

**Expression analysis of transcriptional factor ‘*aflR*’  
during *Aspergillus flavus*-maize kernel interaction**

*Project report submitted in partial fulfilment of the requirement for the  
degree of*

**BACHELOR OF TECHNOLOGY**

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**BIOTECHNOLOGY**

By

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**UNDER THE GUIDANCE OF**

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## **CERTIFICATE**

I hereby declare that the work presented in this report entitled “**Expression analysis of transcriptional factor ‘*aflR*’ during *Aspergillus flavus*-maize kernel interaction**” in partial fulfilment of the requirements for the award of the degree of Bachelor of Technology in Biotechnology submitted in the **department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat** is an authentic record of my own work carried out over a period from January 2019 to May 2019 under the supervision of **Dr. Jata Shankar, Associate Professor, Department of Biotechnology & Bioinformatics**.

The matter embodied in the report has not been submitted for the award of any other degree or diploma.

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This is to certify that the above statement made by the candidate is true to the best of my knowledge.

**Dr. Jata Shankar**

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**Dated:**

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## LIST OF ABBREVIATIONS

<i>A.Niger</i>	<i>Aspergillus niger</i>
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
LCB	Lacto phenol Cotton Blue
PBST 20	Phosphate Buffer Saline Tween 20
Dntp	Deoxy ribonucleoside Triphosphate
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
μL	Microliter
Kb	Kilobytes
Mb	Megabytes
°C	Degree Celsius
B1,G1	Blue1, Green1
B2,G2	Blue2, Green2
<i>A.Flavus</i>	<i>Aspergillus flavus</i>
<i>A.Parasiticus</i>	<i>Aspergillus parasiticus</i>
SDS	Sodium dodecyl sulphate
TAE	Tris-Acetate EDTA
PCR	Polymerase chain reaction
R.T	Room temperature
Ppb	Parts per billion
RT-PCR	Real time-PCR
Rpm	Rotating per minute
cDNA	Complementary DNA
DPEC	Diethyl pyro carbonate
OD	Optical density
<i>A. terreus</i>	<i>Aspergillus terreus</i>
Mrna	Messenger Ribonucleic acid
Bp	Base pair
Etbr	Ethidium bromide

V	Volt
i.e.,	That is
TAE	Tris-Acetate EDTA
Seq	Sequence
Min	Minute
Sec	Second
M	Molar
Ct	Cycle Threshold

## ABSTRACT

Maize is vulnerable to contamination with the harmful pathogen *Aspergillus flavus*. Disease results in the decrease in the standard of grains and pollution of bits along with an exceptionally cancer-causing mycotoxin aflatoxin. The pollution of aflatoxin in maize or maize-related items blended by *Aspergillus flavus* makes extreme financial misfortune and danger human well-being. Along these lines, this investigation was complete to comprehend the colonization of *A. flavus* on maize bits. Maize seed was taken for work. *A. flavus* (BT05) refine was tainted with maize part under various condition and time span and after the colonization of *A. flavus* on maize portion the RNA was separated for investigation of quality articulation of *A. flavus* just as the aflatoxin dependable quality will be examined. To comprehend the instrument of *A. flavus* associated with maize portion, gene expression analysis was done by utilizing RT-PCR for chose qualities of two aflatoxin pathway gene i.e., *aflR* and *aflD* and aflatoxin dependable quality will be in powerless in maize part. Our outcome fills in as an establishment for understanding the aflatoxin generation in maize-part and offer new piece of information to oversee aflatoxin defilement in yields. RT-PCR was done with the two aflatoxin gene and look upon the regulation of the gene on maize under various condition. And finally it was observed that in 3<sup>rd</sup> day of treatment it represents the high gene expression of gene *aflR*. Where as in *aflD* there was low gene expression. After 5<sup>th</sup> day of treatment there was low expression of the *aflD* genes but it *aflR* expression was high. And finally we concluded that in 3<sup>rd</sup> day and 5<sup>th</sup> day the expression of *aflR* gene is high and the expression of *aflD* gene is low.

## CHAPTER-1

### INTRODUCTION

*Aspergillus flavus* is a pioneering parasitic pathogen that taints creating maize, assaulting plants that are debilitated by natural burdens, for examples, dry season and warmth. Parasitic colonization of the portion decreases grain quality and results in the gathering of aflatoxin, a poisonous and cancer causing. We will screen the quality articulation of both growth and maize portion [1]. Contamination portions were analysed histologically utilizing a contagious explicit stain. We discovered portion immunized with *A. flavus* to react to pathogen assault by raised articulation of qualities associated with the resistance reaction. Aflatoxin are lethal, exceedingly cancer causing optional metabolites of *Aspergillus flavus*.

Malady diminished grain characteristic and sullies a bit with the cancer causing mycotoxin aflatoxin. Aflatoxin is mycotoxin created by the dirt growth *Aspergillus flavus*.

It exists in the four essential sorts which are B1, B2, G1 and G2. Alf B1 is the most common and generally lethal. *Aspergillus* can be identified on products, for example corn utilizing fluorescence which separated somewhere in the range of B1 and B2 or G1 and G2 [15]. Albeit a lot of the organism might be unmistakable to the unaided eye; aflatoxin are regularly poisonous at such low dimensions that pollution may escape visual identification. Youthful conidia (anaemically delivered contagious spores) of *A. flavus* seem yellow-green in shading and obscure as the parasite age. After ingestion, aflatoxin is utilized by cytochrome p450 bunch proteins in the liver. Of the metabolites created, of most noteworthy concern is aflatoxin 8, 9-epoxide which can be consolidated into DNA. The measure of aflatoxin 8, 9-epoxide framed decides species vulnerability. The objective of one health is to improve the wellbeing and prosperity of plants and creatures through a superior comprehension of complex connection and conditions between people, creatures and nature [3]. Aflatoxin the executives at a worldwide dimension requires such a methodology since aflatoxin happen worldwide and favourable influence both human and creature wellbeing in locales here they happen. Aflatoxin is normally happening mycotoxin by dismissal of produce auxiliary to guidelines against defilement in sustenance. These poisons have a worldwide

conveyance with the best number of medical issue happening in creating nations where presentation isn't enough controlled. Notwithstanding crop harm, inward breath of *Aspergillus* spores can cause lung harm and ingestion of aflatoxin in defiled nourishment can cause genuine wellbeing results in the two people and creature. Control of aflatoxin creation and sustenance tainting can be viewed as a one health issue in view of the cooperation of ecological condition and human that decides impacts and human and creature wellbeing. [1-7]

The separation, portrayal and a portion of the properties of four firmly related parts of aflatoxin, the blend of the lethal metabolites delivered by specific strains of *Aspergillus flavus*, have been depicted previously. These parts were named aflatoxin B1, B2, G1 and G2. Basic formulae have been proposed for aflatoxin B1 and G1 [2] and the connections between these mixed and B2 and G2 have been established, 3 the two fold bond in the terminal dihydrofuran ring in the initial two segment having moved toward becoming hydrogenated in the others.

*Aspergillus flavus* is artful infectious agents of grains. As it is a vital because it makes aflatoxin as an auxiliary metabolite in many of crops seed both sometime recently and as a by-product. Aflatoxin may be with powerful carcinogenic that's exceedingly controlled in major nations. Within the ground, aflatoxin is related to drought-dry oilseed crops counting maize, shelled nut, cottonseed and plants nuts.

Beneath of proper condition, an organism will develop and produce aflatoxin in nearly any put away edit seed. In capacity, aflatoxin can be power by keeping up accessible dampness at levels underneath that which can bolster development of *A. flavus*. A many of field control bolster development of *A. flavus*. Many of field domination value are being used or investigation, counting. Adjusting with social hones, improvement of safe crops across RT-PCR method: competitive avoidance utilizing strains that don't deliver aflatoxin and improvement with field medication it would square aflatoxin generation.

Scientific categorization: *A. flavus* connect (teleomorph obscure) kingdom organism, phylum Ascomycota, arrange eurotiales, lesson eurotiomycetes, all the descendants trichocomacetes, family trichocomaceae, class *Aspergillus*, kind of species called *A. flavus*.

