"**Expression analysis of *Crz1* and *Rho-GDP of Aspergillus flavus* during interaction with *Triticum aestivum***"

Dissertation submitted in partial fulfilment of the requirement for the

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BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

By

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DECLARATION

I, hereby, declare that the thesis entitled "**Expression analysis of *Crz1* and *Rho-GDP of Aspergillus flavus* during interaction with *Triticum aestivum***" submitted at **Jaypee University Of Information And Technology , Waknaghat**, prepared under the guidance of **Dr. Jata Shankar** and **Dr. Sudhir Kumar** is a original work conducted by us.

It is also declared that, this thesis has been prepared for academic purpose along and has not been/will not be submitted elsewhere for any other purpose.

This information and data given in this report is authentic to the best of my knowledge .

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CERTIFICATE

This is to certify that the work which is being presented in the project title, "**Expression analysis of *Crz1* and *Rho-GDP of Aspergillus flavus* during interaction with *Triticum aestivum***" for the end semester (8th semester) of Bachelor of technology and submitted in Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat is an authentic record of work done by Swati Srivastava (151810) and Subhashree Pradhan (151840) during a period from July 2018 to May 2019 under the supervision of Dr. Jata Shankar, Associate Professor, Department Of Biotechnology and Bioinformatics, and Dr. Sudhir Kumar, Professor, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat.

The above statement made is correct to the best of my knowledge.

Date:

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Swati Srivastava (151810)

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LIST OF ABBEREVIATION

µL	**Micro liter**
Kb	**Kilobyte**
Mb	**Megabyte**
ºC	**Degree Celsius**
A. flavus	***Aspergillus flavus***
cDNA	**Complementary DNA**
PCR	**Polymerase chain reaction**
RT-PCR	**Real Time Polymerase chain reaction**
PBS	**Phosphate Buffer Saline**
PBST	**Phosphate Buffer Saline TWEEN**
PDA	**Potato dextrose agar**
PDB	**Potato dextrose broth**
RPM	**Rotation per minute**
LCB	**Lacto phenol cotton blue**
DEPC	**Diethyl pyrocarbonate**
dNTP	**Deoxyribonucleoside Triphosphate**
RT	**Room temperature**
C_T	**Cycle threshold**
TAE	**Tris-Acetate EDTA**
MM	**Minimal Media**
g	**Grams**

ABSTRACT

Fungus is a group of eukaryotic organism which include yeasts, moulds also most famous mushrooms. Majorly yeast and moulds are responsible for the spoilage of food .due to the large amount of spores produced by fungus, these spores when land into some food, it starts reproducing .beside causing spoilage and economic loss some fungal strain also produce mycotoxins .major strain of fungus which infect foods are *Aspergillus, Penicillium*. In this study the objective is to find out genes responsible for the attachment and growth of fungus on wheat. *Aspergillus flavus* was taken for this study. Pure colony of *Aspergillus flavus* was formed using sub-culturing and conidia harvesting. Microscopic and molecular analysis was made for the spores of *Aspergillus flavus* was stained with lacto-phenol cotton blue and observed under compound microscope. Further for the molecular analysis wheat was infected with the spores of *A.flavus*. After the 3^{rd} and 5^{th} day of infection, RNA was isolated and cDNA from the best purified RNA was made. RT-PCR was carried out to check the quantification of gene.

Genes selected for quantifying expression analysis during interacting with host are *Crz1* and *Rho-GDP. Tubulin* was used as housekeeping gene. *Crz1* is a transcriptional factor regulates morphogenesis via calcium signalling pathway. It helps fungal cell to promote growth and their by assist in virulence during infection on suitable host. *Rho-GDP* is a transcriptional inhibitor. Expression of these two genes will reveal the morphogenesis of fungus. There is high level of expression of *Crz1* gene at 5^{th} day of infection as compared to 3^{rd} day and the expression of *Rho-GDP* was higher on 3^{rd} day as compared to 5^{th} day. The expression levels of gene were quantified by RT-PCR and normalized using *tubulin* gene. The expression analysis of transcriptional factor may help to add in our understanding on how *A. flavus* colonizes on wheat.

CHAPTER 1

INTRODUCTION

Grains which are often referred to as cereals are starchy seeds which are suitable for consumptions, they have similar dietary properties that are they are found to be rich in carbohydrate, low in protein and are deficient of calcium and vitamin A. Some of the commonly cultivated cereals are wheat, rice, rye, oat etc. These are often prone to get contaminated by a variety of fungus from its germination to harvesting. Grain quality is often hampered due to these pathogenic invasions as there might be toxin released which can cause intoxication in human and animals [1]. There are certain secondary metabolite produced by fungus found growing on plants and are often referred to as mycotoxins. These mycotoxins include aflatoxins, citrinin, ergot alkaloids, fumonisins etc [2]. It is very important to prevent mycotoxin production from fungus since these metabolites get stable at environmental temperature and even resistant to thermal changes [3]. Mycotoxins are major concern when it comes to storage of grains because they are a result of fungal development on grain due to the prevailed conditions which eventually favours the growth such as moisture content temperature, storage period etc [2]. It is often considered as an indicator of poor storage condition if there is presence of mycotoxins in the grain. There are certain moulds which do not produce mycotoxins but are often found in stored grains and there metabolic activity usually raises the moisture content of the grain giving a favourable condition for the growth of mycotoxigenic species such as *Aspergillus* and *Penicillium* [4].

Aspergillus is one of the most abundant and highly distributed organisms in earth which are responsible for production of mycotoxins. This genus at present comprises of 339 known species and they are important economically also as they are related in the production of soya sauce, organic acids, hydrolytic enzymes, and biologically active metabolite for example lovastatin. These species are responsible for various plants disorders especially as unprincipled storage mould growth [5]. The first isolate of *Aspergillus* was discovered around 1729 and named the former due to the spore bearing structure. They are saprophytic and grow on decaying plant material. *Aspergillus flavus* is a frequently encountered saprophytic soil borne fungus often found in agricultural crops and is a potent producer of mycotoxins, aflatoxins [5, 2]. *Aspergillus flavus* includes conidial head in shades ranging from yellow or green to brown to sclerotia. It is a known pathogen for both humans and animals. *Aspergillus*

flavus and *Aspergillus parasiticus* are considered to be major contaminants of food crops because they produce aflatoxin [2]. After *Aspergillus fumigatus* it is *Aspergillus flavus* which is known to cause aspergillosis in humans [6]. It is estimated that approximately there are 4.5 billion people who are found to be exposed to undetermined amount of aflatoxin in the developing countries and it is known that low doses of this mycotoxin with dietary causes hepatocellular carcinoma [12]. *Aspergillus flavus* has become an important area of research due to its capability of contaminating the pre and post harvested food crop.

Aspergillus flavus is referred to as crop destroying fungi and wheat being a potential crop is found to be prone to this infection. Wheat comes under the grass family, Poaceae and belongs to genus *Triticum*. Wheat is scientifically named as *Triticum aestivum.* Origin of this genus is tropical South west Asia where it is found in both wild and cultivated form and it is even adaptive to harsh environment. They usually require intermediate temperature level for better growth. It was first cultivate in the United States in the year 1602 on an island of Massachusetts coast and now is the most cultivated crop in the whole world. The genetics of this crop is more complicated than many other domestic crops. The complex genetics is due to the presence of two, four or six set of chromosomes. The storage condition greatly affects the grain quality. Factors such as abiotic and biotic significantly affect the chemical and physical properties of wheat. Spoilage and the type of mycotoxins released depends on the fungal culture infecting the wheat [8]. There are signalling pathways such as calcium signalling pathway and some proteins such as heat shock protein which showed up-regulation during the infection on some foods. Studying and observing these genes which are responsible for the infection can give us the potential new targets against *Aspergillus flavus* growth on wheat hence we can prevent the spoilage of wheat.

Genes taken into consideration for this study include *Crz1* and *Rho-GDP*. *Crz1* is a protein responsible for building a bridge between calcium dependent signalling pathways to various cellular metabolisms. It is a heterodimeric protein having catalytic subunit A and regulatory subunit B. It helps in controlling cell morphology and virulence. The inactivation of calcineurin results in the decreased virulence and decreased growth of fungus. *Crz1* is an important gene in studying the morphogenesis of the fungus and its growth, whereas, *Rho-GDP* acts by promoting cytoskeleton rearrangement of protein in fungus.

To carry out this project pure isolates of *Aspergillus flavus* strain was subcultured followed by conidia harvest using PBST/PBS. Before the infection the detailed morphology,

molecular, and microscopic examination of fungus was done. Structure and growth condition were studied by observing the growth of conidial spores on PDA plates after incubation. For the microscopic evaluation; fungal spores were stained using lactophenol cotton blue stain. Further the molecular analysis of infected wheat with *Aspergillus flavus* was carried out and RNA was isolated during the 3rd and 5th day of infection. cDNA was made from the pure RNA followed by the RT-PCR for the gene expression analysis for selected genes during *A flavus*-wheat interaction. Our study demonstrated Crz1 and Rho Gdp showed expression in *A. flavus* during the infected wheat.

CHAPTER 2

REVIEW OF LITERATURE

Aspergillus:

Aspergillus is a major class which comprises of more than 200 species with teleomorphs dispersed in 9 distinct genera. The family is sub-partitioned in 7 subgenera and further put into areas [9]. *Aspergillus* scientific categorization is by all accounts complex and consistently advancing which is valid for all parasites. The types of this class can be effectively distinguished by their trademark conidiophore, recognizing and separating them is a significant undertaking, for it is generally founded on a scope of morphological highlights.

The family *Aspergillus* is among the most plentiful and broadly circulated life form on earth, and right now includes 339 known species. It is a standout amongst the most significant financially contagious sort and the biotechnological utilization of *Aspergillus* species is identified with creation of soy sauce, of various hydrolytic proteins (amylases, lipases) and natural corrosive (citrus extract, gluconic corrosive), just as organically dynamic metabolites, for example, lovastatin. In spite of the fact that they are not viewed as significant reason for plant infections, *Aspergillus* species are in charge of a few issues in different plants and plant items, particularly as pioneering stockpiling molds. The remarkable outcome of their quality is defilement of nourishments and feeds by mycotoxins, among which the most significant are aflatoxins, ochratoxin and, at a less degree, fumonisins.

