Screening of genes encoding for dicer like protein (*Dcl-1* and *Dcl-2*) in *Aspergillus flavus* and their possible role in aflatoxin biosynthesis

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CERTIFICATE

This is to certify that the project report entitled "**Screening of genes encoding for dicer like protein (*Dcl-1* and *Dcl-2*) in *Aspergillus flavus and their possible role in aflatoxin biosynthesis*** " is an authentic record of work done for a month from August 2014 to May 2015 by Ms. Vertika Bajpai student of UG Biotechnology B.tech. VIII semester in the Department of Biotechnology and Bioinformatics of this University**.**

Signature of Supervisor:

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I express my gratitude to the almighty for blessing in successive completion of this dissertation. I take this golden opportunity to express my gratitude and sincere thanks to our respected research guide **Dr. Jata Shankar** Assistant Professor, Department of Biotechnology and Bioinformatics Jaypee University Of Information and Technology, Waknaghat for his constant and valuable guidance, encouragement and support which boosted our confidence.

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Signature of the Student:

Name of Student: Vertika Bajpai

Date: 27/05/15

SUMMARY

Aspergillus flavus is major producer of aflatoxin, is involved in contamination of agronomically important crops like peanuts, corn and maize. Aflatoxins are carcinogenic and after entering body, they are metabolized by liver to reactive epoxide intermediate which affects the liver.

Presence of *DCL-1* and *DCL-2* genes was screened in two fungal isolates MTCC-AF 9367 which is aflatoxin producer and MTCC-11580 which is non aflatoxin producer grown at two temperature to establish relationship between tempertaure and aflatoxin production and expression of these genes. Primers specific to both these genes were used for seeing the differential expression using semi quantitative PCR.

It was seen that DCL-1 was expressed differentially in MTCC-AF9367 grown at 30 °C(temperature which supports the production of aflatoxin) and DCL-2 expression was difficult to differentiate in MTCC-AF9367 grown at 30°C and 37°C but it was more in MTCC-11580 at 37°C giving us insight that DCL-1 is associated with aflatoxin producing strain and DCL-2 was associated with both MTCC-AF9367 and MTCC-11580.

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INTRODUCTION

Aspergillus is a genus which contains hundreds of mold species found in varied climates.It was first characterized and catalogued in 1729 by the **Italian priest** and biologist **Pier Antonio Micheli**.Aspergillus molds are found throughout the world and are the most common type of fungi in the environment. About 16 species of *Aspergillus* molds are known to be dangerous to humans, causing disease and infection[1].

Table 1. Morphological characteristics of colony (Aspergillus species).

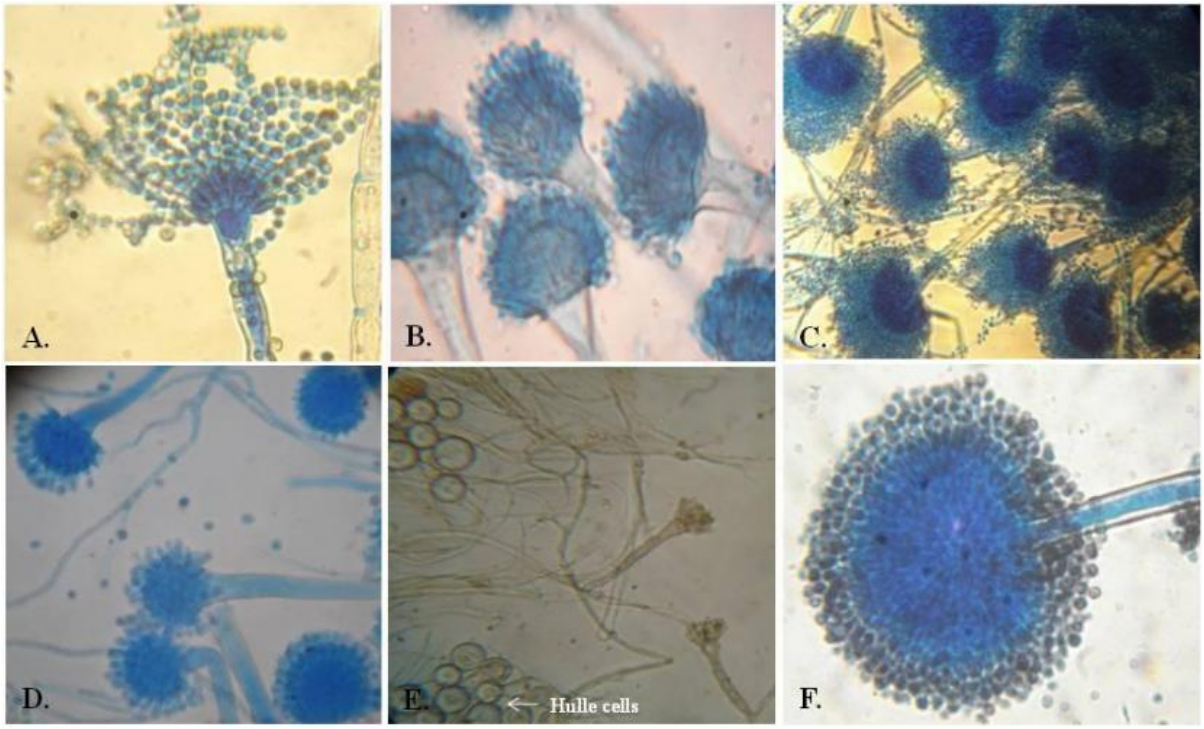
Species	Surface colour	Margins	Reverse side	Elevations	Growth
A.niger	Dark brown to black	Entire	Without colour	Umbonate	Rapid
A.flavus	Yellowish/greyish green	Entire	Colourless to yellow	Umbonate	Moderate to rapid
A.fumigatus	Green to dark green,becoming black with age	Entire	Colourless to yellow	Umbonate	Rapid
A.terreus	Pinkish cinnamon to deeper with age	Entire	Pale to bright yellow to	Umbonate	Moderate to rapid

			deep brown		
A.nidulans	Dark cress green	Entire	Purplish red,brownish dark with age	Umbonate	Slow to moderate

Table 2. Microscopic characteristics of various Aspergillus species

Species	Hyphae	Conidiophore	Conidia	Phialides
A.niger	Branched septate	Length: 200 to 400μm Diameter: 7 to 10 μm Vesicle:Globose	Heads:Blackish brown Diameter: 2.5 to 4 μm Ornamentation: Exine spiny	Biseriate
A.flavus	Branched septate	Length: 600 to 800 μm Diameter: 15 to 20 μm Vesicle: Globose to subglobose	Heads: Yellow/greyish green Diameter: 2 to 6 μm Ornamentation:Almost green	Biseriate

A.fumigatus	Branched septate	Length: 250 to 300 µm Diameter: 2 to 8 µm Vesicle: Dome shaped	Heads:Blue green Diameter: 2.5 to 3 µm Ornamentation:Roughened	Uniseriate
A.terreus	Branched septate	Length: 150 to 250 µm Diameter: 5 to 8 µm Vesicle: Globose	Heads:Creemish Diameter: 2 to 5 µm Ornamentation:Smooth	Biseriate
A.nidulans	Branched septate	Length: 60 to 150 µm Diameter: 8 to 12 µm Vesicle:hemispherical	Heads:Cinnamon brown Diameter: 3 to 3.5 µm Ornamentation:Almost smooth	Biseriate



Microscopic characters of Aspergillus isolates: (A*) A. flavus*; (B)*A. fumigatus*; (C)*A. terreus*; (D)*A. amstelodami*;(E) *A. nidulans* with hulle cells and; (F) *A.niger[1]*

Growth of Aspergillus *flavus*

It grows by producing thread like branching filaments known as hyphae. Filamentous fungi such as *Aspergillus flavus* are sometimes called molds. A network of hyphae known as the mycelium secretes enzymes that break down complex food sources. The resulting small molecules are absorbed by the mycelium to fuel additional fungal growth. The individual hyphae cannot be seen with naked or unaided eyes and only the dense mycelium can be seen. Normally the conidia of aspergillus flavus appear yellowish green when young but the spores turn a darker green with age. In nature, *A. flavus* is capable of growing on many nutrient sources. It is predominately a saprophyte and grows on dead plant and animal tissue in the soil, therefore the significance of this fungus in nutrient recycling. Growth of the fungus on a food source often leads to contamination with aflatoxin, a toxic and carcinogenic compound. *Aspergillus flavus* is also the

second leading cause of aspergillosis in humans. Commonly aspergillus flavus grows on food items such as seeds of corn, peanuts, cotton, and nut trees in nature. Unlike most fungi, Aspergillus flavus is favored by hot dry conditions. The optimum temperature for growth is 37 C (98.6 °F), but the fungus readily grows between the temperatures of 25-42° C (77-108 F), and will grow at temperatures from 12-48°C (54-118° F). Such a high temperature optimum contributes to its pathogenicity on humans.

On media like potato dextrose agar and yeast extract agar, colonies are granular, flat with radial grooves, yellow at first but quickly becoming bright to dark yellow-green with age. Conidial heads are typically radiate, mostly 300-400 um in diameter, later splitting to form loose columns, but having some heads with phialides borne directly on the vesicle. Conidiophores are hyaline and coarsely roughened, the roughness often being more noticeable near the vesicle. Conidia are globose to subglobose (3-6 um in diameter), pale green and conspicuously echinulate. The two strains MTCC AF67 and MTCC 11580 looks similar when grown on same growth media and two temperature conditions [1,8].

Aspergillus species when grown on Potato dextrose agar.