Host run: *Aspergillus flavus* contains wide have extended deft microorganism. *A. flavus* is an amazingly occurring soil organism it result in the major concern of this organism. The main problem with this organism in agribusiness is that it can produces profoundly cancerous poisons named aflatoxin which is a wellbeing creatures risk. Within the soil, *A. flavus* is overwhelmingly an issue within the oilseed crops maize, peanuts and cottonseed and tree nuts. Beneath inappropriate capacity conditions *A. flavus* is competent of developing and shaping aflatoxin in nearly any crop seed. It moreover may be infectious agent of creatures and of creepy crawlies. People as it are transcendently a deft harmful agent of immune suppressed patients.

Few organisms have had as wide a financial affect because of *Aspergillus flavus*. It may be pathogenic on crops, creatures or creepy crawlies cause capacity decays in various cultivated plants and it cause the exceedingly directed mycotoxin, aflatoxin B1. As human harmful organism, *Aspergillus* species has been ended up progressively critical since immunosuppressed individuals bare exceptionally vulnerable to disease by these organisms. Of the aspergilla causing mycoses in people, as it were *A. fumigates* is more critical that *A. Flavus* [20]. *A. flavus* is an additionally an allergen trigger unfavourably susceptible bronchopulmoary aspergillosis. As a creepy crawly infectious agent, it influences a many number of species, counting honeybees, which provoke an illness cry out stone brood [20]. This is not unique within any specific in any plant and creepy crawly. Foremost powerful normally shaped carcinogen is aflatoxin B1. It is one of the most few mycotoxins that have been dive.

*Aspergillus flavus* could be seriousness infective agents of cereal crop, peanuts, rice, maize etc. *A. flavus* is responsible in corn as an ear destroy [1]. In peanuts & corn it is responsible for tree infection called as yellow form of seedlings or “alumroot”. Many side effect incorporate necrotic injuries, chlorosis in high-ground combine and there is a need in improvement for auxiliary roots, 'alumroot' [6]. The root impact can be an outcome of aflatoxin harmfulness because it's been appeared with hinder plant organ improvement inward tobacco [7]. *A Flavus* might too something bad to decay to develop peanut within that ground. In cotton, *A. flavus* influences cotton suitability past creating boll spoil.

Contamination of fibre is notable known as “yellow spot illness”. This ‘yellow’ eludes shinning greenish yellow (BGY) florescence seeing at the cotton filaments beneath long

-wave high intensity light. *A. flavus* disease infecting in the cotton plant brings down Seeds practicality with approximately 60% [15]. Within identical ponder, 38% of aflatoxin originate with the practical.

*Aspergillus flavus* is an all-inclusive dissemination filamentous, saprophytic growth that every now and again contaminates oil abundant seeds of different yield species amid pre as well as post-harvest gather, with consequent creation result in formation of mycotoxin, for example, cyclopiazonic corrosive, aflutter, and the notable aflatoxin [1,2]. Aflatoxin is incredibly cancer-causing, mutagenic, teratogenic mycotoxin related with both intense and perpetual danger in people and creatures [3, 4]. This malicious effect on wellbeing's are most unmistakable in creating nations lacking innovations to screen and decrease aflatoxin amount in harvests and was poor stockpiling state frequently solution is more prominent aflatoxin gathering in the nourishment supply. Other than the wellbeing suggestion in people and creatures. *A. flavus* colonization in yields causing critical monetary misfortunes on account of obliterated/marked down use and lower cost of aflatoxin-debased grains [5, 6].

RNA-seq are quick and higher-through put innovation of transcriptomic for transcriptomic profile investigation, which have been utilized for study succession varieties and complicated transcriptomes with lower false-positively rates and higher affectability and duplicate [20, 21]. Use of RNA- Seq have significantly quickened the comprehension at the intricacy at quality articulation, guideline or systems of creature under different spatial-fleeting condition or quality articulation could be more precisely evaluated utilizing RNA-Seq approached then by the ordinary transcriptomics investigation [22]. Over the previous decade, an advance at the various organisms has been contemplated seriously utilizing RNA-Seq [5-27]. For a life form at the well-Clarified genome, a mapped read grouping to the relating reference genome is the top and fundamental advance for RNA-Seq information investigation [23]. The entire genom sequencing of *A. flavus* was finished [27], and explanation at the gene at the organism indicated different qualities that are possibly relating at the conidial advancement and Aflatoxin biosynthesis [28].Furthermore, RNA-Seq innovation have been utilized in transcriptomic examination of mycotoxin which is aflatoxin that take place in biosynthesis and mycelia advancement in *A. flavus* reaction to its property [5], 5-azacytidinr [23, 29], menadione [30], water movement [31] and temperature [32].

To exhaustively comprehend atomic component of *A. flavus* association with the nut, a RNA-Seq progress were connected in that examination to get or think about transcriptomic profiles of *A. flavus* which colonised at the guarded and defenceless shelled nut seed at the entire genome level. The kinetic contrast of *A. flavus* transcriptome profile from *A. flavus* safe or helpless shelled nut systems was recognized. The conceivable jobs at different communicated qualities and metabolic pathways were talked about, or the component of *A. flavus* communication at the safe or the helpless nuts were additionally found. [14] Likewise the huge transcriptomic data would be useful for further explanation of the genome of *A. flavus*. These investigation would likewise help further comprehension of aflatoxin defilement and add to the plan of the plan of new procedure at oversee aflatoxin training at the nut or different harvests.

Different cluster of gene are responsible for the production of aflatoxin biosynthesis pathway. It will include unassigned gene, regular and structural gene. [12] This current study was done to predict the gene expression of genes like *aflR* and *aflD* which is one of the important genes which donate in the aflatoxin cycle B1 in *A. flavus*.

There are 18 enzymes which involve in the aflatoxin biosynthesis pathway and are responsible to produce toxin from acetyl coenzyme A [12]. Gene involve in this study is *aflR* (regulatory gene) and *aflD* (*Nor-1*) these cluster in 75 Kb region of fungal chromosome-III. In other aflatoxin biosynthetic pathway the activity of other structure gene is regulated by the *aflR* gene [12]. Whereas on the other hand, the conversion of non solorimic acid into averantin is done by *aflD* (*Nor-1*) this is the important role of *aflD* gene. Although there are lots of reports on this study but we will explain the host pathogen interaction and the involvement of gene *aflR* and *aflD* and its expression. Gene like *aflR* and *aflD* is considering as the early indicator off aflatoxin production [12, 14].



## CHAPTER-2

### REVIEW OF LITERATURE

#### 2.1 Maize seed contamination

Eight to sixty maize swards was surveyed in 1994 and 1995, solely, of screen *Aspergillus* infirmity and Aflatoxin impurity of pre-harvest maize in Benin. Three *Aspergillus* species was disengaged from various agroecological zones, with *A. flavus* being most broadly perceived. The country wide mean components to piece contamination were about 20% in the two years. Aflatoxin was removed from maize at any rate 30% of the field attempted. Poison fixation demonstrated a particular zonal collection, with fairly strange states in the Guinea Savannah. There were model toward higher rate of the aflatoxin absolute per rate *A. flavus* disease from the south toward north and it was reported that aflatoxin assembling in maize. Therefore, the geographic point of reference found in the event of *A. flavus* and aflatoxin might be identified with high rate [22].

Despite the way that *Aspergillus flavus* is delineated as a limit structure, it has been comprehensively recognized that malady of maize at this development occurred at the field. Pre-harvest pollution of the maize with *A. flavus* were noted as on schedule as 1920 by taubenhaus and it have been had all the earmarks of being abundant all the through piece improvement. For quite a while, regardless, it was envisioned that pre-harvest maize was without aflatoxin. In 1973 it simply [24] showed the closeness at the aflatoxin B1 in maize before assembles. Since these examinations were coordinated broad work have been done to assess the essentialness of pre-harvest sullyng of the maize and to choose the factor impacting the illness technique by toxic substance conveying life forms, especially in the United States. Field tainting are as of now especially recorded but both *A. parasiticus* Speared and *A. Niger* Tiega Spoil maize in that field, *A. flavus* appears, apparently, of be the staggering aflatoxin- conveying development, especially in tropical zones [30]. In tropical Africa, couple of the examinations have been done to choose the noteworthiness of *A. flavus* in pre-harvest maize. In Benin, maize builds up a staple sustenance to the people and biological conditions are sensible of the parasitic improvement. Pre-harvest tainting of maize by *A. flavus* and ensuing aflatoxin creation might contain a troublesome issue. [26, 37]

definite that maize in Benin were seriously polluted at a starting time of limit. This may have been as result of high pre-harvest tainting. This examination was endeavoured to clear up the assumption. This objective was to choose the scattering and dimension of the maize divide ailment by parasite in the Benin. Also association between *A. flavus* ailment and the proportion of aflatoxin made at pre-harvest maize were developed and incorporation of freighting little creature mischief to the ear amid the time spent aflatoxin corrupting in pre-harvest maize was investigated. Bugs hurting maize grains have been had all the earmarks of being generally corresponded to the rate and measurement of the aflatoxin polluting in pre-harvest maize.