The *Aspergillus* species contain the major number of species which are capable of producing the toxins, mycotoxins which are the harmful secondary metabolites in plants and animals [3, 4]. Mycotoxins are a reason for worry in grain capacity, being one of the numerous significant components, out of this world growth advancement due to already existent conditions, for example, dampness content, temperature, stockpiling period, sullying rate, broken grain and polluting influences, creepy crawly nearness, oxygen rate, harms amid collect handling and grain and seed transport [2-10]. These mycotoxins are hepatocarcinogenic and nephrogenic especially in immunodeficient patients [15].

Aspergillus flavus:

Aspergillus flavus is a saprophytic, soil-borne shape as often as possible experienced in farming yields amid pre-gather and post-collect stage by delivering most strong mycotoxin, aflatoxin (AFB1 and AFB2) [1,3]. Roughly 4.5 billion individuals living in creating nations are presented to unchecked measure of aflatoxin which results in intense aflatoxicosis [14]. The world wellbeing association guidance that low dosages with dietary presentation to aflatoxin is a noteworthy hazard as they can prompt hepatocellular carcinoma [34]. Likewise, *A. flavus* is one of the significant reasons for aspergillosis in people [5]. To confine presentation of aflatoxin, a farthest point of 20 ppb by most nations, and 8 ppb for AFB1 by European commission has been set up. Because of confinements, huge monetary misfortune in the farming business has been accounted for Maize [12] is a prime sustenance source after rice and wheat [12].

Aspergillus flavus and *Aspergillus parasiticus* are the significant contaminants known to create aflatoxins in nourishment crops (maize, cotton, corn, peanuts and oil-seed). These days different modified social practices are being used and investigated to defeat nourishment defilement issue.

Aspergillus species are clinically important as it infects immunocompromised individuals. Aspergilli cause allergic or invasive aspergillosis depending upon the host immunity status [16] though non-irresistible Aspergilli have added to comprehend the science of eukaryotic creatures and fill in as a model living being. Morphotypes of Aspergilli, for example, conidia or mycelia/hyphae helped them to get by in good or horrible ecological conditions. These morphotypes add to destructiveness, pathogenicity and attack into hosts by discharging proteins, chemicals or poisons. Morphological progress of *Aspergillus* species has been a basic advance to taint or to colonize on nourishment items (1).

A. flavus is the second real reason for invasive aspergillosis in people. The abundance of *A. flavus* spores inward breath or contact with tainted yields may cause a majority of parts of the human lung aspergillosis and skin, nails and other contamination. These diseases are essentially predominant in regions with high temperatures, for example, the Middle East, where the organism is the most widely recognized reason for sinusitis and keratitis. Under proper conditions, *A. flavus* will create an assortment of profoundly harmful and cancer-causing auxiliary metabolites, for example, aflatoxin (AF) and cyclopiazonic corrosive. The

World Health Organization (WHO) has assigned aflatoxins as a characteristic presence of class I cancer-causing agent. Because of stable physical and synthetic properties of aflatoxin, straightforward preparing cannot dispose of the yellow *Aspergillus* poison. Along these lines, it is anything but difficult to take aflatoxin from defiled harvests into the natural way of life, bringing about progressively wide scope of sullying and unavoidable monetary misfortunes.

Aspergillus flavus has picked up significance because of the tainting in pre and post collected sustenance crops. In spite of more extensive conveyance of A. *flavus*, no eco-accommodating anti-aflatoxigenic specialist is accessible against this growth. Phytochemical, for example, quercetin has been accounted for as an anti-aflatoxigenic property.

Wheat:

Wheat belongs to the Triticum of the family Poaceae. This genus is originated within the tropical South west Asia, where it happens in wild in addition as uncultivated forms. Wheat is well custom-made to harsh environments and is generally grown up on windswept back square measures that are too dry and too cold for the additional tropically inclined rice and corn, that do best at intermediate temperature levels. Wheat was initially grown within U.S. in 1602 on the island of the Massachusetts coast. Man has been dependent upon the wheat plant for thousands of years. Wheat genetics is additional sophisticated than that of most different domesticated species. Some wheat species square measure diploid, with 2 sets of chromosomes, however several square measure stable polyploids, with four sets of chromosomes (tetraploid) or six (hexaploid) set of chromosomes [9]. Globally, wheat is that the most-produced food among the cereal crops when rice. The development of a contemporary classification relied on the invention, in the 1918s, of the very fact that wheat was classified in keeping with 3 ploidy levels [9, 12]. The classification of Van Slageren (1994) is maybe the foremost wide used genetic-based classification at this time. Each approaches square measure equally valid and each square measure wide used .Wheat is cultivated worldwide. Globally, wheat is that the most produced food among the cereal crops when rice. Wheat grain could be a staple food won't to make flour for sourdough, flat and steamed, breads; cookies, cakes, breakfast cereal, pasta, noodles; and for fermentation to make beer, alcohol, hard liquor or perhaps biofuel. Durum is that the most ordinarily want to make pasta. The foremost *Triticum aestivum* is the hexaploid wheat and includes *Triticum spelta*, modern bread wheat and corn used for cookies and cakes. Wheat is grown up to a limited extent as a forage crop for livestock, and therefore the straw are often used as fodder

for placental or as a construction material for roofing thatch. Although wheat provides abundant of the world's dietary protein and food provide, as several joined in every one hundred to two hundred folks suffers from Coeliac unwellness, a condition which ends from associate degree system response to a protein found in wheat: protein. Grain fed to placental whole or coarsely ground. Starch is employed for pastes and filler textiles. Straw is changed into mats, carpets, baskets, and used for material, cattle bedding, and paper producing. Some wheat is cut for fodder. Wheat grown up for grain crop is additionally used for pasture before the stems elongate and as a temporary pasturage; it's wholesome and eatable.

Spoilage in wheat:

Molds or fungi growths that develop in grains and seeds amid capacity and transport cause germination decline, obvious mouldiness, staining, smelly or harsh scents, solidifying, synthetic and wholesome changes, decrease in handling quality, and type of mycotoxins [16]. These deteriorative changes influence the evaluation and cost of grain and add to client disappointment when the grain is promoted. The breath of grain and parasites results in a misfortune in dry issue just as the creation of warmth and dampness which add to assist decay. Net changes in dietary benefit and the danger of mycotoxin tainting are hard to foresee in light of the fact that they rely upon an intricate connection of components, for example, temperature, dampness, stockpiling time, parasitic species arrangement, sort of grain, and past capacity history. Dampness is the most significant variable deciding the rate of crumbling brought about by organisms, with temperature being the second main consideration. Issues with crumbling of grain in fare shipments are not an ongoing improvement. They are connected fundamentally to grain dampness at stacking, and furthermore to the degree of past shape intrusion [15].

Aspergillus flavus misuses assorted systems to endure introduction to antifungal specialists including morphogenesis.The morphological progress of *Aspergillus flavus* from torpid conidial to mycelial arrange is basic for it to be an effective pathogen. *A. flavus* conidia colonize on yields under the good condition and on effective germination produce cancer-causing aflatoxin. It exists in three structures viz. conidia, mycelia, and hyphae and metabolic adjustment are required for the isotropic development of the conidia to sprout into the hyphae [1, 3]. The fundamental mycotoxins classes that happen in cereals are the aflatoxins (AFB1, AFB2 and AFG1, G2), the tricotecens, deoxinivalenol (DON) and (T-2 poison), the fumonisins (FB1, FB2 and FB3), the zearalenone (ZON), ochratoxin An (OTA)

and the ergot alkaloids, most of the mycotoxins in these gatherings are delivered by three parasites sort:*Aspergillus*, *Penicillium* and *Fusarium* in Council for Agricultural Science and Technology. The types of the *Aspergillus* and *Penicillium* family are the ones that multiply simpler in put away grain [17]. Some parasite create in various temperatures and produce poisons in actually low temperatures, for example, the ones from the Fusarium species sort, this is one reason why in some Brazilian districts the winter crop grain have various poisons from those created amid the warm seasons [16].

CHAPTER 3

AIM AND OBJECTIVE

- To study the host pathogen interaction during infected *Triticum aestivum* with *Aspergillus flavus* under laboratory conditions
- To study the gene expression profile of *Crz1*, *Rho-GDP* of *Aspergillus flavus* during the interaction with *Triticum aestivum* at 3^{rd} and 5^{th} day post-infection

CHAPTER 4

MATERIAL AND METHODS

4.1. Sourcing:

Wheat grains which commonly used on the regular basis were bought from the market. This particular grain was selected to be the host as they are cereals which are usually stored for long term. Stored wheat grains usually show loss in both quality and quantity. These are commonly influenced by both abiotic and biotic factors and *Aspergillus flavus* is one of the most common grain pathogen which leads to mycotoxin production [4]. Hence *Triticum aestivum*, commonly known as Wheat is used as host here for checking the signalling pathway of the fungal growth.

The strain used to infect the wheat grains was *Aspergillus flavus* (MTCC 11866)

4.2. Isolation of pure isolates:

4.2.1 Conidia harvesting

Asexual propogule were formed in various microorganisms which are termed as conidia. Asexual propogule were formed in sac like tissues called as sporangia. After maturation in sporangia, conidia are exposed to outer environment. Conidia are capable of germination and developing a new generation.

Harvesting of conidia is needed as it will give rise to pure colony of respective conidia spores, easy to handle and spores can be easily counted for optimized inoculation in media. Best method to harvest conidia is from phosphate buffer saline solution having TWEEN.

Chemical Reagents:

A) Phosphate buffer saline

Table 1: Composition of PBS

S.No.	Chemical name	For 1000ml
1	Sodium chloride (NaCl)	8.0 g
2	Potassium Chloride (KCl)	0.2 g
3	Sodium phosphate, dibasic anhydrous ($Na_2HPO_4.2H_2O$)	1.44 g
4	Potassium phosphate monobasic (KH_2PO_4)	0.24 g
5	Distilled water	1000 ml

B) Phosphate buffer saline tween

- The PBST was prepared by adding 200µl of tween detergent in 100 ml of PBS followed by autoclaving.