Infection caused by *Aspergillus Species*

Aspergillosis is the group of diseases caused by *Aspergillus* spores. The amount of *Aspergillus* spores in the air is usually within our natural tolerance levels and hence does not affect our health.Symptoms associated with the disease are:

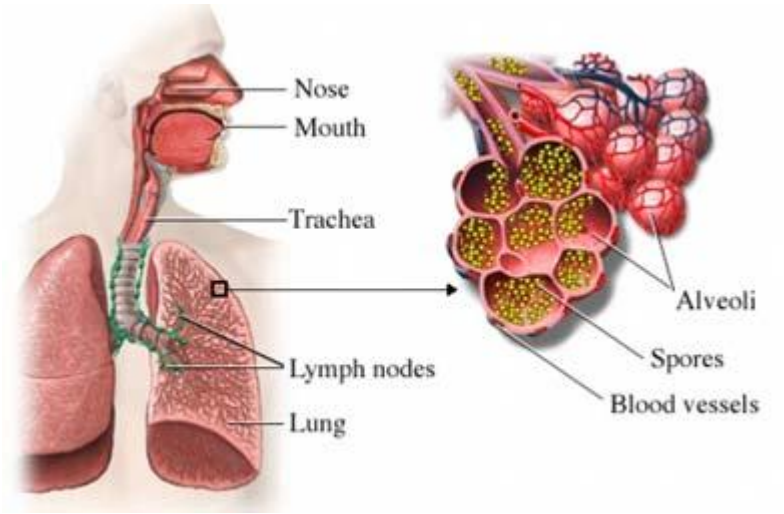
- **Allergic symptoms**: Rseults from exposure to large amounts of *Aspergillus* spores. People with severe asthma or those who are immunocompromised are often sensitive to Aspergillus and can suffer asthma attacks because of the spores.
- **Toxic symptoms**. These are caused by the mycotoxins produced by some species of Aspergillus, especially Aspergillus flavus. One such mycotoxin is aflatoxin, a very potent carcinogen.
- **Infection**. Aspergillus species can infect people and animals and begin to grow inside them, especially in the lungs.People who fall in immunocompromised state are likely to have the infetion.[8,9]

Aspergillosis

Aspergillosis is the name given to group of diseases caused by *Aspergillus* exposure. The main diseases of Aspergillosis are:

- Allergic bronchopulmonary aspergillosis
- Acute invasive aspergillosis
- Disseminated invasive aspergillosis

Allergic Bronchopulmonary Aspergillosis (ABPA)



When a person's immune system is hypersensitive to Aspergillus spores then person is likely to have Allergic bronchopulmonary aspergillosis (ABPA). As a result of this hypersensitivity there are allergic reactions in the person when they are exposed to Aspergillus as the immune system tries to expel the spores from the body. People with cystic fibrosis or asthma are especially vulnerable to Allergic bronchopulmonary aspergillosis, with approximately 5% of asthmatics suffering this disease at some point in their life.

Allergic bronchopulmonary aspergillosis is usually caused by *Aspergillus fumigatus*.

Symptoms:

- Difficulty breathing, shortness of breath and breathlessness
- Wheezing
- Symptoms of asthma, asthma attacks
- Coughing up mucus
- Coughing blood
- Sinusitis (infection or inflammation of the sinuses)
- Loss of appetite

- Fever
- General malaise and feeling unwell
- In later stages can cause lung damage (fibrosis)

Tests for allergic bronchopulmonary aspergillosis can be performed through x-rays, skin tests or blood tests. The treatment for allergic bronchopulmonary aspergillosis is for steroids to be taken through mouth or nasal spray. An antifungal drug called itraconazole can also help to treat the disease in conjunction with steroids.

Chronic Pulmonary Aspergillosis and Aspergillomas.

This disease is caused by Aspergillus infecting the body and growing in cavities in the lungs. These cavities in the lungs would usually have to be created by a previous health problem such as tuberculosis. Once the Aspergillus mold has infected the lungs it begins to grow into a fungal ball (called an aspergilloma or mycetoma) which then makes the person sick because of the allergens or toxins it puts out into the person's body.

At first the symptoms of an aspergilloma might not be noticeable but as time goes on it can show in health problems such as:

- Breathing problems
- Chronic coughing
- Coughing up blood
- Losing weight
- Tiredness
- Fatigue

Aspergillomas can be detected through x-rays or blood tests. Once a person is diagnosed with an aspergilloma it is usually treated through taking drugs such as itraconazole or voriconazole. Anti-fungal drugs may even be injected into the cavity where the aspergilloma is growing in order to

fight it. In some cases surgery might even have to be performed to remove the aspergilloma from the lungs.

Invasive disseminated aspergillosis

Invasive aspergillosis is a major cause of morbidity and mortality in immunocompromised patients. Many cases of pulmonary, cutaneous, cerebral, and paranasal sinus aspergillosis in immunocompetent patient have been defined but disseminated aspergillosis is very rare. However, extra-pulmonary aspergillosis occurs in 25–60% of cases and may involve the central nervous system, liver, skin, and gastrointestinal system[8,9,10].

Identification of Aspergillus *species*

Aspergillus molds have a powdery texture. However the color of the mold's surface differs from species to species and can be used to identify the type of *Aspergillus.* The rate of growth can also be used to identify *Aspergillus,* with most species growing quite quickly. After one week of growth at around 25 degrees Celsius an *Aspergillus* colony will generally be 1-9 cm in diameter, however *Aspergillus glaucus* and *Aspergillus nidulans* grow more slowly and will generally be 0.5-1 cm after the same time[1].

OBJECTIVE

Aflatoxin is found to be most common contaminant of major crop such as corn, peanuts, cotton and other pre/post-harvested crops .It is carcinogenic in nature and is classified as most toxic compound by the International Agency for Research on Cancer (IARC) in 1994. Regular consumption of low level of aflatoxin through contaminated food products causes aflatoxicosis which is characterized by jaundice, rapidly developing ascites etc.

The objective of this study was to screen *DCL-1* and *DCL-2* genes which encodes dicer like protein in *Aspergillus flavus* and their possible role if there is any in aflatoxin biosynthesis. The presence of these genes have been characterized in *Penicillium marneffei* which is phylogenitically close to *A.flavus.* The differential expression of these genes can help in determining the involvement of genes in aflatoxin production as aflatoxin is produced at lower temperature and is seen to be inhibited at higher temperature.

By evaluation of presence of these genes aflatoxin production can be controlled with the help of RNA interference involving genes *DCL-1* and *DCL-2* can help in preventing the contamination caused by the aflatoxin.

REVIEW OF LITERATURE

In filamentous fungi, RNA interference (RNAi) is the most extensively used RNA-based tool for controlled downregulation of gene expression. In this system, doublestranded RNAs (dsRNAs) trigger the degradation of homologous mRNA post-transcriptionally, leading to decreased gene expression. For this purpose, dsRNAs are processed into small interfering RNAs (siRNAs) by the Dicer polypeptide, an RNaseIII-type endonuclease. Subsequently, these 21–26 bp siRNAs are incorporated into the Argonaute-containing RNA-induced silencing complex (RISC) as single-stranded siRNAs for the identification and degradation of complementary mRNAs[5].

MicroRNAs are a class of regulatory small non-coding RNAs, with about 22 nucleotides in length .They possess a seed region at position 2-7 or 2-8 nucleotides sequences from the 5' terminal and is important for target recognition in animals .MicroRNAs are involved in regulation of target gene expression by inducing mRNA cleavage or translational repression in a posttranscriptional manner .There are a variety of small RNAs which also includes the Dicer-dependent miRNAs and the Dicer-independent Piwi-interacting RNAs,(are further involved with Argonaute family proteins to regulate gene expression in diverse cellular processes)[3].

Though extensively catalogued in plants and animals, recent work in filamentous fungi has resulted in identification of eukaryotic small RNAs with the discovery of QDE-2-interacting small RNAs (qiRNAs), microRNA-like RNAs (milRNAs), Dicer-independent small Interfering RNAs (disiRNAs) in N. crassa, endogenous short RNAs (esRNAs) in Mucor circinelloides, and LTR retrotransposon-siRNAs (LTR-siRNAs) and tRNA-derived RNA fragments (tRFs) in Magnaporthe oryzae and Aspergillus fumigatus.

Small RNA molecules being mobile can easily be taken up by cells. When fungal protoplasts were treated with synthetic siRNAs directed toward *Aspergillus flavus* and *Aspergillus parasiticus* aflD (nor-1), it resulted in effectively silencing of gene expression. In Aspergillus nidulans, siRNAs can also be up-taken during spore germination resulting in RNA silencing.Such studies were limited but some of these findings strongly suggest use of synthetic molecules as means to study RNA silencing in vivo as well as to deduce possible role and targets of fungal small RNAs in growth, development and pathogenesis.

Host induced gene silencing (HIGS) has been seen as a promising strategy to control fungal infection. Studies regarding HIGS showed the expression of dsRNA molecules in plants, and further targetting these transcripts resulted in RNA silencing of fungal targets and limited fungal infection[2,4].

Identification of RNAi proteins such as Dicer and Argonaute have been done in many fungi, which include the filamentous fungus *Neurospora crassa* and the fission yeast *Schizosaccharomyces pombe* .

There are two RNA silencing-related phenomena found in the fungus *Neurospora crassa* namely quelling and meiotic silencing by unpaired DNA (MSUD) The protein components (like including RNA-dependent RNA polymerase,argonaute and dicer) used in quelling and MSUD pathways ehxibit high similarity to siRNA and miRNA mechanisms in Drosophilla .There is also presence of orthologs of RNA silencing phenomenon in public databases of fungal genomes directly pointing to the fact that fungi also possess the silencing machinery.

The RNAi pathway of *N. crassa* has been studied extensively.Two pathways has been characterized in this filamentous fungus which are:

1. Quelling pathway:

Quelling is a gene silencing phenomenon identified in *N.crassa* in 1992.Quelling falls in the category of RNA mediated gene silencing mechanisms as per its genetic and biochemical features.It occurs due to transformation with repeated homologous sequences resulting in reversible gene inactivation.

Quelling occurs during the vegetative phase of growth and it affects both transgenes and endogenous genes. There is no requirement of methylation for the process; it is associated though. The process is dominant in heterokaryons with nuclei from ‘‘quelled’’ and wild-type strains, which suggests that a mobile signal, e.g., RNA, acts in trans to cause silencing.

Also a series of studies on quelling-deficient (qde) mutants of N. crassa has indicated the presence of small interfering RNAs in the quelling mechanism.On showing that the qde-1 mutant was defective in an RNAdependent RNA polymerase (RdRP) , implicated existence of RNA components for the quelling pathway and related eukaryotic RNA silencing

pathways. Further support to this was provided by the findings that the SDE1/SGS2 gene in Arabidopsis and the ego-1 gene in Caenorhabditis elegans, both of which encode RdRP, are required for PTGS and RNAi, respectively.Thus, the above genetic evidence suggested that RNA silencing phenomena share evolutionarily conserved machinery.