## **2.2 Threats to Aflatoxin**

The point of confinement of two non-Alfa toxigenic *Aspergillus flavus* related to disconnects (CT3 and K49) to diminish aflatoxin defilement with corn surveyed in four years filed consider (2001-2004). Soil were serving at 6 wheat substance prescriptions: Alfa toxigenic restrict F3W4; two non-Alfa toxigenic pulls back (CT3 and K49) two blends of CT3 or K49 with F3W4 and a sterilize wheat power, related to 20kg ha<sup>-1</sup>. In 2001, immunization Alfa toxigenic pull back broadened corn gain aflatoxin levels by 188% showed up contrastingly in connection with the non-inoculum limited while CT3 and K49 inoculation less in amount of aflatoxin levels in grain by 86 and 60% autonomously[15,16]. In 2002, the non-toxigenic CT3 and K49 diminished aflatoxin limited by sixty on percent and seventy six percent separated from non-inoculated power, only. In 2001, blends of the Aflatoxigenic and non-Aflatoxigenic impact limit on aflatoxin levels in any case 2002 [27], vaccination with blends of K49 and CT3 lessened with aflatoxin quantity of 68 and 37% showed up diversely in connection to non-immunization benchmark independently. In 2003 and 2004, a low segment which is essential aflatoxin sullyng watched (8ng-1) notwithstanding, immunization with blends of K49 + F3W4 and CT3 + F3W4, decreased components of aflatoxin sixty five to ninety four percent showed up diversely in connection to the Alfa toxigenic strain alone. Showed up distinctively in connection [21] to the non-sclerotic passing on CT3, strain K49 makes impressive sclerotic, has logically quick in vitro winding headway and an inexorably unquestionable capacity to colonize corn when unscrupulously immunized potentially displaying continuously significant trademark wellbeing. Results show that non – aflatoxigenic, endemic *A. flavus* isolate, for example, strain K49, has potential

uses of bio control of aflatoxin tainting in southern US corn. In 2003, unprecedented for Italy, basic issues rose with group and debasement of maize headed for animal feed with *Aspergillus* portion *Flavi* and Aflatoxin. These realized milk and decided things being degraded with AFM1 at levels over beyond what many would consider possible. There was small learning and experience of this issue in the Italy [27]. These objectives of these investigation was along these lines to consider the peoples of *Aspergillus* zone *Flavi* in six northern Italian territories and secure information at the general employment of the key species, abilities to make sclertioa age at the essential toxic assistant metabolites, aflatoxin and cyclopiazonic destructive and obstruction of the key characteristics parameters. An entirety of seventy strains was separated and they joined the toxigenic species *A. Flavus*, *A. parasitic*. And *A. flavus* were wining of the peoples inspected, addressing ninety percent of the strains. Seventy percent of strains of *Aspergillus* portion *Flavi* conveyed the Afs [24], with the half of the strains in like manner making cyclopiazonic destructive. Sixty two percent of *A. flavus* strains and the 80% of the *A. parasiticus* had the ability to convey the sclerotic at 30°C [27]. And Using 5/2 agar, only the one strain made S sclerotic and 19 L sclerotic concerning ecological examinations, advancement of *Aspergillus* territory *Flavi* was perfect at some place in the scope of 25 and 30°C, while AFB1 age was perfect concerning openness, 0.99 aw were a perfect for the both advancement and AFs, age, while primary the aflatoxin made in the driest condition attempted (0.83 aw) was the AFB1. This information would be useful in recognizing districts in threat in the northern Italy by associating climatic regional information to measurements of the infectious sullyng present and potential for aflatoxin age in maize headed for the animal feed. These would be beneficial as a component of an expectation strategy for restricting Afs in thing. Parasitic society in the soils of Nigerian maize fields examined to choose allotments of aflatoxin – conveying life forms and to perceive endemic toxicogenic strains of potential motivating force as regular control administrations for compelling aflatoxin contamination in West African harvests. More than 1000 with draws having a spot with *Aspergillus* zone *Flavi* [24] were assembled from soil of 55 Nigerian maize fields arranged in three agro ecological zones by debilitating plating onto balanced rose Bengal agar. The most outstanding individual from *Aspergillus* portion *Flavi* was the *A. flavus* L- strain sought after by mysterious toxin known as strain SBG. *A. Tamari* and *A. parasiticus*. Most shocking rate of SBG was in Zaria region, or least was in

Ogbomosho and Ado- Ekiti regions. Only 44% of 492 *A Flavus* limits conveyed aflatoxin in liquid development. Thirty two percent of the *A Flavus* withdraws made > 1 µg g<sup>-1</sup> complete aflatoxin anyway no *A. flavus* separate made G Alfa toxins. Right when the agro ecological zone were contemplated, inside and out progressively unmistakable degrees of Alfa toxigenic *A. flavus* detaches was found in Northern Guinea Savannah than in Southern.[12, 28]

### 2.3 Aflatoxin

Aflatoxin were to begin with found and characterized within the early the 1960 s after the more than 1,000,000 turkey pouts in Britain passed on to clear harming from mould-contamination shelled nuts supper. There are the two common shapes of the malady causes by the presentation to Aflatoxin. Intense aflatoxicosis comes about in passing. Inveterate aflatoxicosis causes the cancer, in liver and as it the essential target organ, resistant concealment, the teratogenicity and the other side effects [3, 4]. There's moreover a few prove that inhalator introduction to aflatoxin increments the event of inhalator and other the cancers. Chemically, aflatoxin is the Difuranocoumarin subsidiaries created by the "Polyketide Pathway". There are the four major aflatoxin B1, B2, G1 and G2 with their letters alluding on the fluorescence colour beneath bright light (blue or green) and the numbers demonstrating a Comparative relocation separate on the small layer of the Chromatographic plates [4].

The qualities for the Aflatoxin which take in biosynthesis, like these of the numerous auxiliary metabolites, are clusters [3]. The total cluster has been sequenced and explained [11]. The quality cluster is the 82 kb in the length, and contains 25 qualities. Direction of the pathway has have been the subject of the seriously consider [10]. It is trusted that the understandings the bio-synthesis of aflatoxin will encourage improvement of control technique and give an understandings of the how and why aflatoxin advanced, As of late , the genome of the *A. Flavus* have been sequenced [26]. The data in that will give valuable instruments for grip both the organism and aflatoxin. Restrictive amount for the aflatoxin are in the put in at the slightest 99 nations [10, 11]. An administrative level shift to the nation to the other nation. The USA FDA activity levels are set at the 20 parts per billion (p.p.b., µg/kg) for the people nourishment, 0.5 p.p.b for drip and up to 300 p.p.b for the corn and cotton seed in creature nourish. Within the EU, the levels are 4p.p.b. while in India the administrative levels are the set

at the 30 p.p.b for all the foods. In the joined together the state alone, the cruel financial misfortune from the mycotoxins is evaluated to be the \$932 million (CAST 2003). Aflatoxin may be the major supporter to this problem. Economical cost of aflatoxin is troublesome to assess since such add up to gauges ought to incorporate misfortune of compassion creatures to aflatoxisis. Evaluating the cost of the Aflatoxicosis in people around the world is indeed more troublesome. In term of trim misfortune alone, gauges of yearly costs within the US developing regions helpless to aflatoxin were \$ 36,700,000 for California walnuts \$23,000,000 to \$ 47,000,000 for the California almonds [34]. In addition, morphotypes of *Aspergillus* species including *A. fumigatus*, *A. niger*, *A. terreus* producing mycotoxin need further investigation to their role in food crop contamination or their role in the pathogenesis in health host or immune compromised patients or providing resistance against antifungal drugs[35, 36].