  Note: Do not mix it thoroughly so as to avoid foam formation and keep headspace for avoiding overflowing during autoclave.

Procedure:

- Plate containing fully grown conidia was taken.
- 5-6 ml of PBST was added to the culture.
- The PBST was mixed gently so as to obtain only the upper conidia part without harming the mat.
- Approximately 2ml of the mixture was taken in eppendorf.
- The eppendorf was then centrifuged at 10,000 rpm for 5 minutes at 4°C.
- The supernatant was then discarded and the pellets were obtained.
- Equal volume of PBS was added to each eppendorf and mixed well.
- It was again centrifuged at 10,000 rpm for 5 minutes at 4°C.
- Again the supernatant was discarded and equal volume of PBS was added again.
- This was then further stored for future use.
- 200µl of the obtained conidia was put on PDA plates followed by incubation at 37°C.

4.2.2 Subculturing

For subculturing microorganisms need an environment which can mimic actual growth condition of that microbe *in-vitro*. Composition of media were altered and adjusted to make the growth of cells *in-vitro*. Subculturing is done by inoculating single colony of cells into a fresh plate. Subculturing is necessary as after a interval of time period there is accumulation of toxic metabolites in the media, nutrient deficiency is also there and the cell number increased due to growth which eventually prevent further growth of cell in that media. The cell number increase due to growth eventually prevents further growth of cells. Subculturing is also important to note down the appropriate passage number.

Subculturing of *Aspergillus flavus* was made in Potato dextrose agar (PDA) plates. PDA plates were used for the cultivation of fungus. It is a general media for growing fungus with supplements such as antibiotics which help in inhibiting bacterial growth. It is composed of potato infusion (Dehydrated) and dextrose which help in encouraging fungus growth. Solidifying agent is agar. Chloramphenicol can be added for inhibiting bacterial growth.

Potato dextrose agar: pH should be maintained at 5.6±0.2

Table 2: Composition of PDA

S.NO	CHEMICAL NAME	For 1000ml
1	Potato infusion/potato extract	200g/4g
2	Dextrose	20g
3	Agar	20g
4	Distilled water	1000ml

Procedure:

- Conidia from previously harvested vials were taken.
- 200µL of conidia were taken and inoculated into the fresh PDA plates.
- Plates were kept in the incubator at 37°C for growth.

4.3. Microscopy

4.3.1 Chemical Reagent

- Lacto phenol cotton blue(LCB)

4.3.2 Staining

- A clean glass slide was taken.
- Drop of LCB was placed on the slide.
- Fungal culture from the PDA plate were taken and put on the drop of LCB.
- It was then gently teased to mix the fungal culture with LCB.
- Cover slip was placed and kept undisturbed.
- Prepared slide was then observed under microscope at 100, 400 and 1000 magnification.

4.4. Preparation for infecting wheat

4.4.1 Serial dilution:

- From the previously stored conidia in PBS, 100µL was transferred to the new vial and 900µL of PBS was added into it.
- This process of serial dilution was done till 10^{-6} times.

4.4.2 Spore counting:

To count cells/spores in a sample solution hemocytometer is used. It is a device which helps to count the number of spores present in the solution. The area under the lines and the depth of chamber was known which make it possible to count the cells per chamber under microscope.

Formula for counting cells in haemocytometer:

a) Count number of spores in each large chamber in the corner.

b) Total number of spores is calculated.

Spores per ml = Average spores counted $*10^4$.

Procedure:

- Hemocytometer was cleaned using 70% ethanol.
- The cover slip was properly placed on the hemocytometer.
- Cell suspension was applied from the edge of the hemocytometer.
- Hemocytometer was observed under microscope.
- Spores present in each square were counted.

4.3 Infection of wheat seeds:

- Total numbers of seeds were counted to be taken for infection.
- Seeds were soaked with autoclaved distilled water for 3 hours.
- After soaking, seeds were autoclaved.
- 1ml of suspended conidia from 10^6 dilutions were taken and spread on the autoclaved seeds taken in two separate plates.
- Plates were kept in incubator at 37°C.
- After 3rd and 5th day growth of fungus was observed on the seeds.
- Total RNA isolation was made from the 3rd and 5th day of infection.

4.5. RNA isolation using TRIzol method:

Ribonucleic acid is the one strand nucleic acid found in both eukaryotes and prokaryotes. Total RNA isolation is a vital step in the study of genomics. The total RNA includes mRNA, tRNA and mRNA. The total RNA isolation is important for the gene expression analysis which includes cDNA library synthesis, microarray analysis, northern analysis and RT-PCR. Organic purification includes TRIzol, acidified phenol and chloroform. The TRIzol reagent is basically composed of phenol and guanidinium isothiocyanate which has the property of solubilizing biological material and denaturing protein whereas in the later steps addition of chloroform leads to phase separation into aqueous, interphase and organic phase [35].

Reagents required:

- PBST prepared with DEPC water
- PBS
- Liquid nitrogen
- TRIzol reagent
- Chloroform

- Isopropanol
- 70% ethanol
- Absolute ethanol

The following flow chart represents the steps followed for total RNA isolation using TRIzol reagent

To the infected seed 5ml of PBST was added and conidia was recovered

↓

The mixture was divided into different vials

↓

The vials were centrifuged at 10000 rpm for 5 minutes and supernatant was discarded

↓

PBS was added to the pellets and again centrifuged at 10,000 rpm for 5 min

↓

Now the pellets were put into mortar pestle and liquid nitrogen was added to it

↓

After crushing, 5ml of TRIzol reagent was added to the crushed cells

↓

The mixture was put into different vials and incubated for 5 min at RT.

↓

500µL of chloroform was added and the vials were vortexed for about 15 seconds

↓

The vials were incubated at RT for 2-3 min. followed by centrifuge at 4,000 rpm at 4 $^{\circ}$C for 30 min.

The mixture was then divided into three phases from which the upper clear phase was transferred into another vial

250µL of isopropanol was added to the freshly prepared vials followed by inverting it for 10 mins. at RT

The RNA was recovered by centrifugation at 12,000 rpm at 4 $^{\circ}$C for 5 mins. and the supernatant was discarded

Pellets were washed twice using 70% ethanol by centrifuging the sample at 10,000 rpm for 2mins.

The final ethanol was removed and the pellets were kept to dry

20µL of RNase free DEPC water was added to the pellets

The extracted RNA was visualized on 1.5% agarose gel

4.6. cDNA synthesis:

cDNA synthesis is a major step in the gene expression analysis. The cDNA is the DNA which is synthesized from single stranded RNA using reverse transcriptase enzyme. The enzyme reverse transcriptase uses the single stranded mRNA and oligo (dt) primers complementary to the mRNA strand in order to synthesize the first cDNA strand which further act as template

for PCR. Low abundance of RNA is usually detected by the combination of the enzyme and the PCR. The reverse transcriptase enzyme has different activities which includes firstly, the RNA-dependent DNA polymerase activity required for the production of the first strand of cDNA. Secondly, DNA-dependent DNA polymerase and finally, nuclease activity of RNase H which helps in degrading the RNA-DNA hybrid. The sequence of cDNA produced is complementary to the mRNA sequence [36].

The synthesis of cDNA was done using Verso cDNA synthesis kit which includes the following:

Table 3: Compounds in cDNA synthesis kit

S.No.	Components
1	Verso enzyme mix
2	5× cDNA synthesis buffer
3	Anchored oligo dt(500 ng/µL)
4	Random hexamer (400 ng/µL)
5	dNTP Mix (5mM each)
6	RT enhancer

The reaction mixture per vial is kept 20µL. The amount of each component required for 1 reaction is as follows (table 4):

Table 4-Amount needed for 1× PCR reaction

S.No	Component	Amount(in µL)
1	5x cDNA synthesis buffer	4
2	dNTPS mix	2
3	RNA primer/ Oligo dT	1
4	RT enhancer	1
5	Verso enzyme mix	1

6	Template (RNA)	2
7	Nuclease free water	9
	TOTAL	**20µL**

Master mix which did not include template was prepared according the number of samples and then divided into different vials. Template was added to each vial and then it was vortexed in order to mix the template with the master mix. The PCR machine was set with the PCR cycle required for the cDNA synthesis. The cycle was as followed(table 5):

Table 5- Condition for cDNA Synthesis

S.No.	**Objective**	**Temperature (°C)**	**Time (min.)**	**No. of cycles**
1	cDNA synthesis	42	30	1
2	Inactivation	95	2	1

4.7. Polymerase Chain Reaction:

Polymerase chain reaction is a simple and enzymatic process for amplification of DNA fragments. Mentioned compounds were taken in appropriate amount and placed in thermo cycler machine for amplification which occurs in three basic steps. First the reaction mixture was heated above the melting temperature of two strands for the denaturation. After denaturation, temperature was lowered to attach the primers it is known as annealing temperature. After annealing the temperature was again raised for DNA polymerase to add nucleotide in the developing DNA strand. After each cycle the amount of DNA was doubled.

To check the proper cDNA synthesis, semi quantitative PCR was run using *tubulin* as housekeeping gene. The total volume was 12.5ml. The composition for 1× PCR reaction is represented in table 6 while the PCR condition is represented in table 7.

Table 6- Composition in 1× PCR reaction

S.No.	Reagent	Volume(µL)
1	10× Buffer	2
2	dNTPs	0.5
3	Forward primer tubulin	0.5
4	Reverse primer tubulin	0.5
5	Taq polymerase	0.25
6	cDNA	1
7	Nuclease free water	7.75

Table 7- Temperature duration and cycle for PCR

Steps	Temperature(°C)	Duration	Cycle
Initial denaturation	95	4:00	x 1
Final denaturation	95	0:30	x 35
Annealing	56.2	0:30	x 35
Initial extension	72	1:00	x 35
Final extension	72	7:00	x 1

4.8. RT-PCR

RT-PCR is a technique useful in studying the gene expression quality and also RNA quantification. In this technique SYBR green is used in RT-PCR as it is a double stranded DNA binding dye which intercalates non-specifically into the amplified DNA strand. This intercalation eventually helps in measurement of the amount of the PCR product after every cycle. After every PCR cycle the amount of the DNA increases and hence the number of SYBR green molecule which binds to the product also increases. Since the amount of product is directly proportional to the increase in fluorescence, SYBR green can be utilized for quantification. Since the assay is done by detection of accumulated fluorescent signal, a threshold is set for the detection. The cycle threshold value is obtained after the whole PCR cycle which represents the number of cycle which is required for the fluorescent signal to cross the threshold [41]. Every peak formed has a different C_T value. The lesser is the C_T value the higher is the expression of the gene in the template provided.