Similarly, the second qde gene, qde-2 encoded the protein product which was shown to be a piwi-PAZ domain (PPD orArgonaute) protein, is an essential and conserved component of the RNA silencing pathway in a variety of eukaryotic organisms. The third qde-3 encodes a putative RecQ-type DNA helicase. Recently, the Neurospora QDE-3 RecQ helicase and its homologue, RecQ-2, have been shown to play a role in recombination repair, suggesting that QDE-3 RecQ helicases may have a dual role in N. crassa.[3,4,5]

2. MSUD pathway:

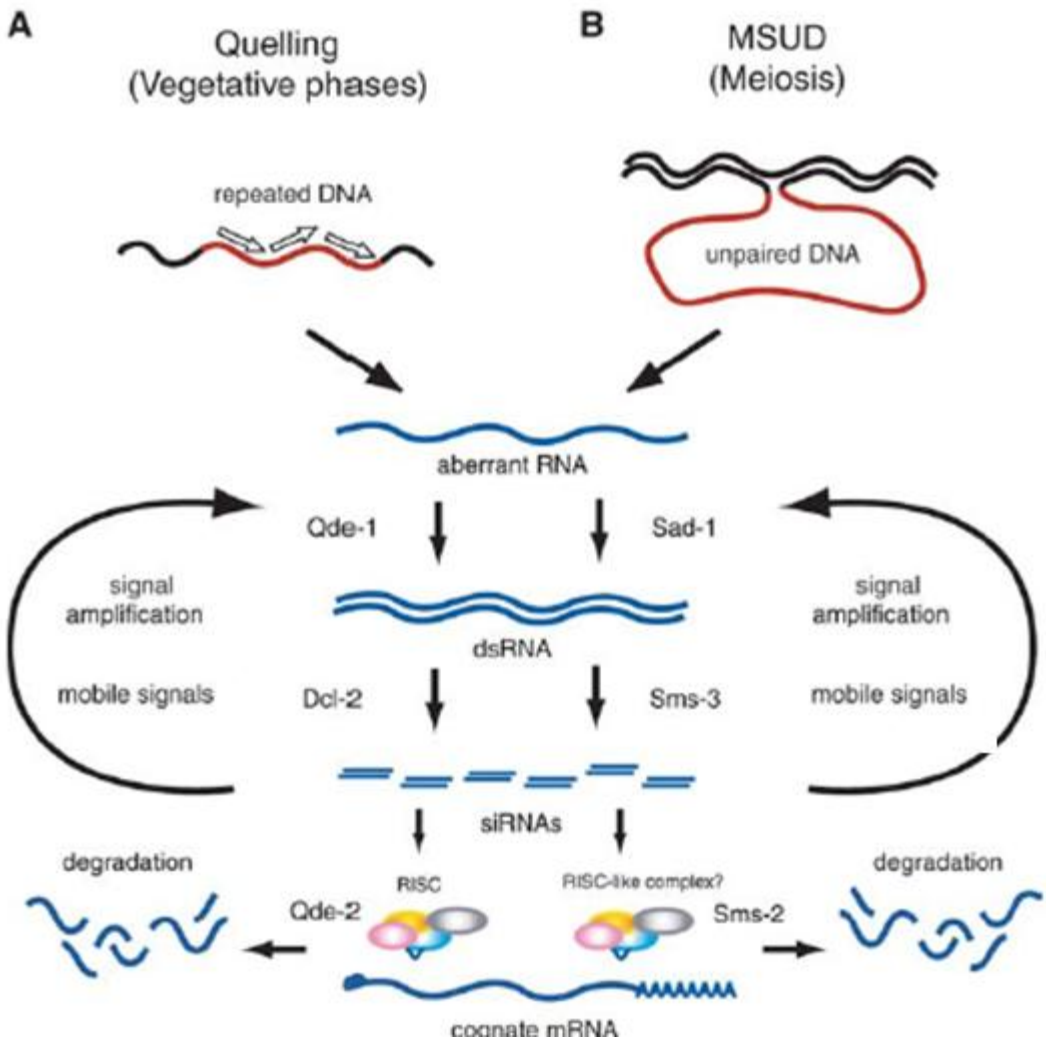
Another RNA silencing-related phenomenon, called meiotic silencing by unpaired DNA (MSUD), from the study on meiotic transvection of the Asm-1 gene, was uncovered in N. crassa. During vegetative growth, N. *crassa* is haploid and a transient diploid cell, when two haploid nuclei of opposite mating type fuse to form the zygote. The zygote formed undergoes meiosis by pairing of homologous chromosomes, followed by post-meiotic mitosis that results in the production of asci with eight haploid ascospores. MSUD abates the expression of genes in one parental chromosome but not in its pairing partner and, therefore, cause unpaired DNA during meiosis.

MSUD affects not only the unpaired copies but also any copy of the unpaired gene in the genome even if the additional copies are paired .This suggested that a mobile trans-acting signal is involved in MSUD as is the case in quelling.

Also one semidominant *Neurospora* mutant, Sad-1 (suppressor of ascus dominance-1) is shown to be deficient in MSUD. This mutation also suppresses the sexual phenotypes of many ascus-dominant mutants that might be caused by failure of meiotic paring, and even complements, albeit partially, the sterility of interspecific crosses that may involve unpaired DNA due to chromosomal variation. Using the UV-induced sad-1 mutant, the Sad-1 gene was isolated and revealed to encode RdRP similar to QDE-1, suggesting that MSUD involves a molecular mechanism similar to RNA silencing. This is consistent with the fact that MSUD is

reversible and involves a mobile trans-acting signal for silencing. The isolation of two additional suppressor loci of MSUD, Sms-2 (suppressor of meiotic silencing-2) and Sms-3 (suppressor of meiotic silencing-3), lend support to this because Sms-2 and Sms-3 encode paralogs of QDE-2 (PPD protein) and DCL-2 (Dicer), respectively Intriguingly,different sets of protein components are required for MSUD and quelling, which indicate that two separate silencing pathwaysexist in N. crassa). In N. crassa, the two dicer- like proteins, DCL-2 and SMS-3 (DCL-1) were reported to be redundantly involved in the quelling pathway. However, DCL-2 appeared to have stronger activity to produce siRNAs in vitro.

In Magnaporthe oryzae (formerly M. grisea), a fungus closely related to N. crassa, it has been shown that one dicer-like protein, MDL-2 (DCL-2 ortholog) is solely responsible for siRNA biogenesis. Therefore, it appears possible that DCL-2 is the primary dicer protein responsible for the quelling pathway in N. crassa even though DCL-1 can compensate when DCL-2 is lost[4,5].



Two silencing pathways in *N.crassa*.

(A) Repeated genome can induce quelling during the vegetative phase.In this, dsRNA produced by Qde-1 RNA dependent RNA polymerase is diced into siRNAs by action of Dcl-2. Further siRNAs lead to degardation of cognate mRNA by incorporation into RISC (RNA induced silencing complex) involving Qde-2.

(B) During meiosis, a DNA fragment that has failed in pairing (unpaired DNA) triggers the second RNA silencing pathway in N. crassa, called MSUD. Mechanisms in MSUD are thought to be quite similar to those in quelling except that MSUD uses a different set of silencing protein components (paralogs) from those in the quelling pathway[3,4,6].

MATERIALS AND METHODS

FUNGAL ISOLATES USED:

Two strains of *Aspergillus flavus* were used, AF67 is aflatoxin producing strain and BT01 is non aflatoxin producing strain so that a comparison can be made between them. Both the strains are grown at two different temperatures (30°C and 37°C) in an appropriate growth medium.

Sl. No.	Isolate name	MTCC No.	NCBI gene bank accession no.	Afltoxin production
1	BT 01	11580	KC907365	No
2	AF 67	9367	Not available	Yes

Table 1: *Aspergillus flavus isolates.*

A. MEDIA USED :

Streaking of fungal cultures were done on agar slants containing potato dextrose agar and then they were grown at two different temperature conditions (30ºC and 37ºC). PDA is the most common media used for growing fungal and bacterial culture because it contains all prerequisitess nutrients required for these cultures to grow. Media was autoclaved and slants were prepared. After preparation of slants, both producing and non producing strains were

streaked and incubated at two different temperatures. These slants served as the mother culture throughout the experiment.

For both DNA and RNA preparation, fungi were grown in 100 ml of glucose minimal medium (1gm of ammonium phosphate monobasic, 5gms of sodium chloride, 0.2gm of magnesium sulfate, 1 gm of potassium phosphate dibasic and 5gms of glucose) in 250-ml conical flasks kept at two different tempartures (30ºC and 37ºC)at 150 rpm. Minimal Media contains the basic nutrients for fungal species to grow. The media is often used to define if a particular microbial species is a heterotroph, namely an organism that does not have any nutritional requirements beyond core sources of carbon (sugars) and nitrogen. Auxotrophs or organisms with nutritional requirements will not be able to grow on minimal media and as fungus is heterotrophy this media is suitable.

B. CONIDIAL SUSPENSION PREPARATION-

- **Phosphate Buffer Saline Tween-80 (PBST)** was prepared and added to the agar slants.
- Each slant was shaken well so that the conidia could be washed out with the PBST and suspensions were prepared and transferred to eppendorf.
- PBST acts as a washing solution, tween-80 acts as a detergent for conidia since they are of hydrophobic nature.

C. MICROSCOPIC ANALYSIS OF ISOLATES-

Macro-morphological characteristics like colour of conidia, mycelial growth of plates were noted. Slides were prepared using Lactophenol cotton blue and observed under microscope.

Lactophenol cotton blue stain is formulated with Lactophenol, which serves as mounting fluid. Organisms suspended in the stain are killed due to the presence of phenol. High concentration of phenol deactivates the lytic cellular enzyme thus the cell donot lyse. It is an acid dye that stains chitin present in the cells of fungi.

D. DETECTION OF AFLATOXIN PRODUCTION AT 30°C and 37°C USING MEDIA YEAST EXTRACT AGAR.

Fungal isolates were screened in YES agar medium supplemented with 3.0% β-cyclodextrin as a fluorescence enhancer and were grown at two different temperatures (30ºC and 37ºC). These YES agar plates were aseptically inoculated at the centre of culture plates with 2.0 µL of working conidial suspension (10^6 conidia/mL) with help of a micropipette. Plates were incubated in the dark at 27°C and 37°C for 3 days. The presence or absence of a fluorescence ring in the agar plate surrounding the mycelia, observed under UV light (365 nm), was termed as positive or negative.

E. TOTAL GENOMIC DNA ISOLATION

The aim of the isolation process was to obtain DNA of high purity so that it can be used for further experiments to get reproducible results.

1) **Isolation of gDNA from *Aspergillus flavus* MTCC-AF9367 and MTCC-11580 by SDS (freeze thawing):**

Freeze thawing helps in lysis of fungal cells. Purification of DNA from complex mixture of cellular molecules is accomplished by removal of proteins and other molecules into an organic solvent. Use of phenol chloroform leads to denaturation of proteins and phase separation in which DNA and RNA are not soluble in the organic solvents, thus remain in aqueous phases of mixture that contains solvent for Protein extraction.

