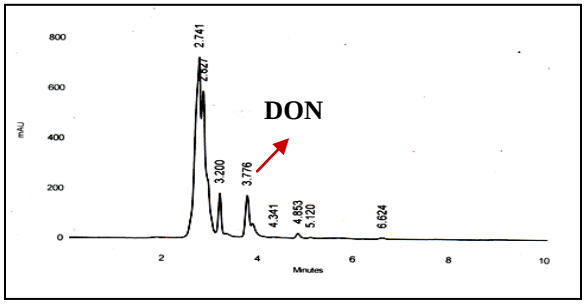
A.2.13. ICR-PQ-15



A.2.14. ICR61



A.2.15. FM306



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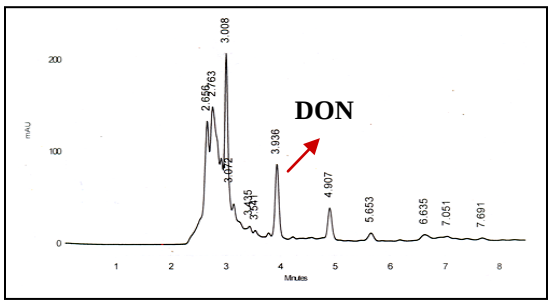
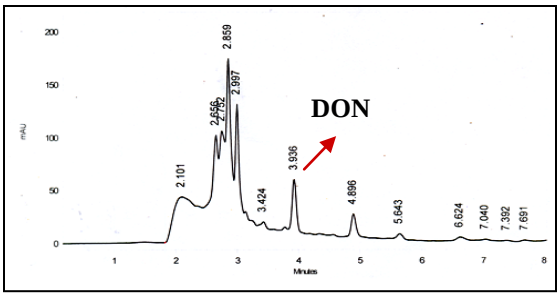
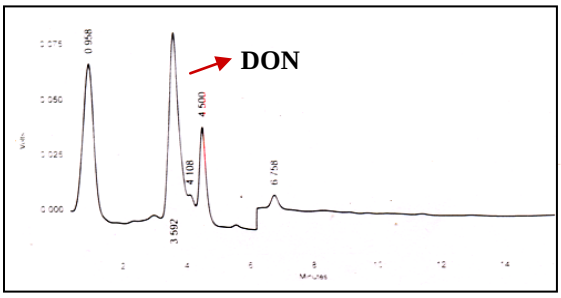