Table 8 shows the component and the amount required for 1x reaction of the RT-PCR, where the volume of one vial is 12.5µL.

Table 8- Reaction Mixture for RT-PCR

S.No.	Component	Amount(µL)
1	SYBR green	6.5
2	Reverse primer	0.5
3	Forward primer	0.5
4	cDNA template	1
5	Nuclease free water	4
	TOTAL	**12.5µL**

Master Mix which did not include the template was prepared according to the number of sample with primers of different gene separately. Once the master mix was prepared, they were now divided according to the gene and sample. Master Mix was also prepared for NTC which did not include any cDNA template. Every reaction was run with duplicates. Once template is added to each of the vial, they were thoroughly mixed and the RT-PCR was set.

It was kept in mind that the primers used had same annealing temperature for normal functioning. We used two gene *Crz1* & *Rho-GDP*. Two different reactions were set up because the annealing temperature was 54° and 52° respectively [2].

The sequences of the former primers are (table 9):

Table 9- Forward and reverse sequence of the Primer for gene of interest

S.No.	PRIMER	Tm(°C)	*Forward and Reverse sequence*
1	***Crz1***	54	5'-CCACCATCCATTAACGTGG-3' 5'-CGGATCAGATTTGCTACGC-3'
2	***Rho-GDP***	52	5'-CGAGCTATAAATCCCGAGG-3' 5'-GTCGTTAAGAGGAAGGGTG-3'

The C_T value was obtained for the different reactions after the RT-PCR cycles were completed.

CHAPTER 5

RESULT AND DISCUSSION

5.1 Subculturing

After the subculturing with harvested conidia of *Aspergillus flavus* BT05 on PDA plates, it was observed that intially within three days of subculturing there were while colour of mycelia or the mat formed on the plates. With time the colour changed from white to olive to dark green colour. The colonies observed in figure 1 were found to be merged due to fact that we did spread the harvested conidia on the plate, they were flat on the edges but wrinkled in the middle with some droplets of liquid which were somwhat brown in color.

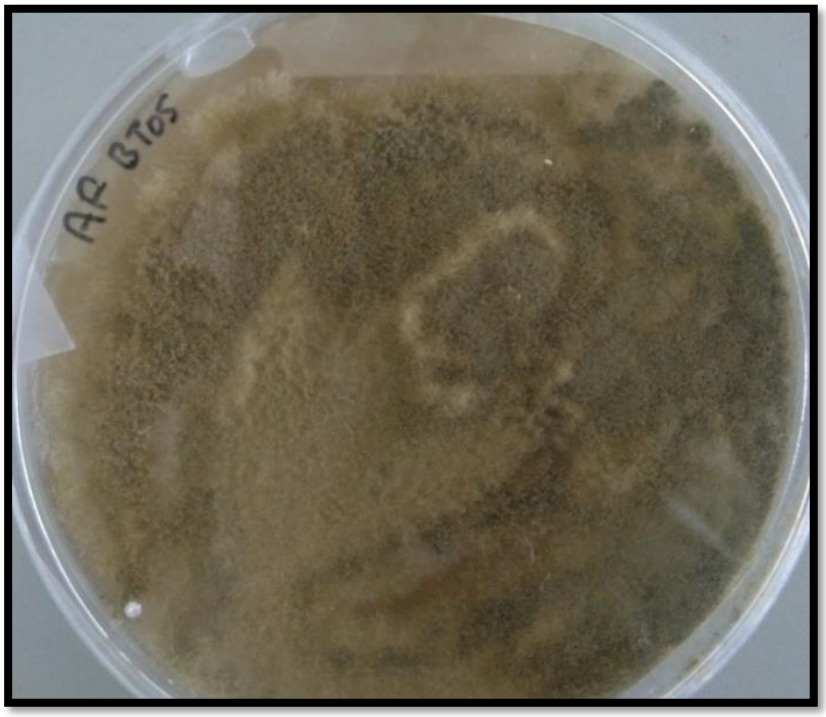


Figure 1 – *Aspergillus flavus* MTCCBT05 subcultured on PDA Plates

5.2 Microscopy

LCB was used to stain and observe the hyphae growth and also the conidia for both the third and the fifth day of infection (figure 2 & 3). Conidia growth was observed to be increasing with the number of days. Dense growh of fungus was observed on the fifth day in comparison to the third day of the infection on the wheat grain.

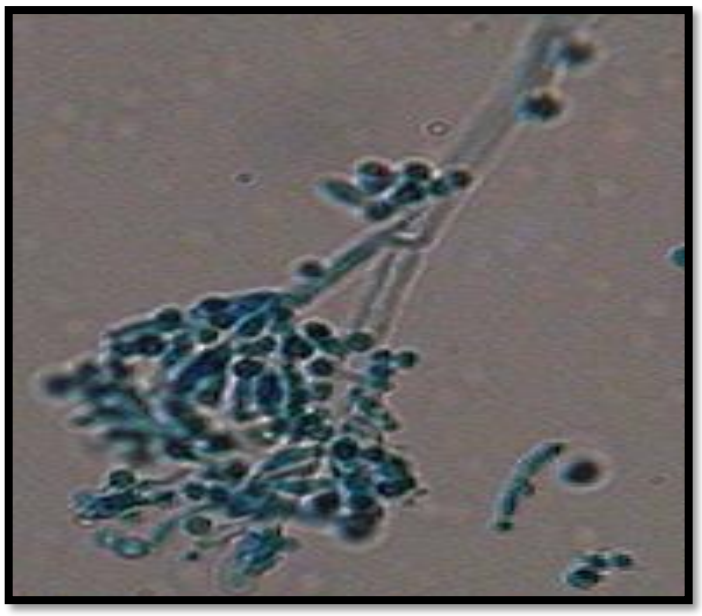


Figure 2 – *Aspergillus flavus* stained using LCB observed under 400 magnification of compound microscope

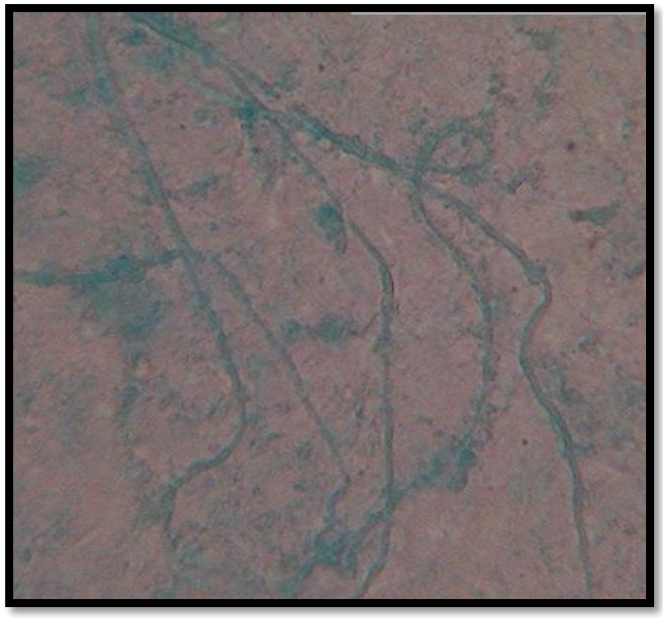


Figure 3- *Aspergillus flavus* stained using LCB and observed under 100 magnification of compound microscope

5.3 Preparation for infecting wheat seeds:

On infection of wheat grains with the conidia, the plates were kept in incubator at 30ºC and observed regularly. Fungal growth was observed on third and fifth day of infection of wheat seeds.

5.3.1 Spore counting

After the serial dilution of the conidia hemocytometer was used to count the number of cells per ml of solution. The following number of spores were counted in each square of the hemocytometer as represented in table 10.

Table 10- Numbers of spores in each square of hemocytometer

S.No.	Square	No.of spores
1	Top left	72
2	Top right	60
3	Down left	41
4	Down right	68
	TOTAL	**241**

The average number of spores = **60.2**

Cells/ml= average no. of cell* 10^4

Cells/ml= **$6.02*10^5$ cells/ml**

5.3.2 Infection of wheat seeds:

Previously soaked and autoclaved wheat grains (figure 4 & 5) were infected with the conidia as shown in figure 6, the plates were kept in incubator at 30 °C and observed regularly. Fungal growth was observed each day and we noticed the transition of color from white to the dark green.

Figure 4-*Triticum aestivum* seeds were soaked in autoclved distilled water for 3 hours

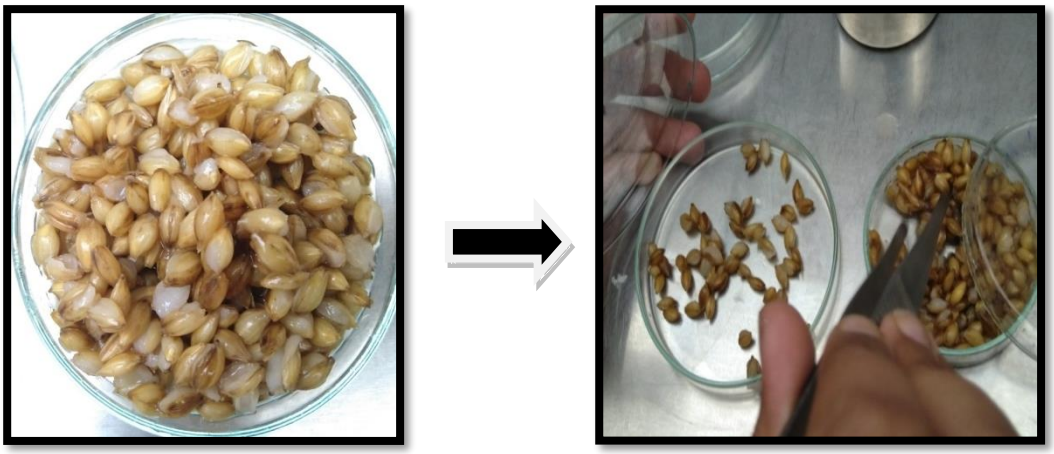


Figure 5- *Triticum aestivum* seeds after autoclave

Figure 6- Infection was made

Growth after 3rd day:

On the third day of infection, white mycelia growth was observed on the wheat grains as shown in figure 7. All the grains were properly covered with the white mycelia coat and indicated infection of all the seeds.