#### **2.4 Variable affecting Aflatoxin arrangement within the field:**

Aflatoxin is most as often as possible detailed within the field in the oil seed crops counting Maize, Cotton, Peanuts and Tree nuts. Portion of the reason for this might be biogeographically those crops are developed within scopes where the *A. flavus* is most regularly detailed. Other conceivable reason might be the carbon utilisation design of *A. flavus*, in the cotton seed and corn, *A. flavus* to begin with utilises free cloying and after that Oil some time recently utilizing starch [31].

Expulsion of lipoid from the cotton seed decreased Aflatoxin generation 800 fold [33]. Drought and the temperature stretch are common variables for the field defilement of the push crop. In the peanuts, test with the dry season stretch and manage the soil temperature 85-100 day after the Planting illustrated that the dry spell stretch and temperature of the 29°C yield most noteworthy number of the inhabited eatable review the peanut and tall Aflatoxin level.

Other natural combination driven to lower the colonization level and no Aflatoxin [31]. An arrangement of ponders illustrated that water system will control the Aflatoxin in the Corn [33]. In the cotton, modestly tall dry season stretch (-1.6 to -1.9 MPA) at time of the inflorescence driven to higher *A. flavus* contamination levels in coming about seeds that lower or higher the water push level at the blossoming [34]. In the corn, as in the

other influenced crop, tall temperature and dry spell push is nearly continuously antecedents to the Aflatoxin episodes [11].

## **2.5 Metabolism in Aflatoxin**

Aflatoxin are perilous, significantly disease causing discretionary metabolites of *Aspergillus flavus* and *A. parasiticus*, which when made in the midst of infectious sickness of a helpless relent in the field or after procure pollute sustenance and feed and undermine human and animal prosperity [3]. Notwithstanding the way that there is a couple of organization frameworks that may diminish aflatoxin debasement of corn, the staggering philosophy for end of aflatoxin is to make pre-harvest have assurance from aflatoxin collection. This approach has expanded significantly progressively conspicuous detectable quality in light of continuous exposures of standard obstacle in the corn that can be manhandled in plant imitating frameworks. The ability to recognize safe corn genotypes has been redesigned by the improvement of a lab part screening measure and by strain of *A. flavus* genetically intended to make B- glucuronidase, an exacerbate whose development can be seen to review the dimension of infectious illness in bits [4]. Examination of safe corn genotypes have related piece pericarp wax properties with resistance, recognized piece proteins related with security from and limitations of infectious advancement aflatoxin biosynthesis, and perceived chromosome territories related with assurance from *Aspergillus* ear ruin and aflatoxin creation. Such research advances could lead as soon as possible to financially open, ergonomically sufficient corn lines with various pre-harvest insurances from aflatoxin debasement [3]. Maize bits are unprotected to tainting by guileful infectious agents like *Aspergillus flavus*. Infection outcome in abatement of crop status and contamination of bits with astoundingly disease causing mycotoxin, aflatoxin. To interpret host reaction to sully the parasite, interpretation of approximately 9000 maize characteristics was checked in the midst of the host- pathogen association with an extraordinary created Affymetrix Gene Chip DNA group. Many of those 4000 maize characteristics were found differentially imparted at a FDR of 0.05. This joined the up rule of obstruction related characteristics and hailing pathways [16]. Transcriptional changes in like manner were found in basic assimilation characteristics. Starch biosynthetic characteristics were down controlled in the midst of tainting while characteristics encoding maize hydrolytic proteins, evidently drew in with the corruption of host

spares, was up overseen. This data demonstrate that defilement with the maize part of *A. flavus* impelled metabolic changes in some part include in making of an assurance result similarly as an aggravation in bit improvement. *Aspergillus flavus* is a spearheading infectious pathogen that debases making maize parts ambushing plants that are crippled by biological weights for instance drought and warmth [3, 4]. Ailment diminishes grain quality and sullies the segment with the malignancy causing mycotoxin aflatoxin. The improvement of safe maize lines has exhibited troublesome regardless of the way there is verification for wellsprings of hindrance. The non-attendance of strong impediment phenotyping markers the anomaly of disease improvement consistently, and a lacking a perception of host resistance segments all have settled on the decision of restriction troublesome. Advances in development for instance micro arrays make it possible investigate the potential to screen translation on a genome wide measurement and gave a better understanding of how animals respond than their condition on a cell quantity [6]. This Study inquiring about plan quality explanation in the midst of the pathogen strike have been found the defend response goes past PR- proteins and incorporate interpretation changes in both basic and discretionary plant metabolic pathways and detoxification pathways. Pytohormones like salicylic destructive, harmonic destructive, ethylene has for a long while been known to be a vital bit of the opposition response. Anyway starch processing pathways anyways not regularly associated with restriction maize a critical piece of the plant defend response fusing in maize [3-5]. Higher maize stalks starch levels have been connected with extended insurance from stalk sully living beings, a significant parcel of which are more prepared for polluting the ear and piece.

## **2.6 Examining the effect of maize by *A. flavus***

Transcriptional changes of maize bits in the midst of sully by *A. flavus* has been analysed with the help of microarrays and qPCR. Kelly et al took a gander maize combinations that was either impenetrable to aflatoxin gathering. It was found that 16 characteristics very important in the sheltered grouping and 15 in the defenceless change and assumed in various frameworks and likely connected with security from aflatoxin storing up. [15]. uncovered increasingly raised measures of value explanation in stressed related characteristics at safe lined of the maize [18]. Found that more maize characteristics were incited by *A. flavus* in defenceless parts differentiated and safe

segments. In all of these examinations, protect related and authoritative characteristics were connected with the response to *A. flavus*. To give an all more clear appreciation of maize divide insurance from *A. flavus* it was checked the transcriptional reaction of crop i.e., maize bits In the midst of illness with *A. flavus* in field with the help of a custom DNA micro array. We documented difference in enunciation of well depicted gatekeeper hailing pathways and assurance which is related characteristics similarly as the impressive changes in explanation at characteristics regarded to starch absorption. There are couple of stages in infection technique have impediment could restrict infectious advancement and aflatoxin pollution. Piece illness with *A. flavus* starting through silk colonisation [15, 18]. Conidia grow and create on senescing silks going down the silk channel to making pieces which could take as pit-bull as 8 days. Resulting strides in the defilement methodology are less portrayed anyway data recommended that *A. flavus* can strike pieces in the midst of their six stages of their improvement sulking, bother, milk, blend, scratch and physiological advancement, Stating late Reese et al immunized detached bits at stages R2-R5 in lab and it was found that partitions at this four stages is powerless against pollution because of *A. flavus* Parasitic illness have seen in hurt bits as youth in milk arrange. These young bits will when all is said in done gather high combinations of aflatoxin because of postponed colonization by thebe pathogen. Defilement in non-hurt pieced in field happens last, in the midst of scratch developmental stage just before physiological improvement [16]. Once inside, *A. flavus* rarely control over the oil rich germ tissue, Parasitic improvement inside endosperm, has been viewed anyway there are irregularities in the composition with respect to the level of colonization. Our examination fixated on the rearrangement response of making partitions that were the inform with, *A. flavus* through the damage, we comprehend that this strategy could disregard some restriction instruments yet it results in progressively consistent disease headway [18]. Security from tainting of harmed pieces is in like manner relevant as it duplicates frightening little animal harm, which is noteworthy in the investigation of malady transmission of the affection, additionally to get the response of different periods of the bit improvement; we surveyed, *A. flavus* illness of four the section stages R2 and R5. We more over picked a specific time of the Four days after the inoculation to dissect quality enunciation subject to past histological examinations by [29] who exhibit that inside four days after the immunizations *A. flavus* mycelium accomplished the fleuron, endosperm, germ tissue. As needs be, trying at