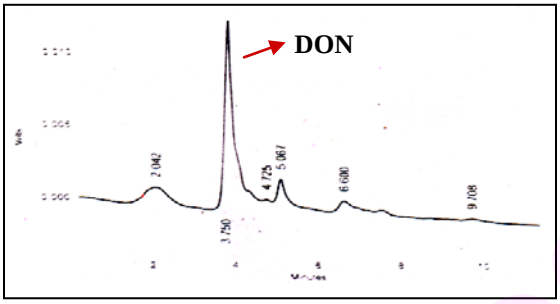
A.2 17. FM247



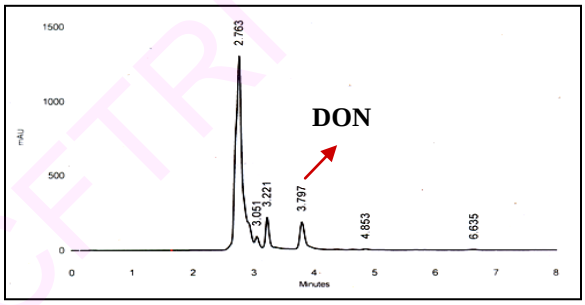
A.2.18. FM246



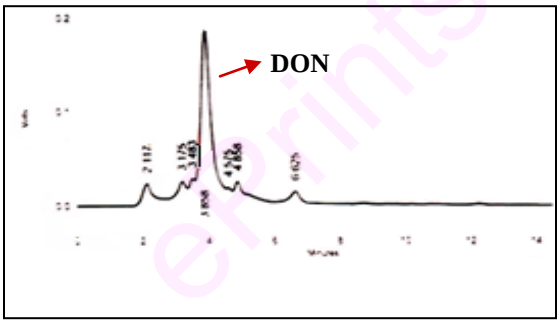
A.2.19. FM006



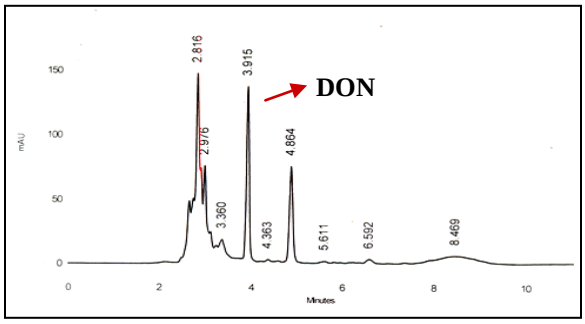
A.2.20. FM311



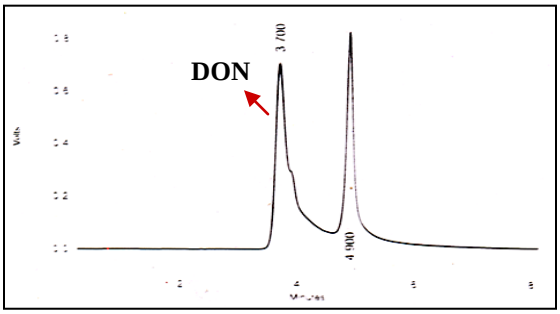
A.2.21. FM245



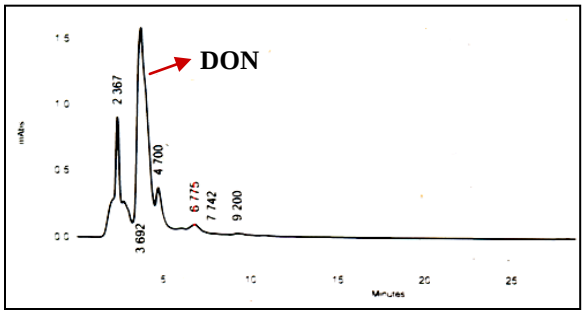
A.2.22. Isolate 6



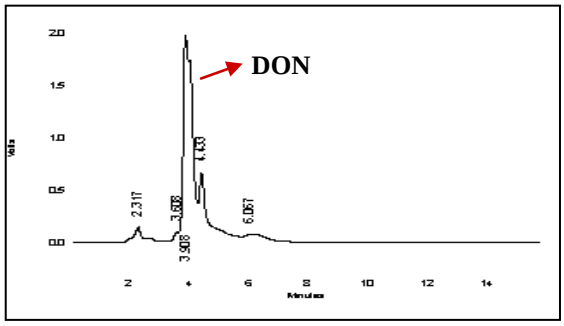
A.2.23. Isolate 2



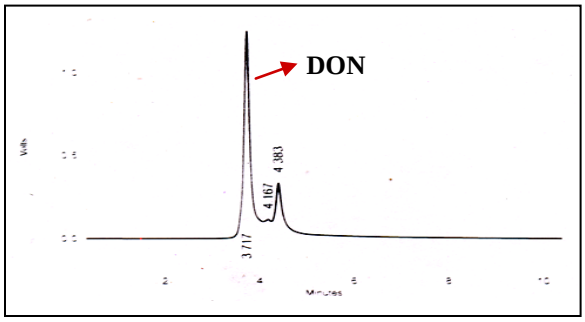
A.2.24. Isolate 3



A.2.25. Isolate 4



A.2.26. ICR-PQ-12



A.2.27. FM242

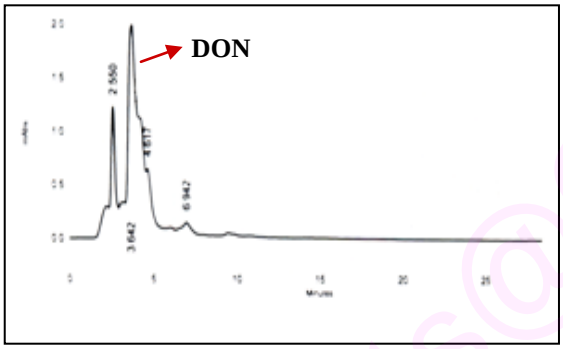
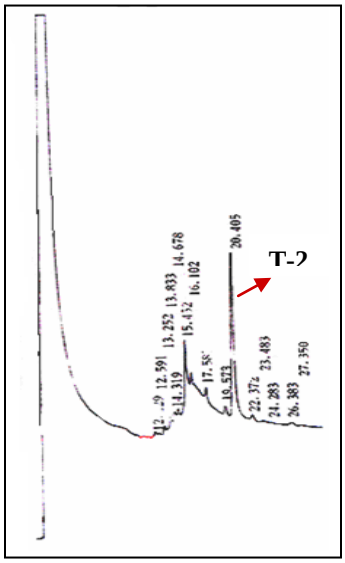
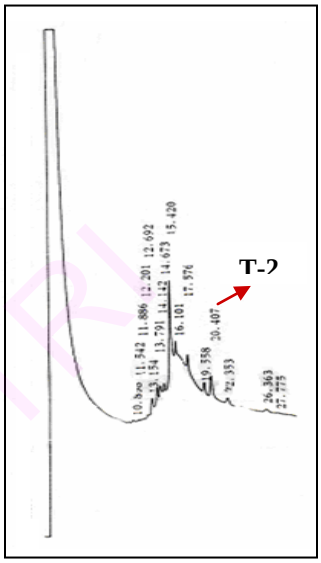