Figure 7-*Aspergillus flavus* growing on wheat seeds after 3rd day of infection

Growth after 5th day:

After five day of infection, fully grown fungus was observed on the seeds with green coloured conidia (figure 8). Every seed was found to have the fungal growth indicating the fat that every grain was infected equally.

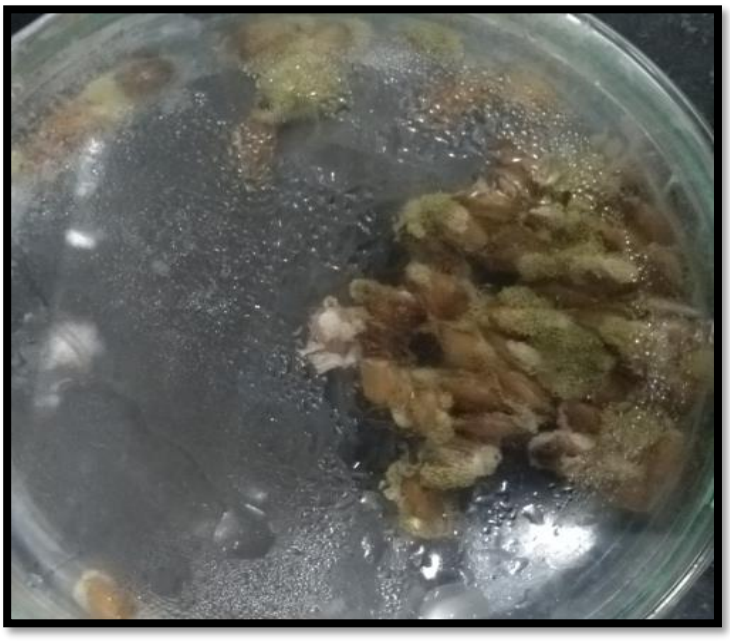


Figure 8 – *Aespergillus flavus* growing on wheat seeds after 5th day

5.4 RNA isolation

Total RNA isolation was done followed by gel electrophoresis of the sample on 1.5% agarose gel. Lane number 1 represents 1kb ladder. Lane number 9 represents RNA isolated from infected grains after 3 day of infection. A slightly clear band was observed (figure 9) in the lane no.9 parallel to the 5000bp band of the ladder which is approximatley 5000bp.

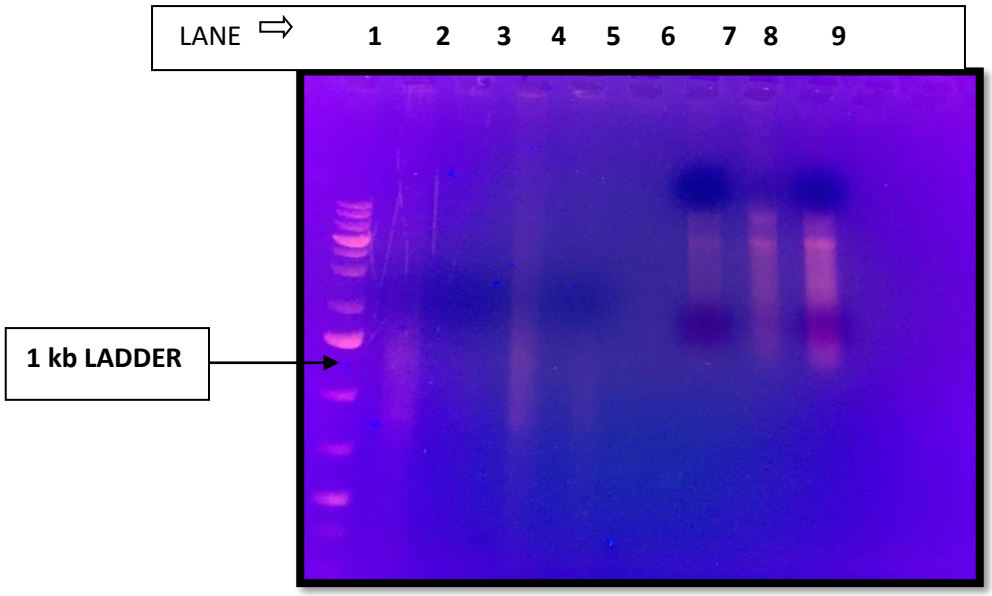


Figure 9- Isolated RNA bands from 3rd day of infection on 1% agarose gel

Total RNA isolation of fungus as on 5th day was observed on 1.5% agarose gel by gel electrophoresis. Lane no. 8,9 and 10 were loaded with RNA sample of 5th day of infection. No clear band was observed in the lane 8 and 10. A slightly clear band was observed in the lane no.9 representing the presence of RNA in the sample as shown in figure 10.

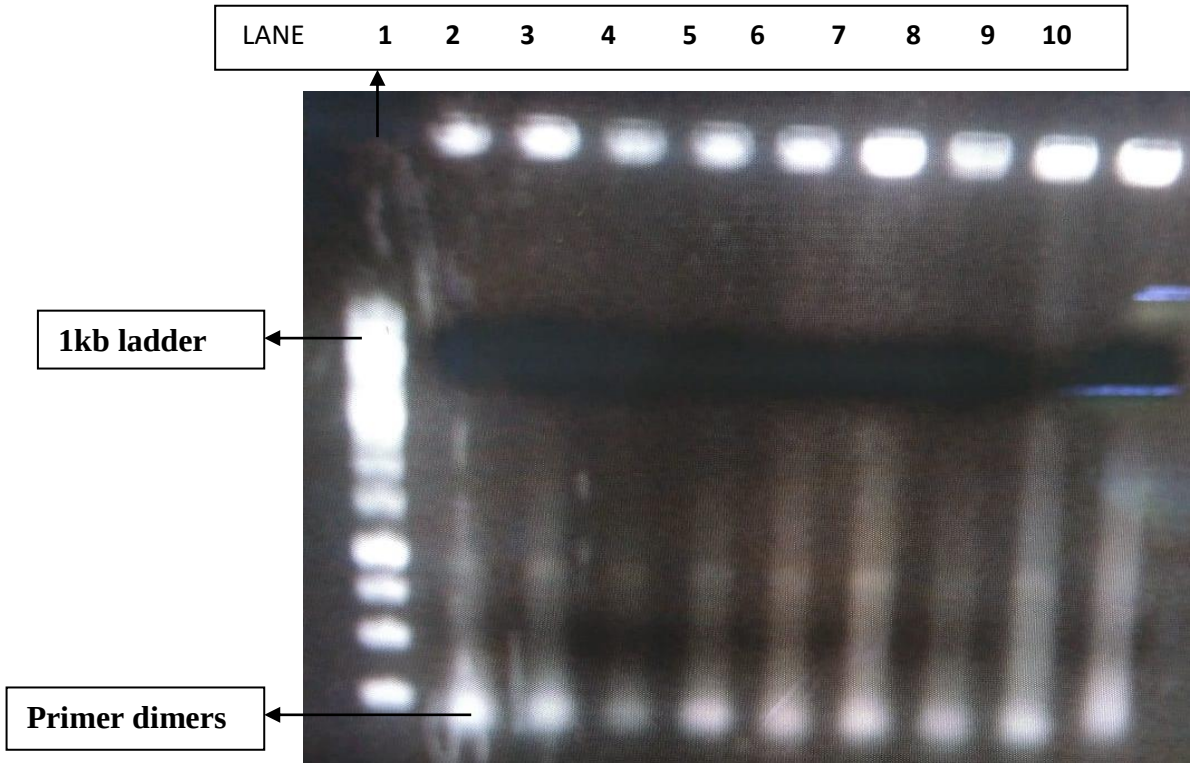


Figure 10- Isolated RNA bands from 5th day of infection visualized on 1% agarose gel

5.5 cDNA synthesis

cDNA synthesis was done through PCR and to check if the quanlity of cDNA synthesized PCR using housekeeping gene such as Tubuline in case if *Aspergillus flavus* is done.

5.6 PCR with tubulin gene:

The expected outcome of cDNA check PCR is two band of 440 and 480 bp [42]. 1.5% agarose gel was used to visualize the PCR products. The cDNA sample for 3rd and 5th day of infecting fungus was put in lane no. 7 and 8. One clear and another slightly clear bands were observed confirming the cDNA synthesis check for the samples.