Four days should be allowed evaluation of the hostess result in a couple of tissues type inside in pieces. Aflatoxin- conveying *Aspergillus flavus* link and *A. parasiticus* spread normally sully harvest for instance corn cotton peanuts particularly in tropical and subtropical extensions. Soil fills in as vital wellspring of inoculum of these living beings in harvests defenceless to aflatoxin sully *A. flavus* is commonly the staggering aflatoxin making parasite in corn and cottonseed. Although *A. flavus* also appear soberer aggressive than *A. parasiticus* in infecting peanut seeds, *A. parasiticus* is more typical input seeds than in various yields. The two species fluctuate in region appearance and small features and are successfully perceived by using organized culture conditions. Additionally aflatoxin isolates of *A. flavus* produce aflatoxin that are b1 and b2, while, *A. parasiticus* isolates additionally produce aflatoxin G1 and G2 [1-2]. *A. flavus* may similarly shape cyclopiazonic destructive which has not been represented *A. parasiticus* degrading the way that *A. flavus* and *A. parasiticus* are solidly related by their DNA homologies they are not vegetative great and are thusly, genetically kept from one another Despite these natural morphological and metabolic. Differences, most soil population studies have failed to distinguish *A. parasiticus* from *A. flavus* as often as possible implying the two species everything considered as *A. flavus* gathering in concretes that isolated the species sizable soil masses of both *A. flavus* and *A. parasiticus* were represented from cornfields despite sickness of corn ears exclusively by *A. flavus*[28] looked into soils all through japan and moreover found mixed masses of *A. flavus* and *A. parasiticus* in the southernmost territories, with the flow of *A. flavus* extending further north than that of *A. parasiticus*. Since peanuts and corn, two essential yields in south western Georgia differentiate in the rate of illness by *A. parasiticus*, this investigation was planned to take a gander at the effects of these harvests on the degree of *A. flavus* to *A. parasiticus* in soil. A further connection was made between these earth people and the masses polluting corn ears and shelled nut seeds from the proportionate fields. In the field plot tests an atoxigenic strain of *Aspergillus flavus* interfered with pre-harvest aflatoxin contamination of corn when associated either at the same time with or one day before toxigenic strain [13]. The atoxigenic strain was furthermore convincing in diminishing postharvest aflatoxin spoiling realized by both an introduced toxicogenic strain and by strains occupant on the parts. The results suggest that atoxigenic strains of *A. flavus* may have potential use as characteristics control administrators composed at diminishing both pre-harvest and postharvest

aflatoxin contamination of corn. Aflatoxin, deadly metabolites of the parasites *Aspergillus flavus* Link Fry additionally *A. parasiticus* Speared, are solid malignant growth causing which present authentic prosperity risks to individuals and private animals since them as frequently as conceivable sully green items. Corn Created in the south eastern United States is even more once in while colonized by high masses of *A. flavus* than corn created in the Midwest where by and large U.S. corn is grown [24].

## **2.7 Anatomical study of Mycotoxin**

Various oat and instinctive harvests are weak to infections ambush either in the field or in the midst of limit. These life forms may make as assistant metabolites a verging social occasion of engineered substances that is called as mycotoxin. These must be wise year after year instabilities in the element of the mycotoxin to sustenance's, dependent upon various factors, As such as troublesome conditions advantage over parasitic assault or improvement [23]. Various mycotoxins were at first perceived after they had caused a grouping of sub-acute medicinal issues in trained creatures similarly as individuals, with many target organs and struts impacted. With present day developing, amassing and getting ready practices, the fact of the matter is to decrease plainly obvious contamination, and a considerable amount of our stress by and by revolves around perpetual effects at low measurements of introduction. In such way , a couple of mycotoxin are incredible animal disease causing operators and had been gathered by the international agency for study in the field of Threatening development [23] as human beings malignant growth causing operators or potential (conceivable and possible) human malignancy causing operators.

In spite of the way that there is the geographical and weather differentiation in creation and occasion of mycotoxin, introduction to those molecules is far and wide, much of the world sustenance supplied spoiled to some degree [25]. Checking for the closeness of the mycotoxinis thusly required. Once in a while the closeness of mycotoxin may render sustenance thing dangerous, required a grouping of the measures to a lessen shot. As these are the toxicants it cannot remove completely the sustenance supply, 4 mycotoxin in the sustenance at various countries have been described dimensions in sustenance (protections, rule levels, generally outrageous development level) those are presumably not going as to be of prosperity concern. At first, the nonattendance of united, direct approached realized wide extent of standards/rules regarding among the various

counties [18] At the point when an issued including mycotoxin had been recognized as a damnation of potential prosperity concerns (issue recognizing evidence), the present strategy in numerous counties is to use an essential initiative frame work for dangers examination, same as to that propped by FAO/WHO (FAO/WHO, 1985). For the mycotoxin, chance examination is an iterative aspect strategy that considers the risk overviewment, chance organization and danger correspondence and is revived as required once essential new information widens up available. It is critical, that there is participation between these huge districts. This technique considers sensible benchmarks related to human and the animal prosperity and joins a relationship with various risks similarly as moneta4ry factors. As to and earnestness of ceaseless illness, especially sickness, mycotoxins appear at present to the speak to a higher risk than anthropogenic contaminants, pesticides (when used by bearings). And sustenance e included substances this relied upon a relationship of tumour power and introduction [32]. Moreover, the consequences of synchronous raised introduction to a couple of mycotoxinsought to be considered. Huge widespread undertaking has been utilized in studying the prosperity risks form just a little mycotoxin. Since the late 1980 s, new mycotoxins has been found and depicted 9fumonisins), and for various mycotoxins have been found and depicted (fumonisins), and for various mycotoxins critical new data related to toxicology, the investigation of infection transmission and human introduction(due to better area procedures and the usage of biomarkers) have ended up being accessibility. This has ache end new and revived legitimate evaluations by the (IARC, IPCS, JECFA, and EU-SCF), similarly as national and diverse associations, for instance, “Scientific Warning gathering for Good, European commission [23]. Different mycotoxin has seen to be disease causing. Cancer causing element is an amazing multistage process that incorporates with both of genetic and epigenetic parts. In this way, accommodating to orchestrate the data released to malignancy causing bend points according to stating late made framework that will help with organizing what is thought about the system and part of operation and the significance of the watched outcome to individuals. Such database information will help future peril evaluations and, point of fact, when gaps are remembered it can strengthen further more research [33]. Throughout the peril examinations, the fact of the matter is to chive sensible courses of action that6 balance the need to guarantee prosperity with fiscal concerns. Coherent appraisals have now all things considered become the explanation behind

recommendation for the worldwide rule on mycotoxin (aflatoxin, ochratoxins, patulin, zearalenone, and fumonisins) [17] by the Codex Committee on the food additives and contaminants (CCFAC) similarly as European Union. Rules may join rules concerning development levels or procedural standards went for expectation various oat and distinctive harvests are weak to infectious ambush either in the field or in the midst of limit. These life forms may make as assistant metabolites a varying social occasion of engineered substance known as mycotoxins [25]. This will be wide year after year instabilities in an elements of mycotoxin as sustenance's, dependent upon various Factors, such as troublesome condition favouring parasitic assault and improvement. Various mycotoxin were at first perceived after they had caused grouping of sub-acute medicinal issues in trained creatures similarly as individuals, with many target organs and structures impacted. With present day developing, amazing and getting ready practices, the fact of the matter is to decrease plainly obvious contamination, and a considerable amount of our stress by and by revolves around perpetual effects at low measurement of introduction. In such way, a couple of mycotoxin [17] are incredible animal disease causing operators and have been gathered by the international agency for the research in threatening development (IARC, 1993) as human malignant growth causing operators or potential (conceivable and possible) human malignancy causing operators. In spite of the way that there is the geographical and weather differentiations in the creating or occasion of mycotoxin, introduction in these molecules is far as well as wide, with too much of world sustenance oversupply spoiled to some degree [25]. Checking for closeness of mycotoxin is thusly required. Once in a while the closeness of mycotoxin may render sustenance things dangerous, necessitate a grouping of the measures to lessen shot. As those toxicants could neither fully eliminate from the sustenance input four my toxins in sustenance various countries has been described dime in sustenance that are presumably not going to of property interest. At first, nonattendance of united and direct progress realised a broad extent to stands. / Rules regarding over the different countries [26].