Fig. A.3. GC Chromatogram of T-2 toxin

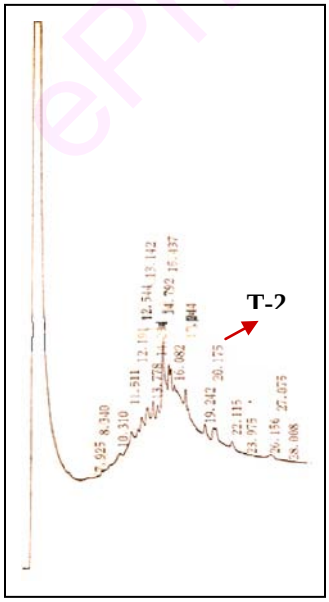
A.3.1. Standard T-2



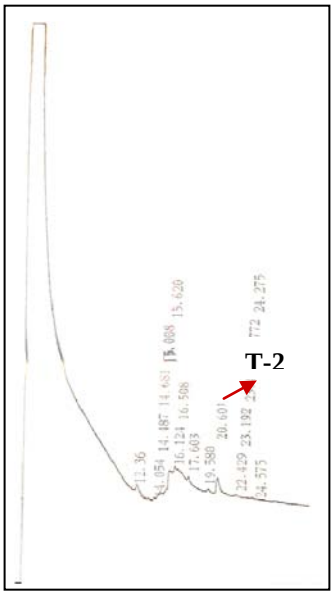
A.3.2. ICR-PQ-10



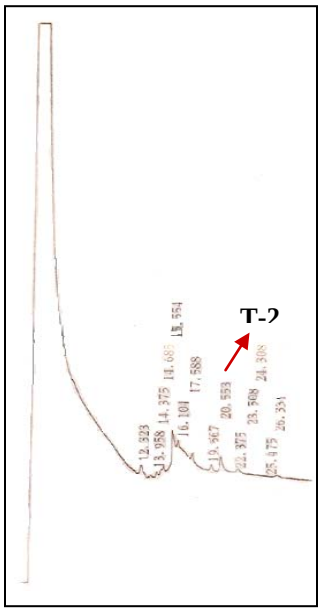
A.3.3. ICR1



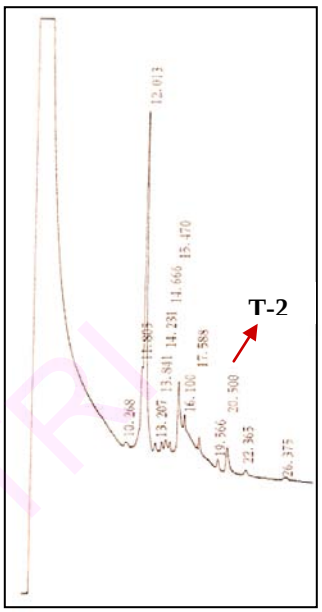
A.3.4. ICR-PQ-11



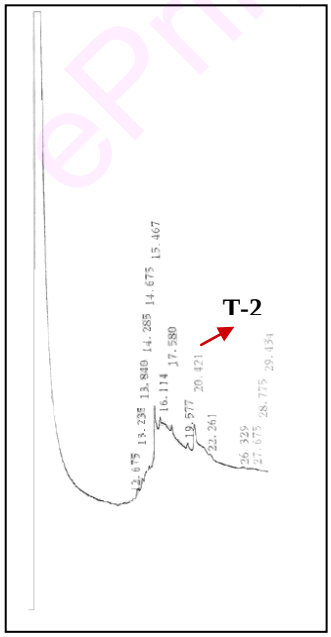
A.3.5. ICR103



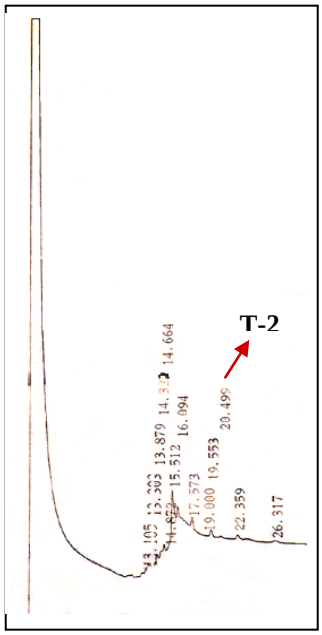
A.3.6. ICR4



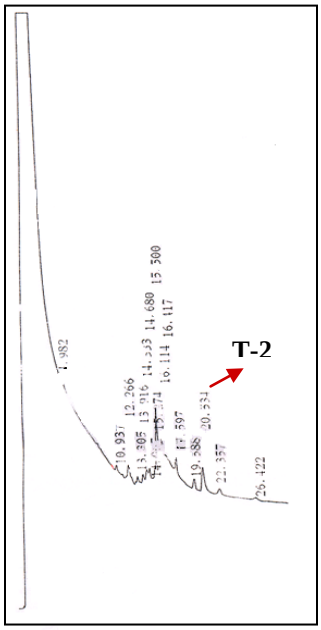