Reagents:

1) Lysis buffer: Tris acetate, EDTA, sodium acetate ,SDS
2) NaCl
3) Chloroform
4) TE buffer: Tris Cl , EDTA

Procedure:

Pre chilled the motar and pestle at -80°C for 15 minutes prior to the start of the experiment

The cultures grown at two temperatures in glucose minimal media is taken and filter out the liquid and moisture content using muslin cloth under LAF hood

Transferred the filtered fungal cultures into chilled motar and pestle and kept at - 80°C for 20 minutes.

Fungal culture was then ground to fine powder with pestle and transferred into a microfuge tube and incubated it at 60°C for 15 minutes.

Again the tube was kept at - 80°C for 15 minutes and let it freeze. And finally thawed the powdered tissue by pouring 500 µl of lysis buffer.

Then 165 µl of 5 M NaCl solution was added and the components were mixed by inverting the tubes several times.

The suspension was then centrifuged at 13,000 rpm for 20 minutes at 4°C.

Eppendorf suspension was immediately transferred to a fresh tube and 400 µl of phenol and chloroform was added (1:1). The solution was mixed by gentle inverting the tube until their solution become milky

Centrifuged for 20 minutes at 4°C and aqueous phase was removed and extracted with equal volumes of chloroform.

DNA in the aqueous supernatant was precipitated with two volumes of 95 % chilled ethanol.

The precipitated DNA was washed 3 times with 70% ethanol at max speed for 5 minutes.

Then air dried the pellet and dissolved in 20 µl of autoclaved water and stored at -20 °C.

2) **DNA isolation of *Aspergillus flavus*; strains MTCC-11580 and MTCC -AF9367 by SDS (liquid nitrogen crushing method):**

Liquid nitrogen results in lysis of thick fungal cell wall and exposes the fungal chromosomes containing genetic material outside. liquid nitrogen (-180 C) which it freeze the tissue to become fragile to be a fine powder which increase the surface area of extraction, and the very low temperature prevent DNase activation.

Reagents:

5) Lysis buffer: Tris acetate, EDTA, sodium acetate ,SDS
6) NaCl
7) Chloroform
8) TE buffer: Tris Cl , EDTA,

Procedure:

30 mg of freeze dried fungal balls was ground to a fine powder in liquid nitrogen using mortar and pestle.

The grounded fungal balls were resuspended and lysed in 500 µl of the lysis buffer by pipetting with pipette until the viscosity of the suspension was significantly reduced and the formation of froth indicated the detachment of DNA polysaccharides.

Then 165 µl of 5 M NaCl solution was added and the components were mixed by inverting the tubes several times

The suspension was then centrifuged at 13,000 rpm for 20 minutes at 4°C.

Eppendorf suspension was immediately transferred to a fresh tube and 400 µl of phenol and chloroform was added (1:1). The solution was mixed by gentle inverting the tube until their solution become milky.

Centrifuge for 20 minutes at 4°C and aqueous phase was removed and extracted with equal volumes of chloroform.

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The precipitated DNA was washed 3 times with 70% ethanol at max speed for 5 minutes.

Then air dry the pellet and dissolve in 20 µl of autoclaved water and stored at -20 °C.

*F. **TOTAL RNA ISOLATION***

Efficient RNA isolation is a prerequisite for gene expression studies and it has an increasingly important role in the study of fungal pathogens in plants especially food crops. But RNA isolation is difficult in filamentous fungi. These organisms have rigid cell walls and the presence of high levels of carbohydrates, excreted from the fungal cells during submerged growth, which interferes with the extraction procedures. Many commercial kits are available but at the same time user developed procedures are also used.

1) **Total RNA isolation from *Aspergillus flavus* MTCC-Af9367 and MTCC-11580 cultured 37°C using CTAB:**

In this method CTAB was used to isolate total RNA. It's a detergent which simultaneously solubilizes the fungal cell wall and lipid membranes of internal organelles and denatures protein and enzymes. Hence the RNA won't get hydrolyzed during the isolation process.

Buffer and solutions:

1. CTAB: cetyl trimethyl ammonium bromide
2. NaCl
3. EDTA (pH 8)
4. 1M Tris
5. Isopropanol
6. 75% ethanol
7. Water

Procedure:

About 200 mg of each sample tissue were ground to the fine powder using liquid nitrogen and transferred into a 2ml centrifuge tubes. 900 µl of the CTAB buffer and 100 µl of beta mercaptoethanol were added.

Then 800 µl of chloroform were added. The mixture was shaken for 30 seconds. Later the mixture was centrifuged at 10,000 RPM for 10 minutes at 4°C. The supernatant was transferred to a new tube.

Next 800 µl of phenol/chloroform (1:1) were added and shaken for 30 seconds. Later the mixture was centrifuged at 10000 RPM for 10 minutes at 4°C.

The supernatant was transferred to a new tube and an equal volume of chloroform /isoamyl alcohol was added. Samples were shaken for 30 seconds and centrifuged at 10000 RPM for 10 minutes at 4°C.

Added equal amounts of isopropanol and mixed well.

Then RNA pellet was washed with 75% ethanol and centrifuged tubes at 7500 RPM for 5 minutes at 4°C. Repeated this step twice.

Discarded supernatant and RNA pellet at bottom was obtained.

Dried pellet at room temperature for 20 minutes and finally dissolved in DEPC treated water.

2) Total RNA isolation from *Aspergillus flavus* MTCC-Af9367 and MTCC-11580 cultured at different temperatures 30°C and 37°C by trizol reagent:

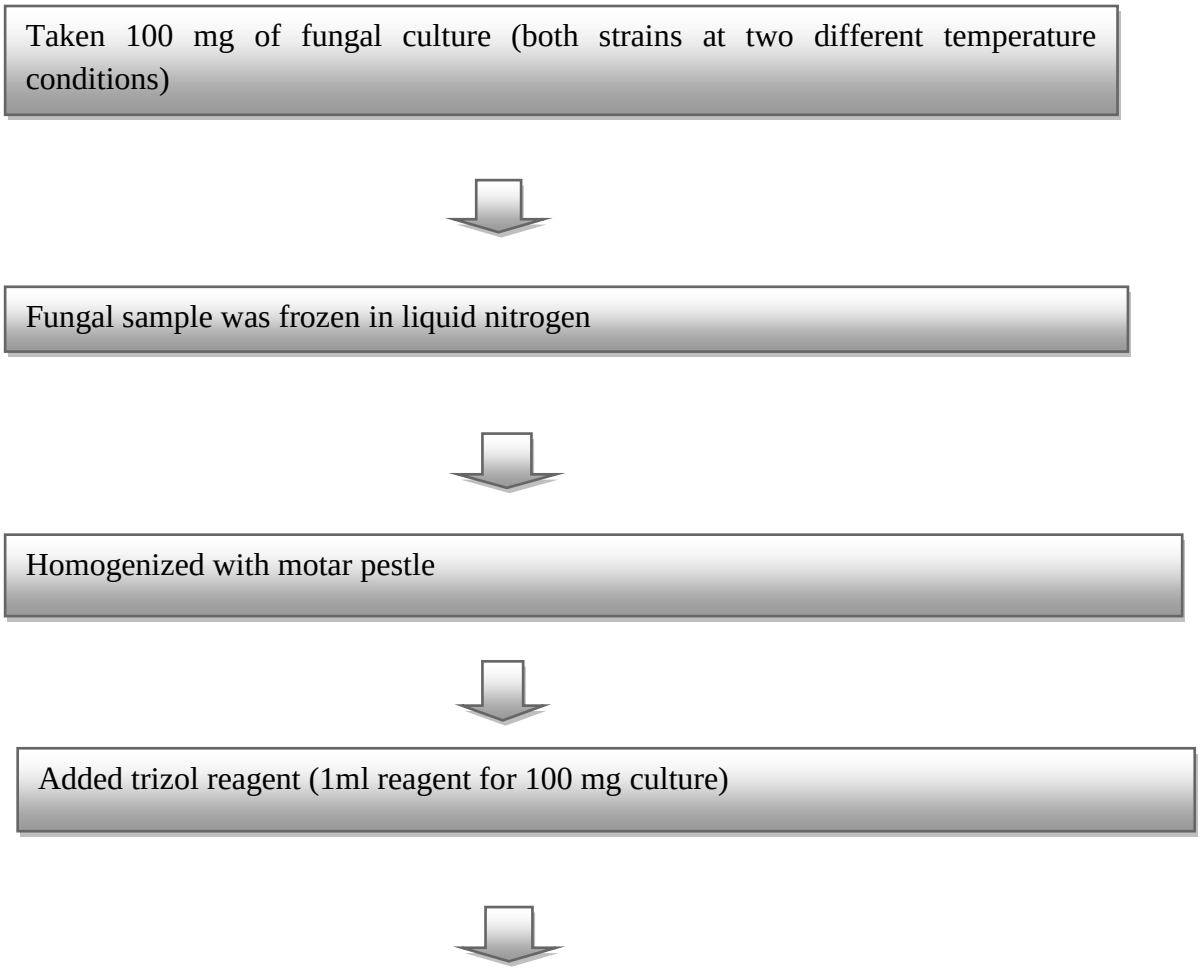
TRIzol Reagent is a ready-to-use reagent used for RNA isolation from cells and tissues. Firstly it consists of guanidinium isothiocyanate, a powerful protein denaturant and helps inactivation of RNases and secondly it has acidic phenol/chloroform which has a role in partitioning of RNA into aqueous supernatant for separation. TRIzol works by maintaining RNA integrity during tissue homogenization, while at the same time disrupting and breaking down cells and cell components. Addition of chloroform, after the centrifugation, separates the solution into aqueous

and organic phases. RNA remains only in the aqueous phase. Total RNA extracted by TRIzol Reagent is free from the contamination of protein and DNA

Reagents:

1) DEPC (diethyl pyro carbonate)
2) Trizol
3) Chloroform
4) Isopropanol
5) 75% ethanol in DEPC water

Procedure:



Incubated for 5 minutes at room temperature to permit complete dissociation of nucleo protein complex

Added .2 ml of chloroform per ml f trizol reagent under the hood and closed the tube

Shaken tube vigorously by hand for 15 seconds and incubated them at room temperature for 2-3 minutes

Centrifuged the sample at 12000 RPM for 15 minutes at 4°C and transferred the supernatant or aqueous phase to the fresh tube.

Precipitated RNA from aqueous phase by mixing with isopropanol (.5ml per 1 ml of trizol)

Incubated the sample at room temperature for 10 minutes and centrifuged at 12000 RPM for 10 minutes at 4°C. Discarded the supernatant.