## 2.8 Toxicity perspective of other *Aspergillus spp.*

*Aspergillus flavus* is the second highest most driving explanation behind aspergillosis. The Limit of *A. flavus* that modify inside the host condition is critical for its accumulation. Starting the growth of conidia is basic events thusly in disposition to get understanding into *A. flavus* nuclear modification while germination protein profile of *A. flavus* was gotten [18]. Approx. 82 % of conidia show growth at 7 h along these lines tests were assembled trained by protein isolation and presented to “nLC- Q- TOF mass spectrometer”. Q-TOF data was dismembered using protein “Lynx global services programming”. A total of 416 proteins were found from Unipart *Aspergillus* species database. Orthologues of *A. flavus* was found in *A. fumigatus*, *A. Nigeria*, *A. terreus*, *A. orate* etc. [20]. A protein was moreover poor down in “NCBI database”, which showed that 27 proteins of *A. flavus* are not declared in Unipart and NCBI database. Helpful depiction in proteins came about fundamentally into cell divider mix or defilement, processing frameworks destructive, protein blend and degradation. Proteins/ synthetic concoctions related with aflatoxin biosynthesis were viewed. It was also observed Dicer like proteins 1, 2 and autophagy connected proteins [2, 9, 11, 13, 18 and 22]. Explanation in protein/ impetuses related with “MAPK hailing pathway” suggests these protein activity in the midst of the germination technique. As a rule this document present a rundown of these proteins/ synthetic compounds related with sprouting of *A. flavus* conidia and it could be in manner which associated with various *Aspergillus* species. *Aspergillus flavus* and *Aspergillus parasiticus* are the genuine contaminations termed which can make aflatoxin in sustenance grains like maize, cotton, corn and oil seed. Currently unique modified social actions are actually effective used and traversed for sustenance sullyng issue. The present examination was required to look upon the effect of photochemical (Ascorbic destructive Gallic destructive Caffeine and Quercetin) on toxicogenic strains of *A. flavus* and *A. parasiticus* [32] by Poisoned sustenance plate strategy and MTT test. In examinations with control a significant decrease being developed was seen with all of the blends. Quercetin demonstrated most grounded inhibitory contact with MIC50 at 36 lg/ml in *A. parasiticus* and MIC50 at 113 lg/ml in *A. flavus*. Caffeine showed extraordinary impede in *A. parasiticus* [33] however Gallic destructive showed incredible impede in *A. flavus*. Ascorbic destructive demonstrated less effect amid phytochemicals attempted in these two *Aspergillus* species. Aflatoxin builds up as social affair of perilous malignant growth causing

metabolites made by *Aspergillus flavus* link Fr., *A. parasiticus* Spear and *A. nomiuskurtzman* [32-33]. Aflatoxin B1, the most hazardous of this social occasion is the most solid malignant growth causing specialist known. Pollution of tree nuts, peanuts corn, and cottonseed by aflatoxin is a veritable sanitation risk to the two individuals and animals. *A. flavus* is the most generally perceived aflatoxin producing development can corn, cotton, tree nuts and peanuts. This development is damage assaulting pathogen that taints plants hurt by dreadful little creature's animals early parts and mechanical gathering. Under conditions extraordinary positive for infection, this fungus can colonize corn kernels directly. Any settled ailment of *A. flavus* will result in fast assembling of aflatoxin in the harvest thing under conditions of warm temperatures and high wetness. The family market of sustenance for human use before long allows a biggest element of 20 p.p.b for AFB1 [27].

## **CHAPTER-3**

### **OBJECTIVE**

- To elucidate host pathogen interaction in maize kernel infection under in-vitro conditions.
- Expression analysis of selected genes from aflatoxin pathway of *Aspergillus flavus* during interaction with maize seed.

## CHAPTER-4

### MATERIAL AND METHODS

#### 4.1. Materials:

**4.1.1 Instruments:** Centrifuge, Incubator, Eppendorf tubes, laminar air flow, Burning lamp, marker, petri dish, fungal culture *A. flavus* (BT05), Maize kernel, Autoclave, Tips, pipette, Flask, Beaker, PCR.

**4.1.2 Chemicals:** Phenol, Chloroform, PBS (phosphate-buffered Saline), PBST (phosphate-buffered saline-twin-20), TAE (tris acetate-EDTA) Buffer, Agarose, 2-mercaptoethanol, RNAiso plus, Isopropanol, DPEC (Diethyl pyro carbonate), 70% ethanol, extraction buffer.

#### 4.2. Methodology:

##### 4.2.1. Media preparation, pouring and sub culturing:

- 19.5gm of PDA powder was added to 500 ml of distilled water.
- It was boiled until it mix properly and dissolve then autoclave the media.
- Prepared media was poured in petri plates and kept undisturbed.
- Fungal strain of *A. flavus* was cultured over media plates.

##### 4.2. 2 Conidia harvesting

- Plate of fungal culture (*A. flavus* –BT05) was taken.
- 5ml-6ml of PBST was added to the plate.
- PBST was gently mixed with plate culture to obtain only the above conidia portion.
- Gently mix and put approx. 2ml of the solution mixture in eppendorf.
- Now centrifuge at 1000 rpm for 5 min at 4°C and discard supernatant.
- PBS was added to pellet up to 2ml.
- Again centrifuge was done at 1000 rpm for 5 min at 4°C.
- Again discard the supernatant and add PBS and store the conidia.



### **4.2. 3. Fungal staining**

- A drop of LCB was placed on a clean dry slide.
- Fungal culture was taken and was gently mix with LCB (lacto phenol cotton blue).
- Cover slip was placed, without the formation of air bubble in it.
- Observe under microscope.
- Maize seed was directly observed under microscope

### **4.2. 4. Spore count**

- Eppendorf was taken with sample (maize seed infected conidia) and dilute the sample i.e. add 900µl of PBS and 100µl of culture total volume make up is 1000µl.
- Last Dilution was taken i.e. 10<sup>4</sup>.
- 10µl of sample was loaded in haemocytometer and calculate the spore under microscope.

### **4.2. 5. Extraction of conidia from maize-kernel:**

1.) Maize seed was taken under various conditions:

- Normal seed.
- Normal seed soaked with autoclaved water.
- Autoclave seed without soaking.
- Autoclave seed soaked with autoclave water and again autoclaved.
- Autoclaved seed soaked with autoclave water.

2.) These seeds were infected with *A. flavus* (BT05) (which working conidial culture is of 10<sup>4</sup> cells/mL was used).

3.) The conidia were harvested from media using 5-6 ml of PBS and PBS-T as washing reagents.

4.) These infected maize seed was incubated for 3<sup>rd</sup> day and 5<sup>th</sup> day and the RNA was isolated from zero day which was uninfected maize seed, 3<sup>rd</sup> day which was taken after 3<sup>rd</sup> days of infection and 5<sup>th</sup> day which was taken after 5<sup>th</sup> day of infection.

5.) These harvested conidia was taken for work from the following day conditions.

6.) The conidia of the fungus was taken out by centrifugation.

7.) After centrifugation the pellet was taken for RNA extraction of zero hour these conidia was taken as uninfected seed conidia.

## **4.2. 6. RNA EXTRACTION PROTOCOL**

### **4.2. 6.1 Homogenization:**

1.) *A. flavus* was extracted from seed samples for homogenizing in 1 ml of RNAiso reagent per 50 to 100mg of tissue applying a glass-bead.

2.) The sample volume was not allowed to increase from 10% of the volume of RNAiso reagent which was used in homogenization of all the samples.

3.) Homogenized sample was incubated for 5 min. at R.T for the complete disconnection of nucleoprotein complexes.

4.) After centrifugation the cell debris was removed out.

5.) In new tubes the supernatant was transferred.

### **4.2. 6.2 Phase separation:**

1) For each 1ml of RNAiso reagent subsequent amount of chloroform was added.

2) The test samples were mixed by vortexing for 15 sec approx. Then followed by incubation maximum for 5 minutes at R.T.

3) For the extraction purpose after incubation the test samples were centrifuged at 4,000 rpm for 30 min at 4°C.

4) The centrifuged samples were then separated into three layers named as phenol chloroform layer; inter phase and upper aqueous layer.

5) The desired was RNA founded in upper aqueous phase.

6) Upper aqueous phase was attentively transferred in new eppendorf without disturbing the other two phase i.e., interphase and organic phase.

#### **4.2. 6.3 RNA precipitation:**

- 1.) RNA precipitation was done with isopropyl alcohol for per ml of RNAiso reagent required for proper mixing.
- 2.) This sample was incubated at 15 to 30°C for 10 minutes at 4°C.
- 3.) Samples were centrifuged for 30 minutes at 4°C but it should not be more than 4,000 rpm.
- 4.) The pale white pellet formed after centrifugation is considered to be RNA and generally can be easily visualised before centrifugation as well.