A.3.7. ICR113



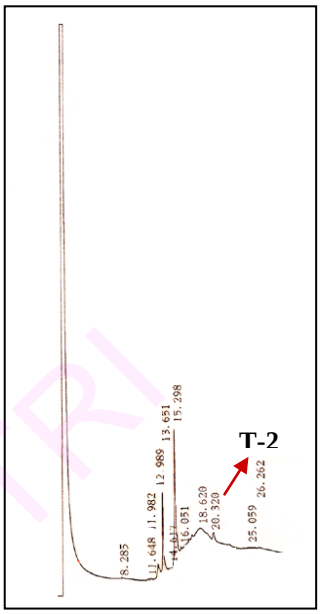
A.3.8. ICR8



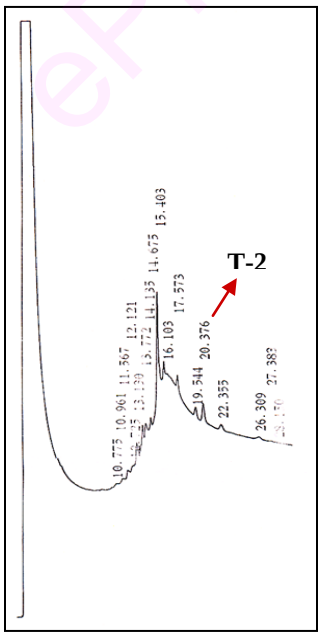
A.3.9. FM553



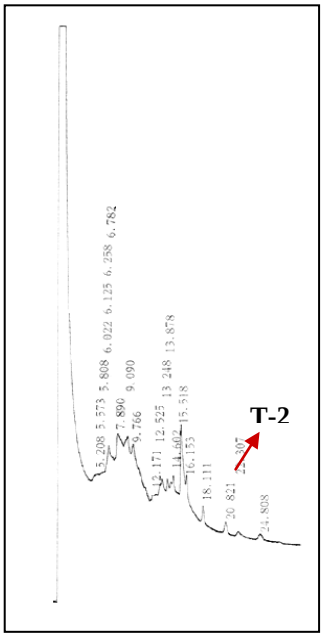
A.3.10. FM556



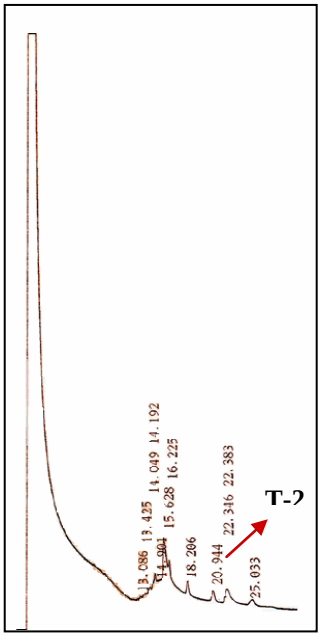
A.3.11. FM307



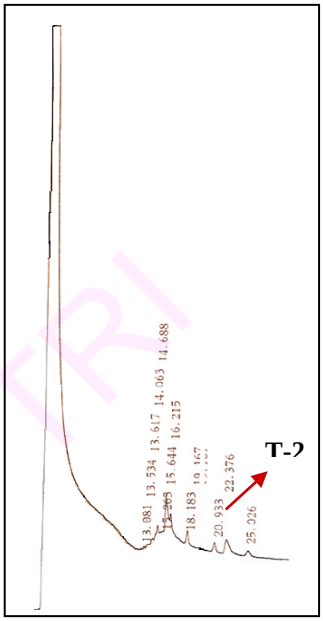
A.3.12. Isolate 5



A.3.13. FM243



A.3.14. FM244



A.3.15. FM299

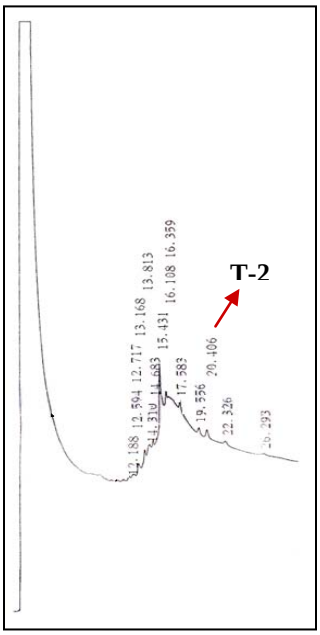
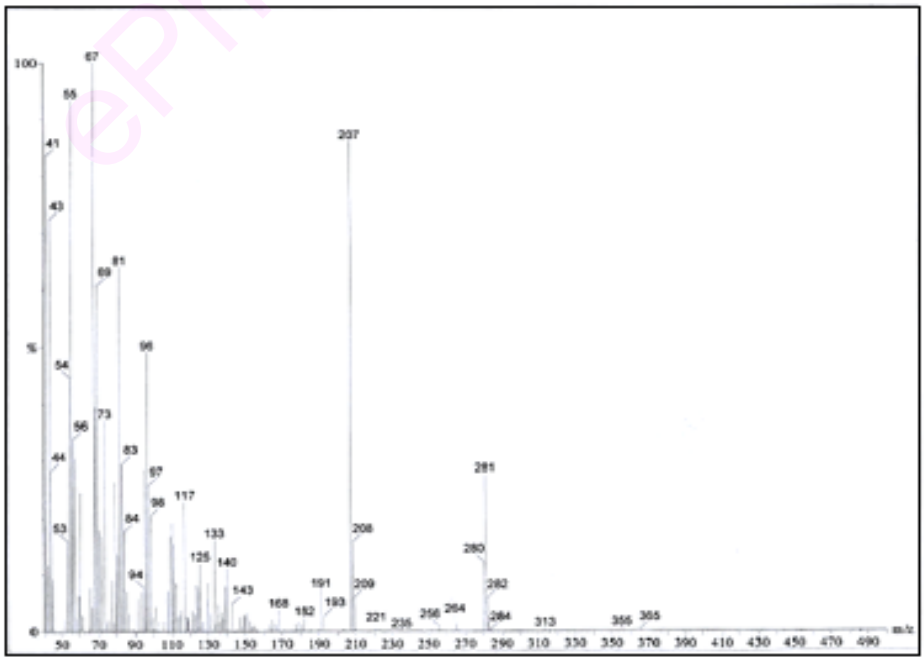