Then RNA pellet was washed with 75% ethanol and mixed well.

Centrifuged tubes at 7500 RPM for 5 minutes at 4°C. Repeated this step twice. Discarded supernatant and RNA pellet at bottom was obtained.

Dried pellet at room temperature for 20 minutes and finally dissolved in DEPC treated water.

G. cDNA SYNTHESIS AND PCR HOUSEKEEPING GENES

Thermo scientific verso cDNA synthesis kit was used to synthesized cDNA

Components:

1) Verso enzyme mix: It includes verso reverse transcriptase which is active at high temperatures, is highly sensitive and can generate long cDNA strands. This mix also contains RNase inhibitor to protect RNA templates from degradation.
2) 5X cDNA synthesis buffer: a proprietary reaction buffer which has been optimized to improve reverse transcription across a wide range of templates.
3) Anchored oligo dT primers and random hexamers: provide flexible RNA priming methods for cDNA synthesis

4) RT enhancer: It is included to remove contaminating DNA, eliminating the need for DNase I treatment. It degrades double stranded DNA during transcription of RNA and inactivated after 2 minutes at 95 °C.

Verso is RNA dependent DNA polymerase with the significantly attenuated RNase H activity. Verso can synthesize long cDNA strands up to 11kb at temperature range of 42 °C to 57 °C. The recommended amount of total RNA to use is between 1 pg to 1 µg. It should be stored at –20 °C until ready for use and repeated freeze thawing should be avoided.

Reaction mixture	**Volume**
5x cDNA synthesis buffer	4 µl
dNTP mix	2 µl
RNA primer	1 µl
RT enhancer	1 µl
Verso enzyme mix	1 µl
Template (RNA)	5 µl
Water , nuclease free	6 µl
Total	20 µl

Table 1: *Reaction mixture for PCR reaction.*

Reverse transcription cycling program:

	Tempertaure	**Time**	**No of cycles**
cDNA synthesis	42°C	30 min	1 cycle

Inactivation	95°C	2 min	1 cycle

Table 2: *PCR conditions*

HOUSEKEEPING GENE, TUBULIN (size: 122 bp):

Gene tubulin was used and then performed PCR to check cDNA. Housekeeping genes are involved in basic cell maintenance and, therefore, are expected to maintain constant expression levels in all cells and conditions (both include HSP6 (size 121bp), HSP70 (size 141bp), HSP 989 (size 102bp) and GAPDH (size 119bp).

Components	**Volume**
Forward primer	0.5 µl
Reverse primer	0.5 µl
DNA	1 µl
PCR mix	6 µl
dH_2O	4 µl
Total	20 µl

Table 3*: PCR reactions for amplification of housekeeping genes.*

H. *DCL-1* GENE AMPLIFICATION:

Reaction mixture	volume
DCL-1forward primer	0.30µl
DCL-1 reverse primer	0.30 µl
Master mix	6.25 µl
cDNA	1.00 µl
water	4.63 µl
Total	12.5 µl

Table 4: *PCR reaction mixtures for the amplification of DCL-1 gene.*

Temperature	Time
95°C	5mins
95°C	45 secs
51.1°C	30 secs
72°C	45 secs
72°C	7 mins

Table 5*: PCR condtions.*

I. DCL-2 GENE AMPLIFICATION:

Reaction mixture	volume
DCL-1forward primer	0.30µl
DCL-1 reverse primer	0.30 µl
Master mix	6.25 µl
cDNA	1.00 µl
water	4.63 µl
Total	12.5 µl

Table 6: *PCR reaction mixtures for the amplification of DCL-2 gene.*

Temperature	Time
95°C	5mins
95°C	45 secs
55.2°C	30 secs
72°C	45 secs
72°C	7 mins

Table 7: *PCR reaction mixtures for the amplification of DCL-2 gene.*

J. SEMI QUANTITATIVE REVERSE TRANSCRIPTASE (RT PCR):

Semi quantitative RT-PCR invloves co amplification of target sequence and an endogenous control sequence whose content is not altered by experimental manipulation.

RT PCR is a highly sensitive method which can be used not only to detect specific mRNAs but also to semi-quantitate their levels of transcripts from a control, house keeping gene (such as actin and GAPDH). Also exogenous primer specific PCR template can be used during PCR. Extraction of total RNA from fungal cells with TRIZOL reagent was done as per the procedure. cDNAs synthesized from verso cDNA synthesis kit were used as a template in this analysis. The RT-PCR was carried out for 35 cycles for both genes DCL-1 and DCL-2 to allow semiquantitative comparisons of cDNAs of two different isolates. The primers and PCR conditions are as follows:

DCL-1

Reaction mixtures	Volumes
DCL-1 forward primer	0.30µl
DCL-1 reverse primer	0.30 µl
Master mix	6.25 µl
cDNA	1.00 µl
Water	4.63 µl
Total	12.5 µl

Table 8: *PCR reaction mixtures.*

Temperature	Time
95°C	5mins
95°C	45 secs
51.1°C	30 secs
72°C	45 secs
72°C	7 mins

Table 9: *PCR conditions.*

DCL-2

Reaction mixtures	Volumes
DCL-2 forward primer	0.30µl
DCL-2 reverse primer	0.30 µl
Master mix	6.25 µl
cDNA	1.00 µl
Water	4.63 µl
Total	12.5 µl

Table 10: *PCR reaction mixtures.*

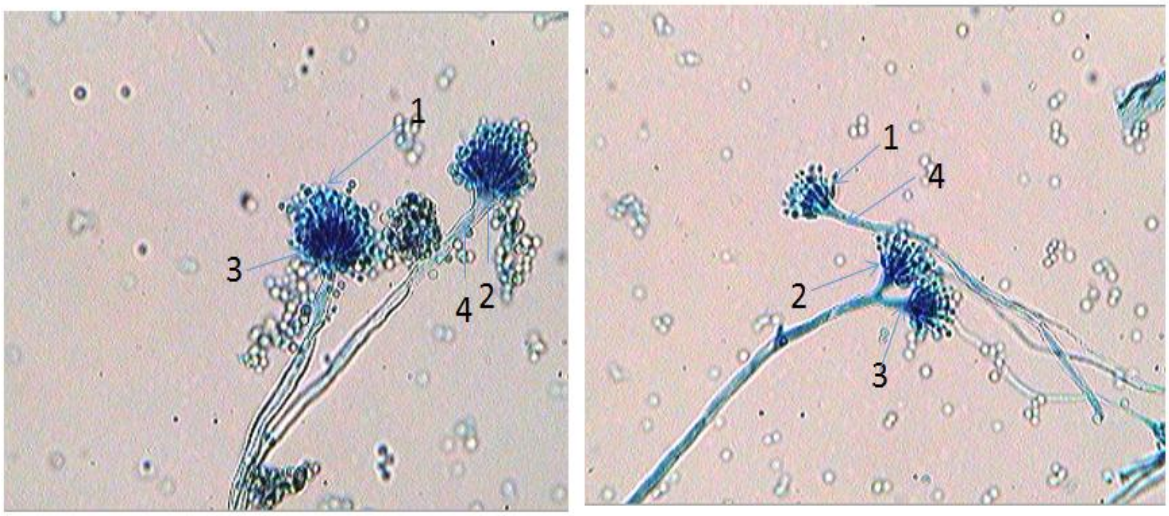
Temperature	Time
95°C	5mins

95°C	45 secs
55.2°C	30 secs
72°C	45 secs
72°C	7 mins

Table 11*: PCR conditions.*

RESULTS AND DISCUSSION

C. MICROSCOPIC ANALYSIS OF ISOLATES: For the identification of conidia and conidiophores of *Aspergillus flavus* in laboratory, lacto phenol cotton blue staining is the simplest method.