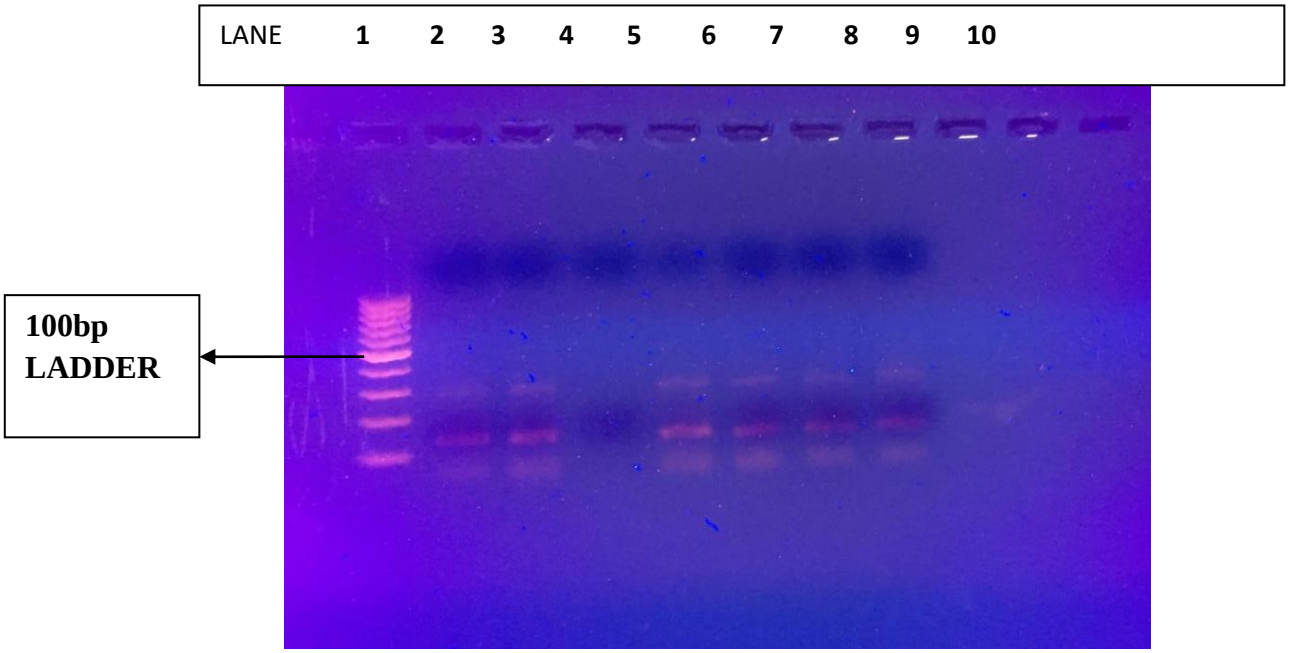


Figure 11: cDNA bands after PCR with *Tubulin*

5.7 RT-PCR

Real time PCR of the synthesized cDNA sample with gene of interest was done. The gene opted for the expression analysis were calcium signalling pathway gene *Crz1* gene and *Rho-GDP* genes. The following C_T value as represented in table 11 & 12 for 3rd and 5th day was obtained for the the following two gene.

Table 11- C_T value of *Crz1* gene normalized using tubulin

Days after infection	**Mean value of tubulin gene**	**Mean value of *Crz1* gene**	**Normalized with tubulin**
3rd day	23.64	31.3	7.66
5th day	24.19	35.44	11.25

- The expression of *Crz1* gene was high on the 3rd day while decreased on the 5th day which means that the expression was down-regulated on the 5th day of infection.

Table 12 – C_T value of *Rho-gdp* gene normalized using tubulin

Days after infection	**Mean value of tubulin gene**	**Mean value of *Rho-GDP* gene**	**Normalized with tubulin**
3rd day	23.64	33.05	9.41
5th day	24.19	26.81	2.62

- The expression of *Rho-GDP* gene was low on the 3rd day in comparison to the 5th day which depicts that there was up-regulation of expression of *Rho-GDP* gene on the 5th day of infection.

Part 2

Characterization and Isolation of unknown fungi growing on formaldehyde solution

1. INTRODUCTION

Volatile organic compounds are multi faceted mixtures of low molecular weight compounds derived from diverse biosynthetic pathways. They are characterized by their high volatility, low vapour pressure and low water solubility. They are present in our daily life; we are constantly exposed to smells from a number of sources such as fragrance and surrounding volatiles from indoor furnishing or biological matter. These compounds are released from burning fuels such as gasoline, wood, coal, or natural gas. They are also released from many consumer products such as cigarettes, solvents, paint, glue, air freshener and pesticides.

When these compounds combine with nitrogen oxides in air they form smog. VOCs commonly enters the body thorough inhalation and skin contact, exposure various VOCs may cause irritation of the eye and respiratory tract, headache, dizziness, memory problem whereas long term exposure to various VOCs may cause fatigue, loss of coordination, damage to liver, kidney, CNS or can even cause cancer. Some of the volatile organic compounds which are eventually harmful to the environment but are used are benzene, ethylene glycol, formaldehyde, methylene chloride, xylene, toluene, etc.

A fungal culture was found to be growing on a plant sample preserved in formaldehyde, which leads to an idea that it has a capability to degrade the volatile organic compounds which have many industrial applications.

Formaldehyde is a colourless, strong smelling gas used in making building materials and many household products. When dissolved in water it is called formalin, which is commonly used as an industrial disinfectant, and as a preservative in funeral homes and medical labs .Formaldehyde also occurs naturally in the environment and human body and other living organisms make small amount as part of normal metabolic processes.

Formaldehyde help in improving everyday life by producing hundreds of items that are necessary ,use in many industries such as chemical , wood , textiles etc . It is the simplest aldehyde (R-CHO) which is volatile in nature and known to be a human carcinogen which

can diffuse easily in many tissues and form cross linking by reacting with many macromolecules such as DNA-DNA, DNA- proteins and protein-protein.

It causes environmental worsening and human diseases when discharged into water bodies untreated. Many bacterial strains are known to be degrading formaldehyde as compared to fungal strain.

Since there was a growth of fungus found on plant sample preserved in formaldehyde it was suspected that it used formaldehyde as its sole carbon source. Having observed this characteristic there came out a big question on the usage of formaldehyde in case of preservation and also the capability of micro organisms to degrade VOCs. Since we know that VOCs are a great threat to environment in today's world this would be a great success if we find any such microbe that could degrade these.

After the sub-culturing of the culture found there were clearly two different species of fungi which were isolated. The growth of this consortium lead to an idea of finding out whether, they are capable of degrading other volatile organic compounds like benzene or xylene. Plates of minimal media and Bushnell Hass agar without any carbon source were used for this experimentation where the pH was maintained to favour fungal growth. The carbon source for the media was benzene and xylene in order to see whether these fungal cultures are capable of degrading them or not.

On successful finding or isolation of fungus which are capable of degrading VOCs would be a great step towards fighting pollution which is the biggest concern in today's world. These fungi can then be used in the area where effluents are released or they can be used to treat the effluent before being discharged in the environment. Hence in this particular way these microbes would help in reducing pollution.

2. REVIEW OF LITERATURE

Formaldehyde is highly reactive chemical compound widely used in industries. It has a toxic effects on all living organisms, it is classified as human carcinogen by IARC (2006) (International Agency for Research on Cancer) .Degradation of formaldehyde was observed by many bacteria. Many microorganisms have been shown to degrade formaldehyde in the sewage, and bacterial degradability has been the research hotspot on formaldehyde

degradation. There is lesser known fungal culture which shows these kind of activities. Filamentous fungus plays an important role in bioremediation in wastewater treatment. Fungi performed oxidation reaction as a prelude to the detoxification and excretion of hydrocarbon rather than using these compounds carbon sources for growth [34]. Interestingly, Hartmens, Van der werf and De bont (1990) isolated two fungi able to grow on styrene as a sole carbon and energy sources.

Formaldehyde is grouped under volatile organic compounds. The previous research was performed using the sample from sewerage of a furniture factory which was then cultivated in sterilized basal medium with supplemented formaldehyde followed by plating for a single colony. Further identification and characterisation of the fungus was carried out by genome sequencing. The genomic DNA was obtained from the mycelia and partial fragments of ITS region (internal transcribed spacer region of the ribosomal DNA containing ITS1, ITS2, and 5.8S rRNA gene) and *β-tubulin* gene were amplified for sequence analysis [37].

Alignment of the determined sequences and those of related ITS regions and *b-tubulin* sequences from the GenBank database. The phylogenetic trees were constructed by the neighbor-joining method with bootstrap analysis.

The optimal temperature for isolates growth was determined by measuring the dry cell mass of mycelia under different temperature. Formaldehyde resistant was observed by inoculating isolates with different initial formaldehyde concentrations were screened in liquid medium. A basal medium supplemented with 300 mg l-1 formaldehyde was set as enzyme-inducing medium, and a basal medium without formaldehyde was used as the control. Mycelia were then collected by filtration and washed well with sodium phosphate buffer. Crude enzyme extract was obtained through sonication. Enzyme activities of GSH-dependent FADH and FDH were assayed at 340 nm using spectrophotometer by monitoring the formation of NADH using a photometer.

One of the strain, *Exophiala jeanselmei,* was used successfully for treating the styrene polluted air in biofilter [33]. More recently, deuteromycete *cladosporium sphaerospermum* was isolated from biofilter that had been used to remove toluene from contaminated air [34, 39].

3. AIMS AND OBJECTIVE

The aim of the study is to characterise and isolate fungal culture found growing on plant sample preserved in formaldehyde solution. The objectives are:

1) To characterise and isolate the fungal cultures found growing on plant sample preserved in formaldehyde solution.

2) To observe the benzene, xylene and formaldehyde degrading capacity of each isolates.

3) To characterise the isolate capable of degrading any one of them by DNA isolation, RNA isolation and sequencing.

4. MATERIALS AND METHODS

4.1 Sourcing

The fungal consortium was found to be growing on a plant sample preserved in formaldehyde solution. The culture was collected plated on potato dextrose agar which was further used as master plate (figure1) for the further experimentation.

Figure 1: PDA master plate of fungal consortium found on formaldehyde

4.2.Subculturing techniques:

4.2.1 Chemical reagents:

1. PBS
2. PBST
3. PDA: pH should be maintained at 5.6±0.2

4. PDB: pH should be maintained at 5.6±0.2

Table 1: Composition of PDB

S.NO	CHEMICAL NAME	Amt for 1000ml
1	Potato infusion/potato extract	200g/4g
2	Dextrose	20g
3	Distilled water	1000ml

The pH was maintained at 5.5 to favour fungal growth.

4.2.2 Techniques:

1. Direct Dusting

- Fungal culture with fully grown conidia was taken.
- With the help of a sterile inoculating loop or autoclaved tip the conidia was slightly taken.
- It was then dusted upon the PDA plates followed by incubation at 37 °C or dusted in PDB followed by their incubation in shaker incubator.

2. Conidia Harvesting

Conidia harvesting was done using the protocol as explained in part I on page number 21.

4.3 Staining and microscopy:

4.3.1 Chemical reagent

❖ LCB

4.3.2 Staining

Staining of each isolates (I & II) was carried out using the protocol as explained in part I on page number 24(3.2).