Fig. A.4. Growth of *Fusarium* on Banana Leaf Agar

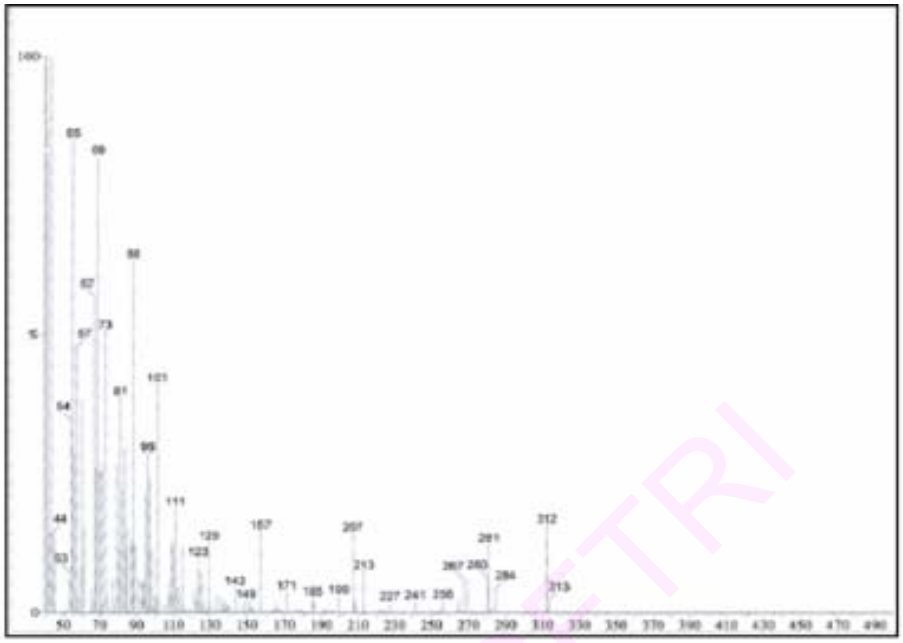
A.5. GC-MS Chromatograms and Mass Spectra of Trichothecenes from *Fusarial* Culture Extracts

A.5.1. DON

A.5.2. DAS



A.5.3. NIV



A.5.4. Fus-X

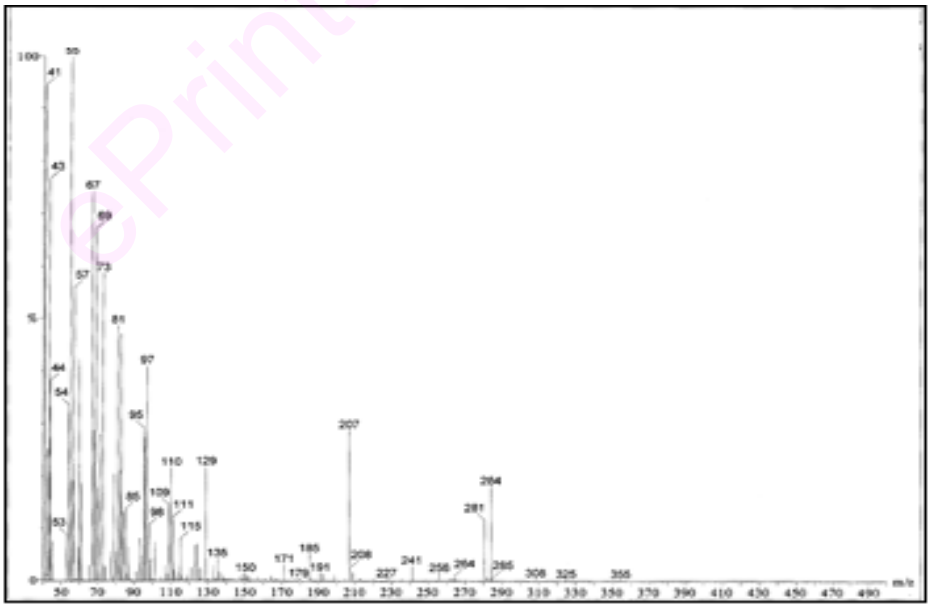


Table A.1. Observations of Morphological Features of the Isolates Obtained as Trichothecene Positives

Sl. No.	Name of isolate	Colony morphology on PDA			Spore morphology after growth on BLA		Group classified
		From above	From below	Pigment production	Macroconidia	Microconidia	
1.	ICR-PQ-10	White turning to pink with age, floccose mycelia	Pink to carmine red	Pigment diffused into media	Not observed	Present (aseptate)	1
2.	ICR-PQ-4	,,	,,	,,	,,	,,	,,
3.	ICR-PQ-11	,,	,,	,,	,,	,,	,,
4.	ICR-PQ-9	,,	,,	,,	,,	,,	,,
5.	ICR103	,,	,,	,,	,,	,,	,,
6.	ICR15	,,	,,	,,	,,	,,	,,
7.	ICR18	,,	,,	,,	,,	,,	,,
8.	ICR11	,,	,,	,,	,,	,,	,,
9.	FM556	,,	,,	,,	,,	,,	,,
10.	FM243	,,	,,	,,	,,	,,	,,
11.	FM244	,,	,,	,,	,,	,,	,,
12.	FM299	,,	,,	,,	,,	,,	,,
13.	ICR-PQ-13	,,	,,	,,	,,	,, (septate)	2
14.	ICR1	Pinkish white, cotton thread like mycelia	-	Red pigment at the agar edges, no diffusion into media	,,	,, (aseptate)	3