#### **4.2. 6.4 Washing of the RNA:**

- 1) Supernatant was completely discarded from tube.
- 2) RNA pellet washing was done twice with 70% ethanol.
- 3) Sample was mixed properly and centrifugation was done but not more than 5,000 rpm for 2 minutes at 4°C.

#### **4.2. 7. RNA QUANTIFICATION**

The appraisal of RNA uprightness is a basic initial phase in getting significant quality articulation information. Working with low quality RNA may emphatically bargain the trial consequences of downstream application which are regularly work concentrated, tedious and exceeding costly. Utilizing flawless RNA is a key component for the effective utilization of current atomic organic techniques, as RT-PCR or miniaturized scale cluster examination. To confirm RNA quality three days economically accessible computerized slim electrophoresis frameworks are accessible which are en route to turn into the standard in RNA quality appraisal.

Profiles produced yield data on RNA focused, permit a visual assessment of RNA trustworthiness and create approximately proportions between the mass of ribosomal sub-units. In this audit the significance of RNA quality for the RT-PCR was dissected by deciding the RNA nature of various cow-like tissue and cell culture. Autonomous examination frameworks are portrayed and looked at (OD estimation, Nano Drop, Bio-analyser 21000 and Experian). Further the examination and relationship between the all-out RNA upright Ned on PCR execution just as on PCR proficiency is depicted. Based on the determined outcomes we can content that RT-PCR execution is influenced by the RNA uprightness and PCR profanely all in

all isn't influenced by the RNA trustworthiness. We can suggest a RIN higher than 5 bases great all out RNA quality and higher than 8 as ideal absolute RNA for downstream application.

#### **4.2. 7.1 Protocol to use Nano drop:**

- 1) Lower and upper surface of micro Spector photometer (thermo scientific multi scanner) was cleaned which is sample retention plates.
- 2) 1 to 2µl of buffer of DEPC water was added this will act as blank. And in rest the samples was being added. This instrument contains seven rows and at a time 14 sample can be loaded.
- 3) The plate was placed inside the Nano drop.
- 4) Nano drop software was opened and nucleic acid module was elected.
- 5) Nucleic acid was measured by clicking on "run".
- 6) After completion of measure the plates is been exit out of the Nano drop instrument.
- 7) Clean the deionized water with tissue paper.
- 8) The 260/280 ration and concentration of the sample is flashed on the screen of computer in tabular form.
- 9) Note down the readings of the samples.

#### **4.2. 8. cDNA SYNTHESIS:**

Complementary DNA (cDNA) special case. Most qualities are single duplicated in DNA (exemption: ribosomal 5S quality in oocytes). mRNA is more often plenteous (i.e. display in numerous duplicates) in certain cells which is ordinarily. Single duplicate DNA quality this can be taken as that of the quality is being communicates within the cells at same time. Hence, this is regularly cashier of discover the quality in this mRNA form e.g., affront mRNA in B cell of islets of lanherhance within the pancreas. Egg whites mRNA which is in oviduct check counter acting agent mRNA in plasma cells. In any case, on the off chance that the quality is frame a eukaryote, it will contain intron which is grafted out some time recently mRNA interpret and the quality could be in a different form real DNA shape. mRNA cannot be wield directly (restriction endonuclease as it were activity two fold strands DNA ) and so mRNA must be changed over in the form of DNA. This is often done utilizing

RNA as format with the help of chemical switch transcriptase which gets from retrovirus. The invert of ordinary transcriptase where the layout is DNA and its mRNA. There are a few strategies of making cDNA. The strategy that takes after is one case and misuses the real that a eukaryotes mRNA have a tail of adenine bases at the 3' end conclusion which result in annealing with an oligo-DT preliminary (it need starting strand blend). The moment DNA strands is synthesized utilizing another preliminary (oligo-DT) which anneals to cytosine already include to the 3' conclusion by a terminal transferase chemical.

#### 4.2. 8.1 Requirements: for 1×

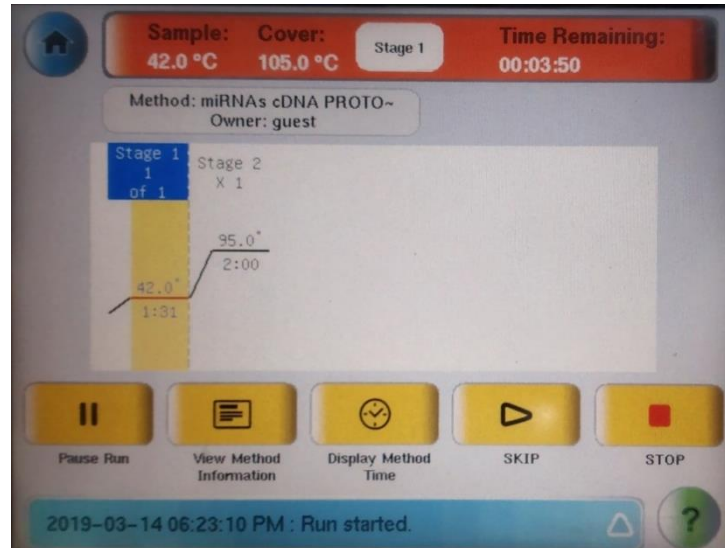
**TABLE 1:** Requirement for 1× cDNA Synthesis

<b>Reagent</b>	<b>Volume (1×)</b>
5× cDNA synthesis buffer	4µl
dNTPs mix	2µl
RNA primer	1µl
RT- enhancer	1µl
RNA Template	1-5µl
Nuclease free water	20µl
Total volume	20µl

#### 4.2. 8.2 Procedure for cDNA synthesis:

- 1mg RNA was added (for the reaction volume of 20µl)
- Tube was placed on ice. The tube was only removed to add the compounds. Add the component one at a time and then again the maser mix was placed on ice.
- Nuclease free water was added and final amount of volume was makeup.
- 5× iScript Reaction was added up to 4µl for a 20µl reaction volume.
- 1ml of iScript was added at R.T.
- The reaction was incubated completely with the required temperature:-
  - a) 25°C for 5 min.
  - b) 42°C for 30 min

- c) 85°C for 5 min.
- The samples was kept at -20°C in refrigerator.
  - Using PCR reaction with housekeeping gene (*tubulin*) cDNA sample was checked where RNA was used to synthesis first stand cDNA synthesis.



**FIGURE 1:** cDNA synthesis cycle from REAL Time PCR machine in the genomic laboratory

#### **4.2. 9. Polymerase chain reaction (PCR)**

Polymerase chain reaction is one of the quick system used for artificial enzymatic intensification to explicit RNA groupings it utilize two oligonucleotide preliminaries that cross-breed to inverse strands and flank of the locale of enthusiasm for main RNA. Monotonous cycles cover layout denaturing, grounding toughening and the expansion amassing of the tempered preliminaries by DNA polymerase, yield in the exponential amassing of a specific piece which ends are characterized by 5' end of the preliminaries. The groundwork multiplication items integrated in one cycle can fill as a layout in the following. A consequence the amount of the target DNA double roughly pairs at each cycle. PCR has a tremendous effect in both essential and indicative parts of science. Like the PCR itself, the bulk of utilization has been assembling expontially. It is along these lines prescribed that applicable research and labs in creating nations like Nigeria ought to gain this basic and generally modest yet rather strong innovation.

#### **4.2. 9.1 METHODS OF PCR:**

##### **Requirements:**

- 1) Thermal cycler (thermo cycler).
- 2) PCR amplification mix.
- 3) RNA samples/cDNA samples.
- 4) Oligonucleotide primer (forward and reverse primer) of desire gene.
- 5) DNTPs (deoxy nucleotide triphosphates).
- 6) 10× Reaction buffer.