4.4 Serial dilution & spore counting

Serial dilution and spore counting was done using the protocol as explained in part I page number 24 & 25.

4.5 . Experimental set-up

4.5.1 Using Bushnell Hass Agar media (carbon free media for bacterial culture)

5.1.1 Chemical reagents:

- Bushnell Hass agar media
- Benzene
- Xylene
- Sucrose

4.5.1.2 Material required

- syringe filter
- hot plate
- autoclave
- pH meter

4.5.1.3 Procedure

- 11.6 grams of Bushnell Hass Agar media was added in 500ml of distilled water.
- pH was set between 5.5 in order to support fungal growth..
- It was then divided into four equal parts.
- The four flasks were then autoclaved.
- Sucrose stock solution was prepared.
- After autoclave benzene, xylene and sucrose was added using syringe filter in LAF in three flasks respectively in 1 % according to the total volume of media.
- One flask was kept as control as it didn't contain any carbon source.
- Plates were prepared using the media containing different carbon source.
- When the plates were solidified inoculation was done using the harvested conidia.
- The plates were then incubated at 37°C until growth was found.

4.5.2 Using minimal media for fungal growth

5.2.1 Media composition

Table 2 – Composition for minimal media

S.NO.	Chemical name	Amt for 1000ml
1	Sodium nitrate ($NaNO_3$)	1.0 g
2	Potassium phosphate, monobasic (KH_2PO_4)	1.8 g
3	Magnesium sulphate($MgSO_4.7H_2O$)	0.9 g
4	Potassium chloride (KCl)	0.5 g
5	Calcium chloride ($CaCl_2$)	0.1 g
6	Ferrous sulphate ($FeSO_4.7H_2O$)	1.0 g
7	Distilled water	1000 ml

4.5.2.2 Media preparation:

- Conical flask was taken.
- 250 ml of water was added to it with a magnetic bead.
- This setup was then set up on magnetic stirrer.
- Each component was then weighed separately and added only after the first one is dissolved.
- The volume was increased if the components did not dissolve.
- Finally the volume was made up to 1000ml.
- pH was set between 5.5.
- Once the media was prepared it was again divided into four parts.
- These were then autoclaved.
- After autoclave benzene, xylene and formaldehyde was added using syringe filter to each flask respectively in 0.5 % according to the total volume of media whereas the fourth one was kept as carbon free media.

4.5.2.3 Procedure

- Harvested conidia were used as inoculums.
- Each flask was inoculated with 200µl of the conidia.
- The flasks were then kept for incubation at 37 $^{\circ}$C in shaker incubator.

4.5.3 Using NCIMB medium (no.18) for unknown fungal growth

NCIMB is an international organization which provides specialist in microbiology, chemical analysis, biomaterial storage product and services to their clients in their respective researches, patents etc. NCIM also recommends media for the revival or subculturing of the microbes where the components specified are the original formulations as prescribed by the depositors in the repositories. The NCIMB medium no.18 was used to subculture the unknown fungal consortium. This particular medium id prescribed for fungal growth and was used in this experiment as they did not contain any specified carbon source which was actually our aim. The carbon less medium composition was used so that formaldehyde and benzene could be used as the sole carbon source here and degradation could be observed. The composition of the media contains mixture of solutions (part I and part II). The following tables show the component of both solution I (table 5) and II (table 6).

Solution part I (for 1000ml)

Table 3 – Amounts needed for preparing solution I

S.No	**Chemicals**	**Amount**
1	$(NH)_2SO_4$	0.5 g
2	K_2HPO_4	0.5 g
3	$MgSO_4.7H_2O$	0.5 g
4	1N H_2SO_4	5 ml
5	Distilled water	1000ml

Solution part II (for 100ml**)**

Table 4 –Amounts needed for preparing solution II

S.No.	Chemical name	Concentration/ amount
1	$FeSO_4.7H_2O$	167mg
2	1N H_2SO_4	50ml
3	Distilled water	100ml

After the preparation of both the solution, solution I was autoclaved while solution II was ultra filtered. Once the sterilization is completed, 4 parts of solution I was added to 1 part of solution II. The pH was set at 3.0. Carbon source was added to the final medium with the concentration of 1mg/ml.

- Three different flask containing three different carbon source was prepared. The carbon sources were formaldehyde, benzene and glucose.
- Colonies from pre-cultured plates were picked.
- Each flask was inoculated with a colony of the unknown fungus.
- The flasks were kept at 30 $^{\circ}$C in shaker incubator.
- Since previously it was observed that the fungus grew at lower temperature the flasks were transferred 4 $^{\circ}$C for better growth.

4.5.2.4 Procedure of inoculation of isolates:

- ❖ Harvested conidia were used as inoculums.
- ❖ Each flask was inoculated with 200µl of the conidia.
- ❖ The flasks were then kept for incubation at 37 $^{\circ}$C in shaker incubator.

4.6. DNA extraction

4.6.1 Chemical reagents:

- Liquid nitrogen
- Extraction buffer
- Phenol: chloroform (1:1)

- Chloroform
- Chilled isopropanol
- 70% ethanol
- TE buffer
- TAE buffer
- Agarose

4.6.1.1 Preparation of extraction buffer:

1. 5M NaCl - 29.2 gm of NaCl was added in 100ml of distilled water.

2. 10% SDS

- 10 gram SDS taken in 250m L conical flask.
- 80 ml of deionised water was taken and mixed it.
- Flask was heated at 68 $^{\circ}$C
- Volume was adjusted to 100 ml.

Table 5 – Composition for making extraction Buffer

Sr no.	Chemicals	Amount for 1000ml
1.	1M Tris HCl (p H 7.5)	200ml
2.	0.5 EDTA(p H – 8)	50ml
3.	10% SDS	50ml
4.	5M NaCl	50ml
5.	Double distilled water	650ml

4.6.1.2 Preparation of 1×TAE buffer:

1 × TAE (for 500 ml 1× TAE): 10 ml TAE was added in 490 ml of distilled water.

4.6.1.3 Preparation of 0.8% Agarose:

- 0.8 gm of agarose was added in 100 ml of 1× TAE.
- Dissolved agarose in TAE using heating.
- Poured it into the gel electrophoresis cassette

- Comb was placed and allowed it to solidify.

4.6.2 Using liquid nitrogen to break cells:

- ❖ Purified colonies of fungal mycelium was inoculated in 100 ml of PDB and kept at 37 °C in a shaker incubator.
- ❖ The mycelium was filtered and allowed to drain for sometime after placed them on muslin cloth.
- ❖ Mycelium was then grind with mortar and pestle in liquid nitrogen.
- ❖ The frozen powder was taken in a vial and 500µL of extraction buffer was added and mixed properly.
- ❖ The reaction mixture was incubated at room temperature for 30 minutes and centrifuged at 14000 rpm for 5 minutes.
- ❖ The supernatant was aspirated to a new vial and equal volume of phenol: chloroform (1:1) was added to it.
- ❖ It was vortexed briefly and centrifuged again at 13,000 rpm for 2 minutes.
- ❖ The supernatant was again transferred to a new vial and re-extracted twice with 300 µL chloroform and centrifuged again at 13,000 rpm for 2 minutes.
- ❖ The final supernatant was then transferred to another vial, 300 µL of chilled isopropanol was added to it and gently mixed by inverting the tube twice or thrice.
- ❖ The reaction mixture was incubated for 30 minutes at -20 °C.
- ❖ The nucleic acid was then recovered by centrifuged at 13,000 rpm for 5 minutes.
- ❖ The supernatant was discarded and the pellet was washed twice with 70% ethanol and dried for 15 minutes at 37 °C.
- ❖ The isolated DNA was resuspended in 20µL TE buffer.
- ❖ 0.8% 100 ml agarose was prepared and genomic DNA was run to observe the bands.

4.6.3 Using glass beads to break cells.

- ❖ Thin glasses were taken and sterilized it using 70% ethanol and break it using mortar and pestle in LAF.
- ❖ After glass beads were formed, extraction buffer was poured into it.
- ❖ When glass beads soaked extraction buffer then mycelium was added and crushed using mortar pestle for about 30-40 min until powder were formed.

- The reaction mixture was incubated at room temperature for 30 minutes and centrifuged at 14000 rpm for 5 minutes.
- The supernatant was aspirated to a new vial and equal volume of phenol: chloroform (1:1) was added to it.
- It was vortexed briefly and centrifuged again at 13,000 rpm for 2 minutes.
- The supernatant was again transferred to a new vial and re-extracted twice with 300 µL chloroform and centrifuged again at 13,000 rpm for 2 minutes.
- The final supernatant was then transferred to another vial, 300 µL of chilled isopropanol was added to it and gently mixed by inverting the tube twice or thrice.
- The reaction mixture was incubated for 30 minutes at -20 °C.
- The nucleic acid was then recovered by centrifuged at 13,000 rpm for 5 minutes.
- The supernatant was discarded and the pellet was washed twice with 70% ethanol and dried for 15 minutes at 37 °C.
- The isolated DNA was resuspended in 20µL TE buffer.
- 0.8% 100 ml agarose was prepared and genomic DNA was run to observe the bands.

5. RESULT AND DISCUSSION

5.1 Sub culturing

After the sub-culturing of the master plate we obtained two different colonies isolates I & II which were morphologically distinguished on the basis of their colony colour which were green and grey (figure 2 & 3). A bacterial culture was also obtained growing symbiotically with fungus culture.