15.	FM302	,,	,,	,,	,,	,,	,,
16.	FM303	,,	,,	,,	,,	,,	,,
17.	FM306	,,	,,	Pigment diffused into media	,,	,,	,,
18.	FM006	,,	,,	,,	,,	,,	,,
19.	FM553	,,	,,	,,	,,	,,	,,
20.	FM247	,,	,,	,,	,,	,,	,,
21.	ICR-PQ-15	,,	,,	,,	,,	,,	,,
22.	ICR50	White turning to pink with age, powdery appearance of mycelia	-	-	Present	Present (two types)	,,
23.	ICR62	,,	,,	,,	,,	,,	,,
24.	ICR4	,,	,,	,,	,,	,,	,,
25.	FM307	,,	,,	,,	,,	,,	,,
26.	ICR57	Pinkish white, cotton thread like mycelia	,,	,,	Not observed	Present (aseptate)	5
27.	ICR110(1)	,,	,,	,,	,,	,,	,,
28.	ICR113	,,	,,	,,	,,	,,	,,
29.	FM550	White, turning to violet with age	,,	,,	,,	,,	6
30.	FM311	,,	,,	,,	,,	,,	,,
31.	FM245	,,	,,	,,	,,	,,	,,
32.	FM242	White , powdery appearance of mycelia	,,	,,	,,	,,	7
33.	Isolate 6	White, floccose mycelia	,,	,,	,,	,,	8
34.	ICR61	White, cotton thread like mycelia	,,	,,	Present	Present (two types)	9
35.	ICR106(1)	,,	,,	,,	,,	,,	,,

36.	ICR8	,,	,,	,,	,,	,,	,,
37.	ICR-PQ-12	Pinkish yellow to orange, profuse velvety mycelia	Red	Pigment diffusing into the media	Present	Mesoconidia present	10
38.	Isolate 2	,,	,,	,,	,,	,,	,,
39.	ICR-PQ-2	Yellowish, profuse velvety mycelia	Red	,,	Present	Mesoconidia present	11
40.	FM246	Orangish white, floccose mycelia	-	-	Present	Present (two types)	12
41.	ICR96	,,	,,	,,	,,	,,	,,
42.	Isolate 1	Pinkish white, profuse velvety growth	Pink	Pigment diffusing into the media	,,	Absent	13
43.	Isolate 3	,,	,,	,,	,,	,,	,,
44	Isolate 4	,,	,,	,,	,,	,,	,,
45.	Isolate 5	,,	,,	,,	,,	,,	,,
46.	NCIM, Pune	Pinkish white, profuse velvety growth	Pink	Pigment diffusing into the media	Present	Absent	14

Table 1.2. Toxins Produced by Common *Fusarium* Species

Species	Group*	Toxin produced	Reference
F. cerealis (=*F. crookwellense*)	Discolor	NIV, 4-ANIV, FUS-X, FUS-C	Eriksen, 2003; Moss and Thrane, 2004
F. culmorum	Discolor	DON, ZEN, NIV, FUS-X, FUS-C, 3-ADON, MON	Moss and Thrane, 2004; Sanhueza and Degrossi, 2004
F. graminearum	Discolor	DON, 15-ADON, ZEN, NIV, FUS-X	Eriksen, 2003; Moss and Thrane, 2004
F. psuedograminearum	Discolor	DON, 3-ADON, ZEA	Moss and Thrane, 2004; Leslie and Summerell, 2006; Mishra et al, 2006
F. sambucinum	Discolor	DAS, MAS	Joffe, 1974; Eriksen, 2003; Moss and Thrane, 2004
F. tumidum	Discolor	DAS	Joffe, 1974; Moss and Thrane, 2004
F. venenatum	Discolor	NIV	Moss and Thrane, 2004
F. torulosum	Discolor	WOR	Logrieco et al, 1998; Eriksen, 2003
F. kyushuense	Sporotrichiella	NIV, Type A trichothecenes	Geiser et al, 2004; Moss and Thrane, 2004; Torp and Nirenberg, 2004
F. sporotrichioides	Sporotrichiella	T-2, HT-2, DAS, NEO, FUS-C, MON	Eriksen, 2003; Moss and Thrane, 2004; Sanhueza and Degrossi, 2004;
F. poae	Sporotrichiella	DAS, MAS, NIV, FUS-X, T-2, HT-2, FUS-C	Joffe, 1974; Eriksen, 2003; Moss and Thrane, 2004
F. tricinctum	Sporotrichiella	FUS-C, T-2, NEO	Burmeister, 1971; Ueno et al, 1975; Eriksen, 2003; Sanhueza and Degrossi, 2004
F. chlamydospoum	Sporotrichiella	MON	Sanhueza and Degrossi, 2004; Leslie and Summerell, 2006
F. acuminatum	Gibbosum	T-2, HT-2, DAS, MAS, MON, NEO	Eriksen, 2003; Sanhueza and Degrossi, 2004