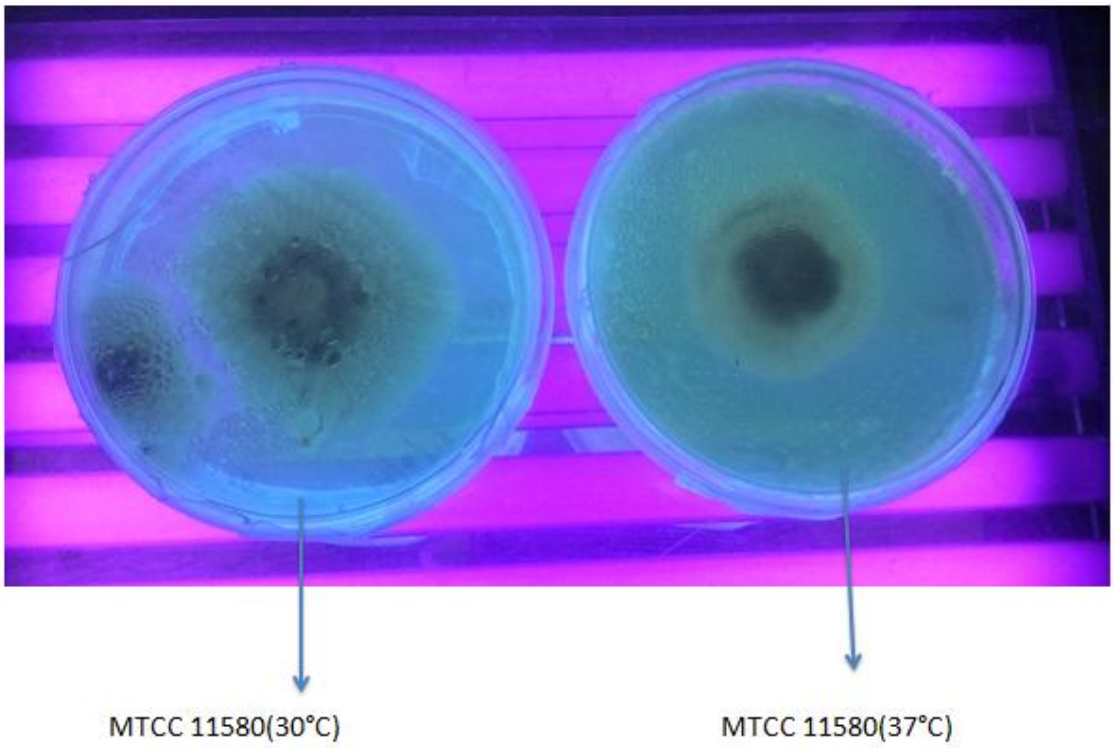


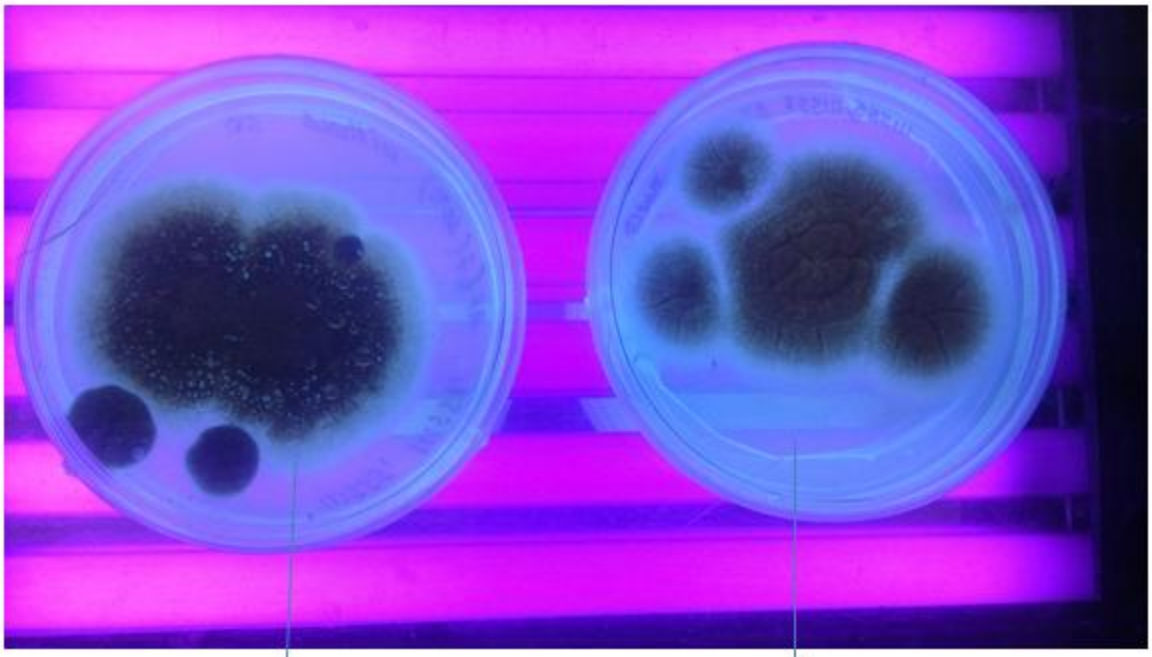
Aspergillus flavus(MTCC AF9367) and MTCC 11580 when viewed under 100 x

Figure 1: *Staining of aspergillus flavus strain MTCC AF9367 and MTCC 11580.following*

1) Conidia
2) Phialides
3) Vesicles
4) Conidiophore

D. DETECTION OF AFLATOXIN PRODUCTION AT 30°C and 37°C USING MEDIA YEAST EXTRACT AGAR. The presence of fluorescence ring around the colony indicates the presence of Aflatoxin production. Presence of fluorescence ring in AF67 and MTCC-11580 at 30°C indicates the Aflatoxin production whereas MTCC-11580, and AF67 at 37°C produces very little or no Aflatoxin (absence of ring).

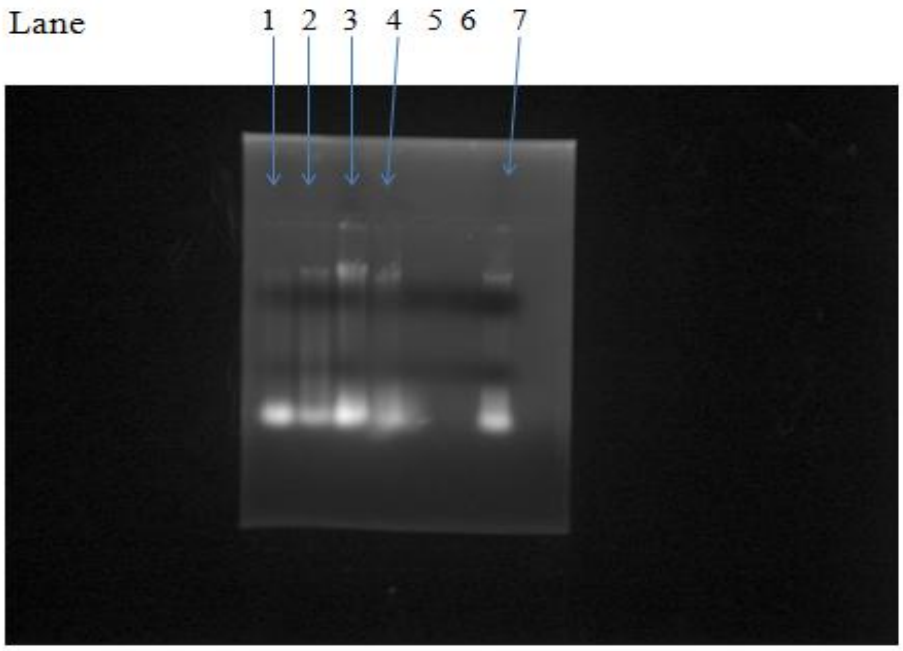




Discussion: In picture 1 there is no production of aflatoxin as there is no fluorescence ring which is seen where as in second picture there is presence of beige ring around the colony which confirms the production of aflatoxin in MTCC-AF9367.

E. TOTAL GENOMIC DNA ISOLATION

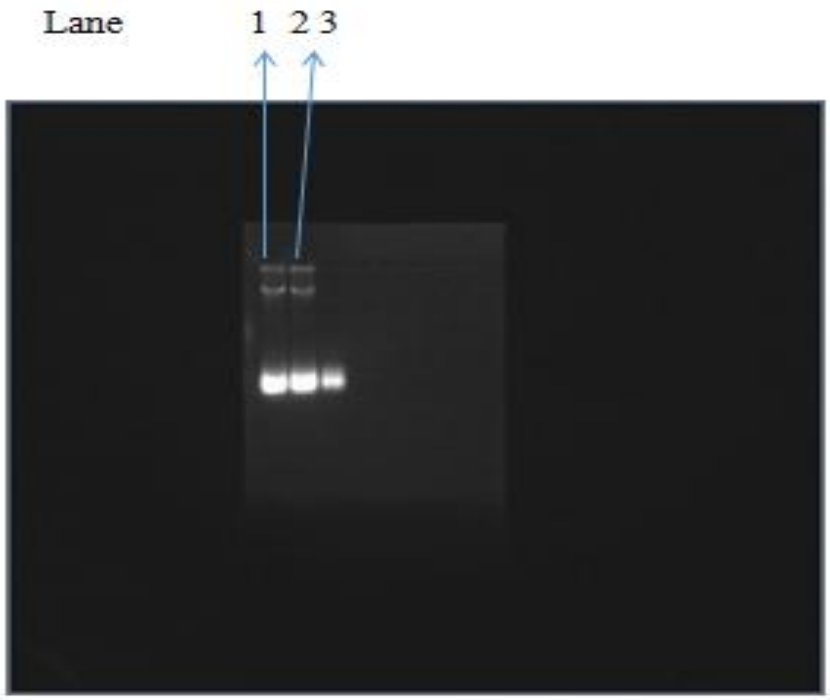
1) **Isolation of gDNA from *Aspergillus flavus* MTCC-Af9367 and MTCC-11580 by SDS (freeze thawing):**



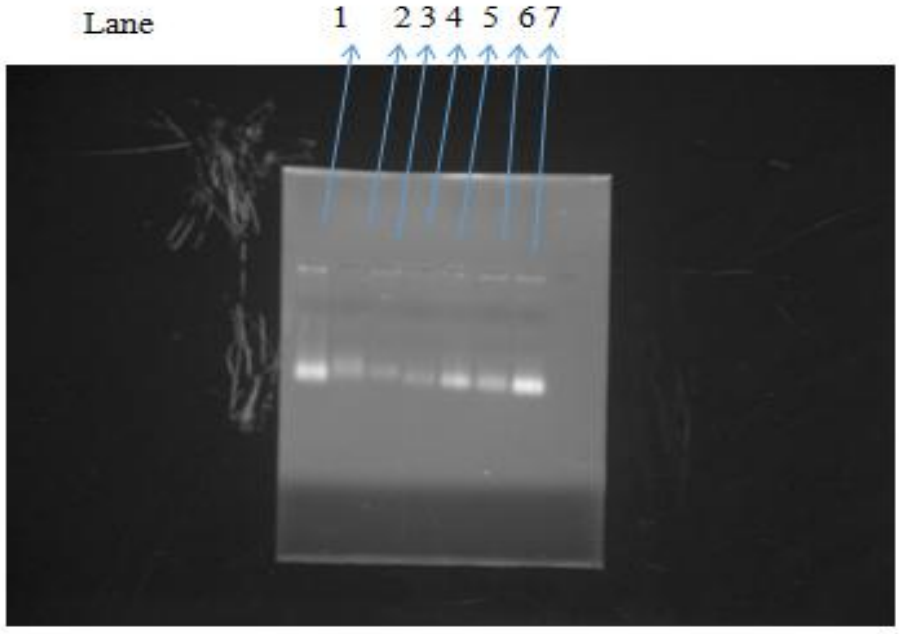
Lane 1: BT01 (30°C)
Lane 2: AF67 (30°C)
Lane 3: BT 01 (37°C)
Lane 4: AF67 (37°C)
Lane 7: BT01 (30°C)

Discussion: The DNA isolationby freeze thawing was not efficient.The possible reasons may be inappropriate lysis of fungal cell wall which is made up of chitin and is very thick. Therefore the bands are not visible of both isolates MTCC AF9367 and MTCC 11580 when grown at two different temperatures.