Figure 2: Isolate I

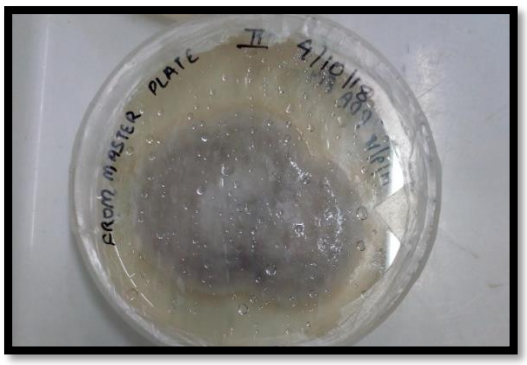


Figure 3: Isolate II

5.2 Staining:

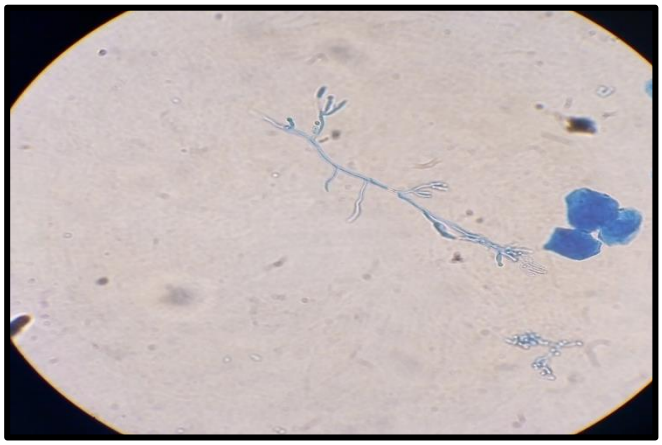


Figure 4 - Staining of Isolate I at 400 magnification under compound microscope

5.3 Spore Counting using Hemocytometer:

Cells /ml = average number of spores * 10^4

Cells in right top - 67

Cells in left top - 72

Cells in right bottom -50

Cells in left bottom -52

Average number of cells -60.2

Cells /ml – 6.02 * 10^5 cells/ml

5.4 Experimental Setup:

5.4.1 – Using Bushnell Hass Agar Media

No growth of isolates I & II was observed on Bushnell Hass agar media where the sole source of carbon was kept benzene, xylene and sucrose. The figures 5 and 6 represent plates with Bushnell Hass agar media where sole carbon source was xylene, benzene, sucrose and negative control with no carbon source.

Figure 5 – Bushnell Hass agar media containing xylene and benzene as their sole carbon sources

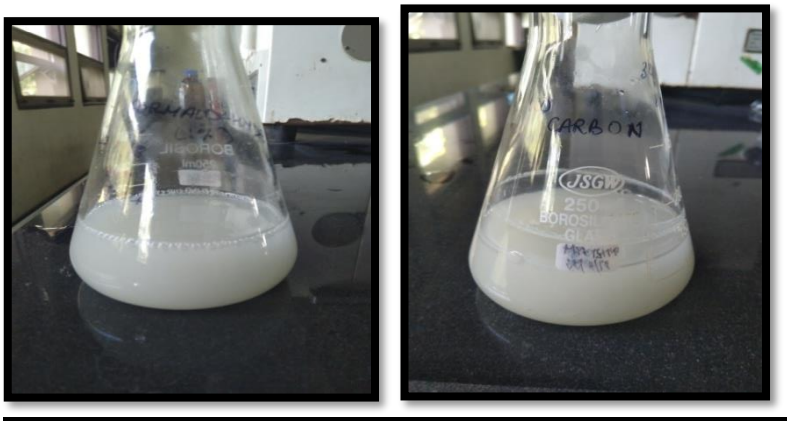
Figure 6 – Bushnell Hass agar media containing sucrose as sole carbon sources and no carbon plate

- Growth of fungal culture was observed in sucrose plates.
- No growth in xylene and no carbon plates.
- Bacterial growth was observed in benzene plate.

5.4.2 – Minimal Media:

In figure 7 minimal media with different carbon source there was no growth found in any of the flask after 7 days of inoculation giving us the idea that there was no degradation.

1) Formaldehyde 2) No carbon



3) Benzene 4) Xylene

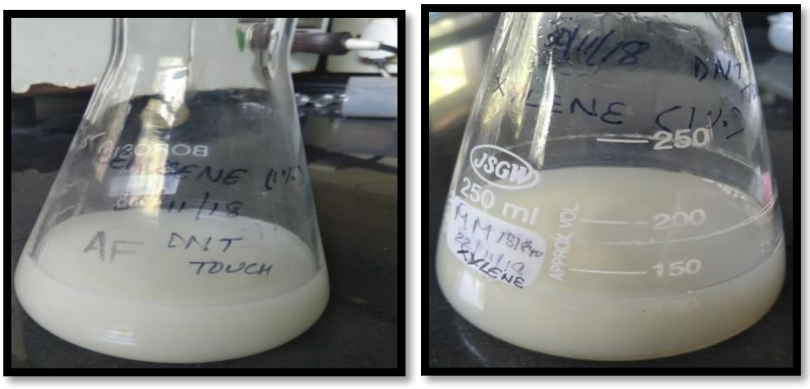


Figure 7- Minimal media with different carbon sources

5.5 DNA extraction:

5.5.1 Using liquid nitrogen to break cells:

1 2 3 4 L

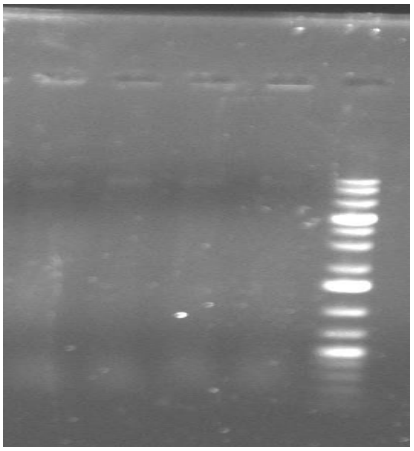


Figure 8 – DNA bands of Isolate I on 0.8 % agarose.

- Lane 1,2,3,4 – Genomic DNA from the Isolate I.
- Lane 5- 1kb ladder
- No clear bands were observed (figure 8)
- RNA and protein contamination was found due to the fact that there was no RNAse treatment.

6.5.2 Using glass beads to break fungal cells:

L 1 2 3 4

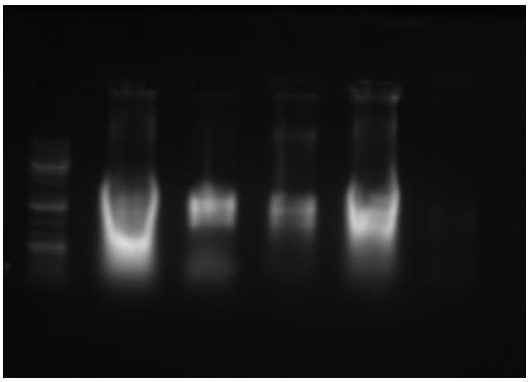


Figure 9-DNA band of Isolate II on 0.8% Agarose gel.

- Lane L – 1kb ladder
- Lane 2-4- Extracted DNA from Isolate II culture.

  There were no clear bands observed (figure 9) due to some handling error while the procedure.

CHAPTER 6

CONCLUSION

The study reports the expression analysis of the calcium signalling pathway gene *Crz1* and *Rho-GDP* of *Aspergillus flavus* during its interaction with *Triticum aestivum (*wheat). *Aspergillus* species is one of the most opportunistic fungi which are responsible for multiple clinical conditions. This genus consists of approximately 339 species which are known. *Aspergillus* and *Penicillium* are two most commonly known genus responsible for infecting the crops both pre and post harvest.

Aspergillus flavus is considered to be one of the most important pathogens in plant which leads to the production of highly toxic mycotoxins which may include aflatoxin, citrinin, fumonisins etc [43]. Their distribution majorly cosmopolitan and hence are known for colonizing on stored or pre-harvested grains and cereals [43]. Their colony characteristics are that they are flat with wrinkled centre where the colony colour ranges between green to dark olive green.

Sub-culturing of the fungus on PDA plates was done in order to isolate pure colonies. Conidia from the pure colonies were used to infect wheat grains. After the 3^{rd} day and 5^{th} day of infection of wheat RNA isolation was done. Successful isolation of RNA was followed by preparation of cDNA. Housekeeping gene *tubulin* was used to check the quality of the cDNA synthesized. The two bands on the gel of the PCR product confirmed the appropriate cDNA synthesis.

Following the checking of cDNA synthesis, RT-PCR was used for the expression analysis of *Crz1* and *Rho-GDP* with both the 3^{rd} and 5^{th} day cDNA templates. C_T values were obtained for *tubulin*, *Crz1* and *Rho-GDP* with cDNA of 3^{rd} and 5^{th} day as template. For the *Crz1* gene, the C_T value was found to be in increasing order with increase in number of days. We know that the C_T value is inversely proportional to gene expression. It can be concluded from the pattern of normalized value that the expression of *Crz1* gene was comparetively more on 3^{rd} day than on 5^{th} day. The C_T value after the RT-PCR in the case of *Rho-GDP* was found to be decreasing with the increase in number of days. Since C_T value is inversely proportinal to the gene expression, it was found that the expression of gene *Rho-GDP* was more on the 5^{th} day than that on 3^{rd} day.

Hence it can be concluded that expression of *Crz1*gene is high in *A. flavus* on 3rd day in comparison to day 5 infected wheat, whereas the expression of *Rho-GDP* gene up-regulated on both days post infection. Further, more functional sudies are required during host pathogen intraction to decipher importance of these genes during the colonization of *A. flavus* on sutaible host.

In other study, we explored fungus that is capable of degrading formaldehyde as they were observed to be growing on plant sample preserved in formaldehyde solution, which gave us the idea that they use formaldehyde as their carbon source. Formaldehyde is a volatile organic compound which are eventually very harmful to living being if they are exposed for short or long term. Volatile organic compounds such as benzene, xylene are found in paint, glue and other industrial solvents, are harmful to living being including environment. Having fungal culture which is capable of degrading organic compounds, possibly provide biological treatment strategies to the industrial discharge materials.

In this study the reported culture growing on plant sample preserved in formaldehyde was found to be having two different isolates of fungus. Out of the two fungal isolates the isolate-I morphologically under microscope seems *Penicillium digitatum*, however, ITS region sequencing is required to confirm the species. It was then cultured on minimal media (MM) supplemented with sucrose, benzene. Formaldehyde and xylene. It did grow on sucrose MM but did not show growth under others conditions. For isolate II, the experiment was carried out in broth instead of agar plate. Our results showed that there was no growth on aromatic carbon source. It may be concluded that fungus isolates were probably using nutrients from the plant preserved in formaldehyde and was probably tolerating the formaldehyde environment and not using them for their growth.

CHAPTER 7

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