F. equiseti	Gibbosum	ZEN, FUC, MON, DAS, DON	Joffe, 1974; Molto et al, 1997; Moss and Thrane, 2004; Sanhueza and Degrossi, 2004
F. armeniacum	Gibbosum	T-2, HT-2	Nagy and Hornok, 1994; Moss and Thrane, 2004
F. semitectum (=*F. incarnatum*)	Arthrospriella	ZEN, DAS, T-2	Rukmini and Bhat, 1978; Molto et al, 1997; Eriksen, 2003
F. nelsonii	Arthrosporiella	Not known	Marasas et al, 1998; Leslie and Summerell, 2006
F. musarum	Arthrosporiella	T-2, HT-2, DAS	Marasas et al, 1998; Moss and Thrane, 2004
F. oxysporum	Elegans	WOR, MON, NIV, FUS-X	Joffe, 1974; Lee et al, 1986; Abbas et al, 1988; Eriksen, 2003
F. verticillioides (=*F. moniliforme*)	Liseola	FUM, FUS-C, Fusaric acid, DON	Joffe, 1974; Ramakrishna et al , 1989; Eriksen, 2003
F. proliferatum	Liseola	FUM, MON, FUS-C	Eriksen, 2003; Leslie et al, 2005
F. globosum	Liseola	FUM	Fandohan et al, 2003; Geiser et al, 2004
F. nygamai	Liseola	FUM. MON	Fandohan et al, 2003; Sanhueza and Degrossi, 2004
F. dlamini	Liseola	FUM. MON	Fandohan et al, 2003; Sanhueza and Degrossi, 2004
F. anthophilum	Liseola	FUM. MON	Fandohan et al, 2003; Sanhueza and Degrossi, 2004
F. napiforme	Liseola	FUM. MON	Fandohan et al, 2003; Sanhueza and Degrossi, 2004
F. thapsinum	Liseola	FUM	Fandohan et al, 2003; Geiser et al, 2004; Moss and Thrane, 2004
F. subglutinans	Liseola	FUM, MON, FUS-C, Fusaric acid	Labuda et al, 2003; Geiser et al, 2004; Sanhueza and Degrossi,2004
F. ramigenum	Liseola	MON, FUM	van Hove and Munaut, 2002; Geiser et al, 2004; Leslie and Summerell, 2006;
F. brevicatenlatum	Liseola	FUM,	Geiser et al, 2004; Leslie and Summerell, 2006
F. sacchari	Liseola	T-2	Štyriak et al, 1994; Geiser et al, 2004;
F. avenaceum	Roseum	MON, FUS-C	Joffe, 1974; Eriksen, 2003
F. solani	Martiella	T-2, NEO, HT-2, DAS, FUS-X	Ueno et al, 1975; Sanhueza and Degrossi, 2004

ADON: 3- and 15-acetyldeoxynivalenol, DAS: diacetoxyscirpenol, DON: deoxynivalenol, FUC: fusarochromanone, FUM: fumonisins, FUS-C: fusarin C, FUS-X: fusarenon X, MAS: monoacetoxyscirpenol, MON: moniliformin, NEO: neosolaneol, NIV: nivalenol, ANIV-acetyl nivalenol; WOR: wortmannin, ZEN: zearalenone
*The groups are discussed in section 1.4.1. Conventional taxonomic identification based on morphology.

Table 1.7. Trichothecene Levels Detected in Different Commodities Worldwide

Sl. No.	Country	Food/Feed	Trichothecene levels detected (mg/kg)					References
			T-2	HT-2	DAS	DON	NIV	
1.	Poland	Wheat/bran			0.05	2-40	0.01	Perkowski et al, 1990
		Barley	Upto 2.4	Upto 0.37		av. 0.12		Perkowski et al, 1997b
		Oats	0.302	0.02	0.02		0.056	Perkowski et al, 1997a
								Goliński et al, 1997
		Maize				4-927		Grabarkiewicz-Szczesna et al, 1996
2.	Germany	Wheat bran	0.006	0.005-0.033		0.319-0.389	0.021-0.065	Schollenberger et al, 2005a
		Wheat	0.006	0.005-0.027		0.031-0.095	0.021-0.03	
		Corn	0.006	Upto 0.026		0.015-0.227	0.021	
		Oats	0.006-0.12			0.011-0.148		
		Maize				Upto 0.5		Obst et al, 1997
		Bread	0.004	0.012		0.134	0.025	Schollenberger et al, 2005b
		Barley				Upto 4.76		Müller and Schwadorf, 1993
3.	Bulgaria	Wheat	0.055			av. 1.8		Vrabcheva et al, 1996
4.	Finland	Oats				1.3-3.6		Hietaniemi and Kumpulainen, 1991
		Feeds /grains				0.007-0.3		
		Rye				0.005-0.05		Bottalico and Perrone, 2002
5.	Norway	Wheat				0.45-4.3	Max. 0.054	Langseth and Elen, 1996
		Barley				2.2-13.33	Max. 0.77	
		Oats				7.3-62.05	Max. 0.67	