3. **DNA isolation of *Aspergillus flavus*; strains MTCC-11580 and MTCC -AF9367 by SDS (liquid nitrogen crushing method):**



Lane 1:AF67 (30°C),
Lane 2: AF67 (37°C)



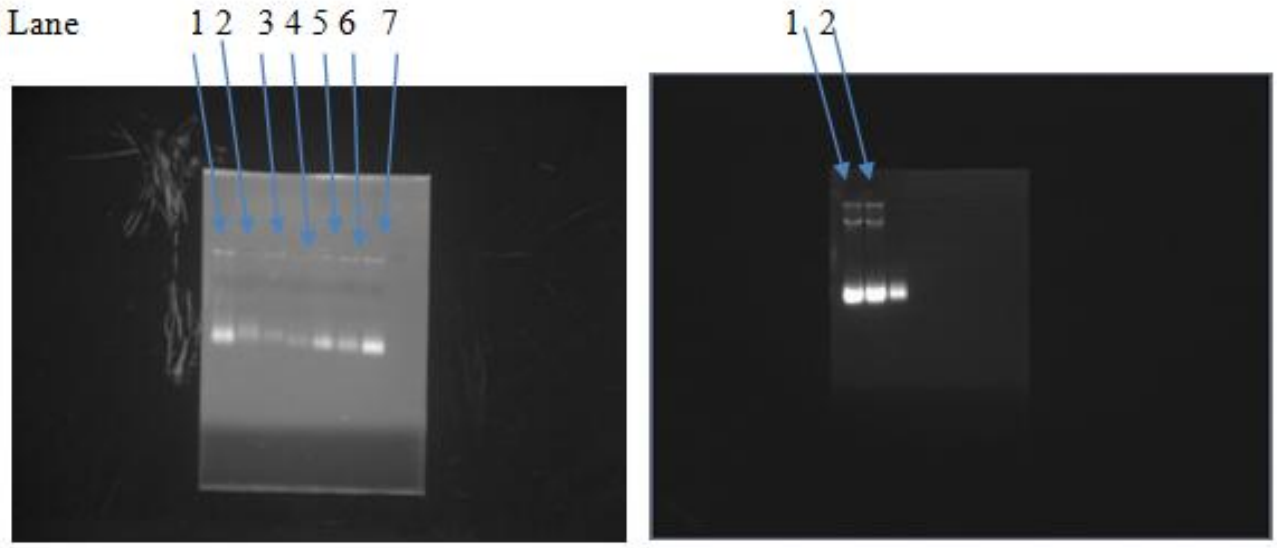
Lane 1: BT01 (30°C)
Lane 2: AF67 (30°C)
Lane 3: BT01 (37°C)
Lane 4: AF67 (37°C)
Lane 6: BT01 (30°C)
Lane 7: BT01 (30°C)

Discussion: DNA bands can be seen in both the strains.In second picture DNA has not moved out of well, degraded RNA and contaminations can also be seen. Inspite of using same procedure

and reagents we got DNA bands in this case due to use of liquid nitrogen. The reason is proper lysis of fungal cell wall exposing the DNA.

F .TOTAL RNA ISOLATION

1. **Total RNA isolation from *Aspergillus flavus* MTCC-Af9367 and MTCC-11580 cultured 37°C using CTAB:**



Lane 1, 2, 3 – BT-01 (30°C)

Lane 4, 5, 6, 7- BT-01 (37°C)

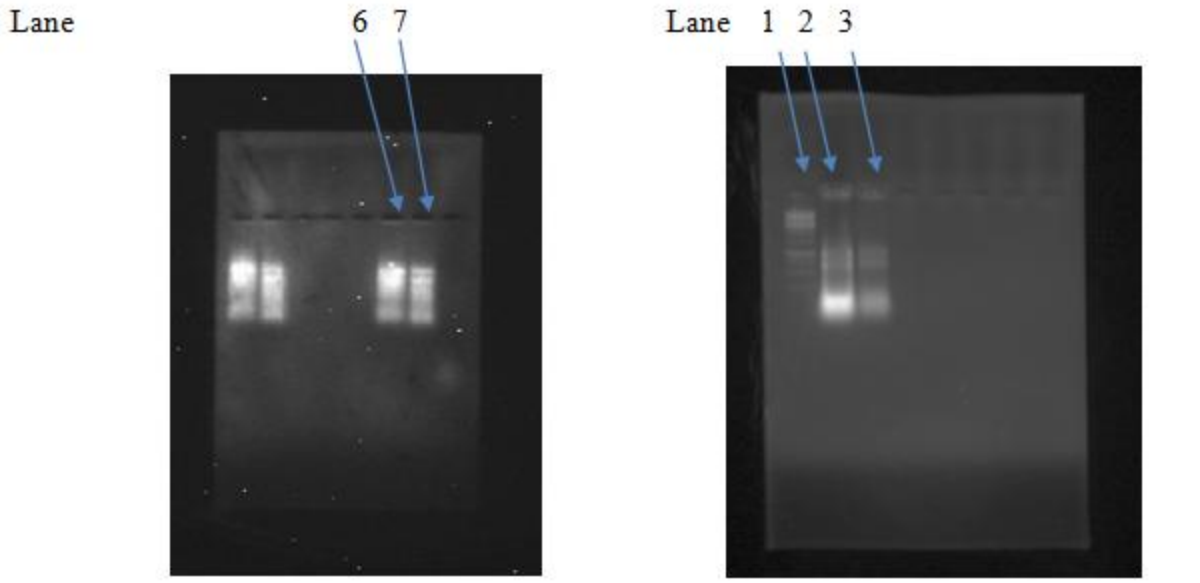
Lane 1- AF-67(30°C)

Lane 2- AF-67(37°C)

Discussion:

The total RNA isolation by CTAB was not efficient as compare to isolation using TRIZOL reagent. One of the reasons behind could be due the improper lysis of fungal cell wall. Fungal cell wall is composed of chitin and it's very thick, it is difficult to lyse using CTAB. Therefore DNA bands are not clear in both isolates, MTCC AF9367 and MTCC 11580 at both temperatures.

2. **Total RNA isolation from *Aspergillus flavus* MTCC-Af9367 and MTCC-11580 cultured at different temperatures 30°C and 37°C by trizol reagent**



Lane 6: MTCC AF9367 (30°C)

Lane 7: MTCC BT-01(30°C)

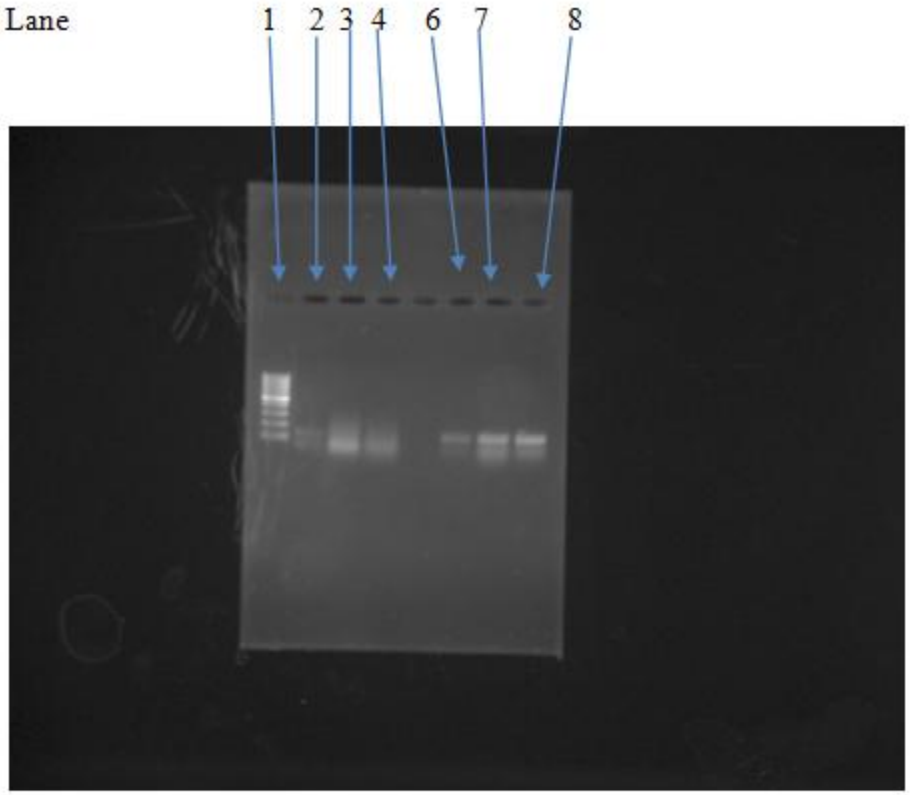
Lane 1: Ladder

Lane 2: MTCC AF9367 (37°C)

Lane 3: MTCC BT-01 (37°C)

Discussion: RNA isolation from TRIZOL was efficient as it resulted in prominent band in both the fungal isolates MTCC AF9367 and MTCC 11580 both grown at two different tempertaures.

G. cDNA SYNTHESIS AND PCR HOUSEKEEPING GENES



Lane 1: Ladder

Lane 2: Control (30°C)

Lane 3: AF-67 (30°C)

Lane 4: BT-01 (30°C)

Lane 5: Control (37°C)

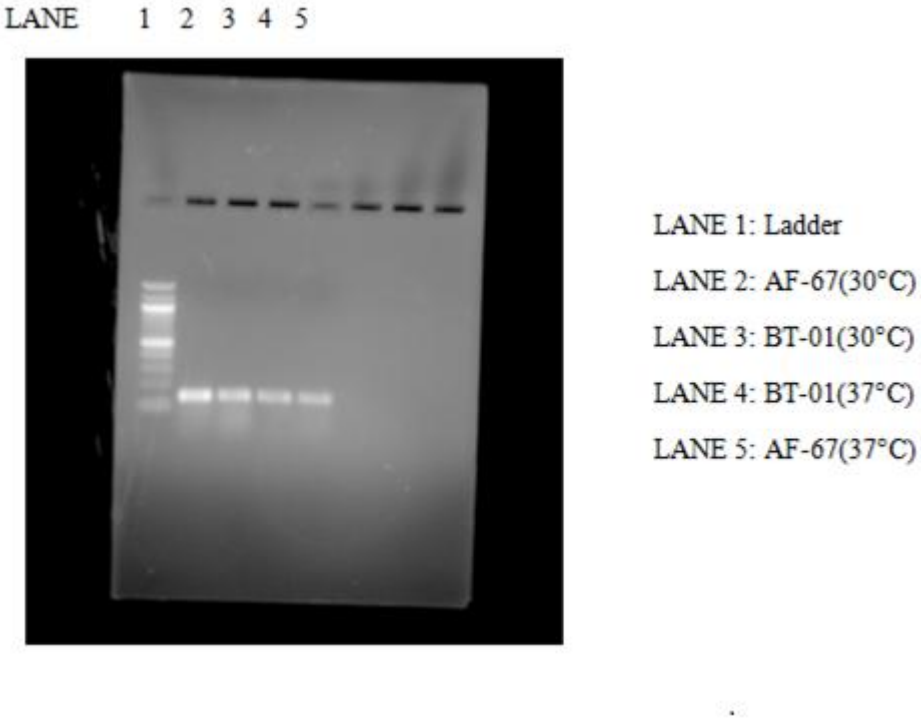
Lane 6: AF-67 (37°C)

Lane 7: BT-01 (37°C)

Discussion:

There was amplification in both the strains MTCC MTCC-AF9367 and MTCC-111580 grown at (30°C and 37°C) when house keeping tubulin primers were used. It means that proper amplification occurred and cDNA was isolated successfully

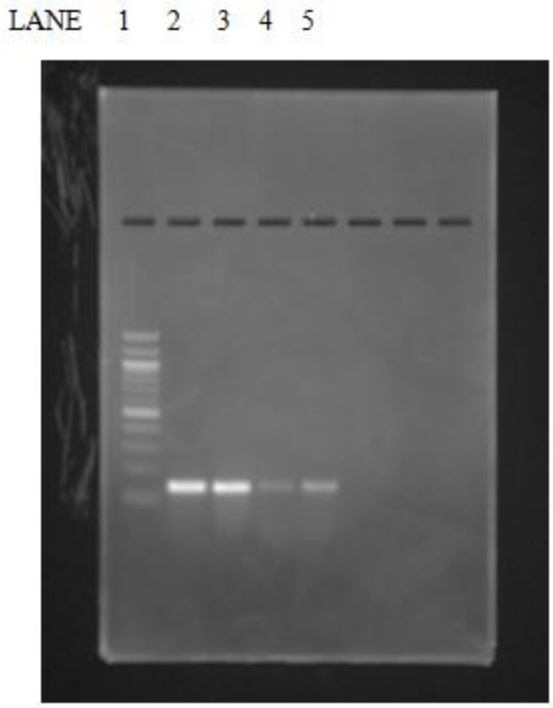
H. *DCL-1 GENE AMPLIFICATION*:



Discussion:

Amplification was seen in both strains MTCC MTCC-AF9367 and MTCC-111580 by using DCL-1 primers. The intensity of bands was high in strains MTCC-AF9367 and MTCC-111580 grown at 30°C as compared to 37°C. This indicates gene DCL-1 is present in high copy in both fungal isolates grown at 30°C.

I. *DCL-2 GENE AMPLIFICATION*:



LANE 1: Ladder

LANE 2: AF-67(30°C)

LANE 3: BT-01 (30°C)

LANE 4: AF-67(37°C)

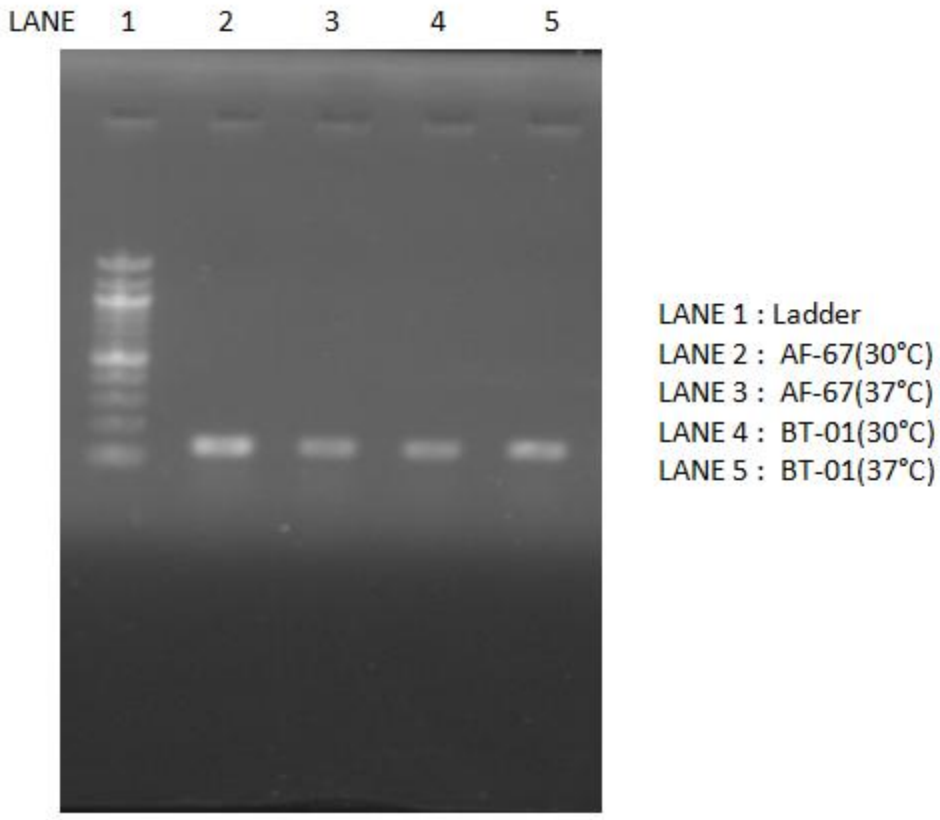
LANE 5: BT-01 (37°C)

Discussion:

Amplification was seen in both strains MTCC MTCC-AF9367 and MTCC-111580 by using DCL-2 primers. The intensity of bands was high in strains MTCC-AF9367 and MTCC-111580 grown at 30°C as compared to 37°C. This indicates gene DCL-2 is present in high copy in both fungal isolates grown at 30°C.

J. SEMI QUANTITATIVE REVERSE TRANSCRIPTASE (RT PCR):

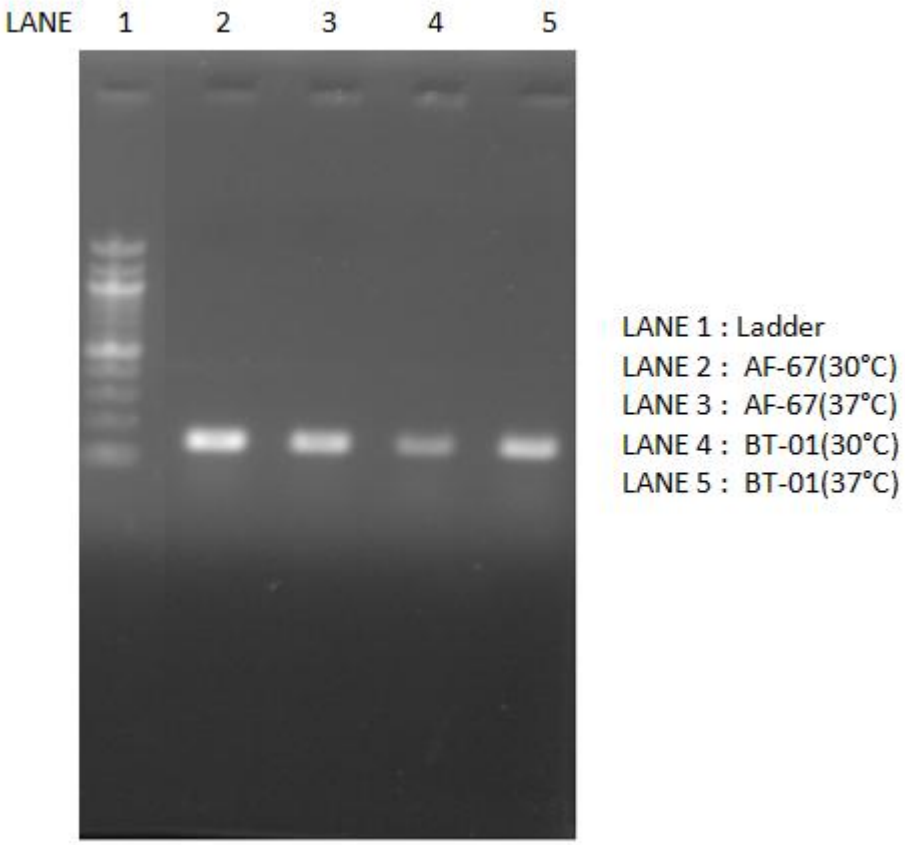
DCL-1 GENE AMPLIFICATION:



Discussion:

DCL-1 amplication band was seen in MTCC-AF9367 (30°C) and the gene was differentialy expressed in the producing strain at the temperature which supports aflatoxin production as compared to non producing strain MTCC-11580 (30°C and 37°C). This confirms its high production in aflatoxin synthesizing strain and possible role in aflatoxin.

DCL-2 GENE AMPLIFICATION:



Discussion:

DCL-2 amplification band was seen in both MTCC-AF9367 (30°C) and MTCC-AF9367 (37°C) as compared to MTCC-11580 strains grown at theses respective temperatures. But it was difficult to make out difference in the expression. DCL-2 was expressed in MTCC-11580 at (37°C).

CONCLUSION

Aflatoxins leads to contamination of food and feed .In most of the developing countries oocurs issue of food safety due to absence of detection techniques,no appropriate methods of monitoring and correct regulating measures to ensure the safety of food supply. Thus , the objective was to determine presence of Dicer like proteins and their involvement(RNA interference) if any in aflatoxin pathway .

Two fungal strains of Aspergillus flavus were taken and screened for genes *DCL-1* and *DCL-2* which are present in closely related species of Ascomycota *Penicillium marneffei* and *Neurospora crassa* (involved in RNA inrterference mechanisms).

cDNA was successfully obtained and amplication was done.Two strains , one aflatoxin producing(MTCC-AF9367) and one aflatoxin non producing(MTCC-111580) were taken and then the results were tried to compare.

Semi quantitative results confirmed the presence of genes(*DCL-1* and *DCL-2*) in high no in MTCC-AF9367 (aflatoxin producing strain) and MTCC 111580(non producing strain) respectively.Thus the results were positive . Their possible may be there but for its confirmation there is need of High Throughput Genome Sequencing as there is no well developed database which is available .

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APPENDICES

POTATO DEXTROSE AGAR MEDIA

S.No.	COMPOUND	AMOUNT(gm/l)
01.	Potatoes, infusion form	200
02.	Dextrose	20
03.	Agar	15

PHOSPHATE BUFFER SALINE TWEEN-80

S.No.	COMPOUND	AMOUNT(1L)
01.	Sodium chloride	8.0 g
02.	Potassium chloride	0.2 g
03.	Disodium hydrogen phosphate	1.44 g
04.	Tween-80	0.05 % (v/v)

GLUCOSE MINIMAL MEDIA COMPOSITION

SL. NO.	**COMPONENTS**	**AMOUNT (gms/100ml)**
1	Ammonium phosphate monobasic	1.0 gm
2	Glucose	5.0 gm
3	Sodium chloride	5.0 gm

4	Magnesium sulfate	0.2 gm
5	Potassium phosphate dibasic	1.0 gm

LACTOPHENOL COTTON BLUE STAIN:

Sl. No.	**COMPONENTS**	**AMOUNT**
1	Phenol crystals	20 gms
2	Cotton blue	0.05 gms
3	Lactic acid	20 ml
4	Glycerol	20 ml
5	Distilled water	20 ml