6.	Netherlands	Wheat				0.020-0.231	0.007-0.203	Tanaka et al, 1990
		Barley				0.004-0.152	0.03-0.145	
		Oats				0.056-0.147	0.017-0.039	
		Rye				0.008-0.384	0.016-0.034	
7.	Italy	Soft wheat				0.055-0.33		Pascale et al, 2001
		Hard wheat				0.06-1		
		Barley				0.07-1.54		
8.	Austria	Durum wheat				Upto 8.2		Adler et al, 1995
9.	Czech Republic	Barley				< 2	> 2	Bottalico and Perrone, 2002
10.	Japan	Wheat				0.029-11.7	0.01-4.4	Yoshizawa, 1997
		Barley				61-71	14-26	
11.	Russia	Wheat				7.25-36.25	0.16-1.25	Leonov et al, 1990
12.	New Zealand	Maize				Max. 3.4-8.5	Max. 4.4-7	Lauren et al, 1996
13.	Canada	Wheat				0.01-10.5		Scott, 1997
		Maize				0.02-4.09		
		Wheat / Barley	0.16-0.31	0.12	0.11	Upto 0.5	0.09-0.81	Stratton et al, 1993
		Animal feed				0.013-0.2	0.065-0.311	Abramson et al, 1997
14.	Argentina	Wheat				0.1-9.25		Pacin et al, 1997
15.	China	Maize				0.49-3.1	0.6	Wang et al, 1995a
16.	Korea	Barley				0.005-0.361	0.04-2.038	Ryu et al, 1996
		Maize				Mean 0.145	Mean 0.168	
17.	USA	Wheat				< 0.1-18		Trucksess et al, 1995

		Barley				< 0.5-26		
18.	France	Corn				0.14-0.6	1.18-4.28	Jelinek et al, 1989
		Wheat				0.02-0.11	0.02-0.14	
19.	Ethiopia	Sorghum				0.04	Upto 0.49	Ayalew et al, 2006
		Wheat				0.04-2.34		
20.	Nigeria	Maize			0.001-0.051	0.009-0.745		Adejumo et al, 2007
21.	Croatia	Feed	0.05-3.4		0.05-3.4	0.05-3.4		Sokolović and Šimpraga, 2006
22.	Slovakia	Poultry feed	av. 0.013	av. 0.018		av. 0.303		Labuda et al, 2005
23.	Zambia	Animal feed				Up to 1		Siame and Lovelace, 2006
		Maize				0.5-16		
24.	Brazil	Wheat	0.04-0.8		0.6	0.47-0.59	0.16-0.4	Furlong et al, 1995
		Corn	0.555	0.767				Milanez et al, 2006
25.	India	Wheat	0.55-0.8			0.346-8.38	0.03-0.4	Bhat et al, 1989
		Maize	20-40			17-21		Janardhana et al, 1999
		Peanut	Upto 38.89		Upto 2.03			Bhavanishankar and Shantha, 1987
		Sorghum	0.064-0.012		0.084-0.014			Lincy et al, 2008 (This study)
		Poultry feed	0.13					

Publications

1. **S .V. Lincy**, R. Latha, A. Chandrashekar and H.K. Manonmani, 2008. Detection of toxigenic fungi and quantification of type A trichothecene levels in some food and feed materials from India. Food Control 19: 962-966.
2. **L. S. Varghese**, A. Chandrashekar, H. K. Manonmani and M. S. Narayan, 2008. Detection of trichothecene producing *Fusaria* isolated from various food materials from India (To be Communicated)
3. **S. V. Lincy** and A. Chandrashekar, 2008. Expression of trichodiene synthase by different *Fusaria* under different growth conditions (manuscript under preparation)

Papers presented in Symposia

1. **Lincy, S. V**., Deepak, C. A., Reeta Davis, Anilkumar, P. K., Prakash, M. H. and Chandrashekar, A. 'Application of PCR and PCR Biosensor for Rapid Analysis of Microorganisms Associated with Food' at International workshop on Biosensors, held at Central Food Technological Research Institute (CFTRI), Mysore on 11-13 August 2003.
2. **Lincy, S. V.**, Deepak, C. A., Chandrashekar, A. and Rati Rao, E.'Rapid detection of Mycotoxin producing *Fusarium*' at International Symposium on Molecular Toxicology and Environmental Health, held at Industrial Toxicology Research Centre (ITRC), Lucknow on 5-8 November 2003
3. **Lincy, S. V.**, Chandrashekar, A. and Rati Rao, E. 'PCR Detection of Trichothecene Producing *Fusarium* Species' at the 6th Indian Convention of Food Scientists & Technologists (ICFOST), held at Defence Food Research Laboratory (DFRL), Mysore on 9-10 December 2004.
4. **Lincy, S. V**., Chandrashekar, A. and Rati Rao, E. 'Identification of Trichothecene Producing Chemotypes of *Fusarium* from Sorghum' at the 46th Annual Conference of Association of Microbiologists of India (AMI), held at Osmania University, Hyderabad on 8-10 December 2005.
5. **Lincy, S. V.** and Chandrashekar, A. 'Potential Trichothecene Producers in Food Samples from Mysore Market' at the National Symposium on Sensors and Instrumentation for Food Processing (NSSIFP-2006), held at CFTRI, Mysore on 20-21 January 2006.
6. **Lincy, S. V.** and Chandrashekar, A. 'Cloning and Expression of *tri 5* in *E. coli* and Purification of the Protein for Raising Antibodies in Poultry' at the 4th Annual Conference of the Biotechnologists of India (Biotech-2006), held at Centre for Cellular and Molecular Biology (CCMB), Hyderabad during 26-28 November 2006.
7. **Lincy, S. V.**, Chandrashekar, A. and Manonmani, H. K. 'Generation of Chicken Antibodies against Tri 5 and their Characterization for the Detection of Trichothecegenic Molds' at the 75th Annual Conference of Society of Biological Chemists of India (SBC-I), held at Jawaharlal Nehru University, Delhi during 8-11 December 2006.