#### **4.2. 9.2 Procedure:**

- 1.) Based on total number of samples a reaction mixture was prepared for amplification and finally the total volume was calculated. The total calculation is based on the 1× of reaction:
  - Taq polymerase (0.2µl)
  - Buffer(2µl)
  - Forward primer(0.5µl)
  - Reverse primer (0.5µl)
  - DNTPs (0.4µl)
  - Distilled water(15.4µl)
  - RNA (1µl)
- 2.) Adding these all to a PCR tube will make a reaction mixture now take 11.5 µl in one PCR tubes.
- 3.) Taq polymerase(5µl) was added at the last in master mix
- 4.) 1µl of RNA was added to each PCR tubes.
- 5.) Gently mixed the PCR tubes or spin the PCR vials and placed the vials in thermal cycler.
- 6.) Now the amplification of RNA take place under various condition:
  - 2 minutes at 94°C
  - 20 second at 94°C
  - 30 second at 44°C
  - 40 second at 72°C

- 7 minutes at 72°C
- 7.) Total number of PCR cycle was 35.
- 8.) After the amplification, the vial was taken for gel electrophoresis for observation i.e. formation of band on gel and the rest was kept in -20°C.

#### **4.2. 10. AGAROSE GEL ELECTROPHORESIS**

A straightforward strategy for agarose gel electrophoresis permitting the synchronous partition of 15 tests in less than 1 hour is depicted. The approach is entirely reasonable for clinical groove investigation of proteins in plasma and other body fluid since a simple goal is receive with examples which are anything's but tough to change. It is additionally helpful in lipoprotein examinations in a somewhat adjusted structure.

Other than the normal utilize the method could fill in as a profitable expository device in protein science. The strategy is effectively adjusted for preparative work on 5-100 mg scale. The utilization of sanitized agarose for partition of proteins not isolated in conventional agarose is additionally portrayed.

The presence of RNA band was checked by agarose gel electrophoresis which was done on agarose extraction gel of 1.5% to check the result for the existence of RNA band on gel.

##### **4.2. 10 .1 Reagent preparations:**

- TAE (tris-acetic acid-EDTA) buffer (50×) — In 500 ml of MQ water 242gm of tris base was dissolved. Then 57.1ml of glacial acetic acid was mixed this solution was followed up by 100 ml of 0.5M EDTA at the PH of 8.0. Before use total make-up of the value was by 1000ml with MQ water and it was diluted as working concentration of 1×.
- Ethidium Bromide (10mg/ml)-in 1ml of MQ water 10 mg of ethidium bromide was added and it was dissolved properly before storing it in a bottle at R.T.
- Loading dye (6×)-for the analysis of RNA, 0.025% of “bromophenol blue” (BPB) with 40% sucrose was used.



- Agarose- 1.5% of agarose gel was used for checking the RNA band which was prepared in 1x TAE buffer.

(Procedure {Russell and Sambrook, 2001})

#### **4.2. 10 .2 Pouring of agarose gel:**

- 1) Concentration of 0.7% to 2% was commonly used for agarose gel but it depends on the size of RNA band which is required for separation.
- 2) For the preparation of gel 1.5% of agarose was measure.
- 3) In 100 ml of 1× TAE agarose powder was poured and heat the solution until it dissolve proper in 1× TAE. Heat for 2-3 min for proper dissolving of agarose in TAE.
- 4) Allow agarose solution was allowed to come at R.T for 3-4 min
- 5) When the agarose solution reaches the R.T, then 3-5µl/100ml of Etbr (Ethidium bromide).
- 6) Etbr was added to gel.
- 7) Pouring of agarose gel was done in the casting tray with the comb.
- 8) Gel was left for solidify at R.T for 15-20 minutes.

#### **4.2. 10 .3. Samples loading and running of agarose gel:**

- 1) 4ul of RNA sample/PCR product was mixed with 3µl of loading dye.
- 2) In electrophoresis unit, the solidified agarose was kept for loading of the samples.
- 3) 1× TAE buffer was poured in electrophoresis unit so that the gel may completely covered by TAE.
- 4) According to the sample the ladder was added in the well of agarose gel which is followed by the samples loading.
- 5) At 100-150V the gel was allowed to run with loading dye for 20-25 minutes.
- 6) Electrode was disconnected and power was turn off after proper running of sample.
- 7) Then removing of gel was done properly from the tray.
- 8) Gel documentation system was used for the observation the gel. Gel was placed under the gel Doc. And look upon the RNA bands. This was the final result.

#### **4.2. 11. RT-PCR:**

Real time PCR is indistinguishable to a basic PCR but that the advance of the response is checked by a camera or finder in “Real time”. There are a number of strategies that are utilized to permit the progress of a PCR to be checked. Each strategy employs a few kind of fluorescent marker which binds to the DNA. Subsequently as the number of quality duplicates increment amid the response so that fluorescence increments. Typically invaluable since the efficiency and the reaction can be seen. There is additionally no ought to run the PCR item out on a gel after the reaction. Methods of observing DNA intensification in “real-time”. Fluorescent dyes intercalating fluorescent colours (e.g. SYBER green) are the best and cheapest way to screen PCR in real-time. These colours fluoresce as it were when bound to two fold strand DNA. So as the number of copies of DNA increment amid the response so the fluorescence increment. The major advantage of using a colour such as this the need of specificity.

This colour will report the enhancement of any DNA not just in gene of interest. Fluorescent probes fluorescent tests are pieces of DNA complimentary to your quality of intrigued that are named with a fluorescent colour. The best and most commonly utilized sort of test is the taqman-type probe. These test are named with a fluorescent columnist particles at one conclusion and a quencher molecule (able of extinguishing the fluorescent of the correspondent) at the other. Consequently beneath normal circumstances the fluorescent outflow from the test is moo. In any case amid the PCR the probe binds to the gene of interest and gotten to be cleaved by the polymerase. Thus the columnist and quencher are physically isolated and the fluorescence increment. Another commonly utilized sort of probe is the “molecule beacon”. Once more these are little pieces of DNA complimentary to gene of interest named with a fluorescence columnist and a quencher atom on inverse close.

#### **4.2. 11.1 Requirements:**

- 1) SYBER green assay 2× SYBER green mix(6.5µl)
- 2) Forward primer (0.5µl)
- 3) Reverse primer(0.5µl)
- 4) Template cDNA(1µl)
- 5) Nuclease free water(4µl)

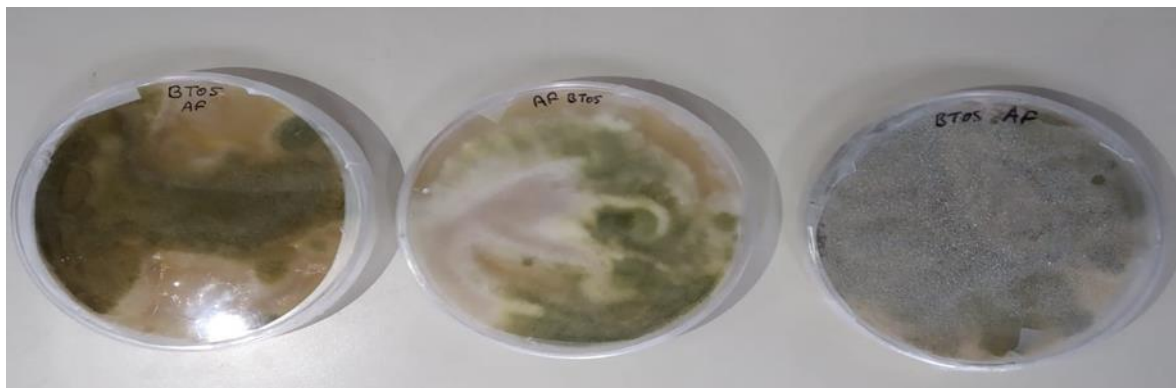
#### **4.2. 11.2 PROCEDURE:**

- 1.) For Real-time PCR: Plate was transferred in the RT- thermal cycle and real time PCR.
- 2.) The following program was used to run the RT-PCR (as per the instrumental manual).
  - 1 cycle: 95°C for 10 min (trigger the host-start taq DNA polymerase)
  - 40 cycles:95°C for 15 sec(data was collected throughout)
  - 60°C for 1 min (data need to be collected throughout)
- 3.) SYBER green dye was used to analyse the fluorescence curve formed with the dissociation of the sample.
  - Temperature: 95°C for 15 second (Denaturation purpose).
  - Temperature: 60°C for 15 second (Hence, data was collected).
  - Gradient increase in temperature from 60°C to 95°C at +/- 2% temperature error rate (Data was collected).
  - 15 sec: 95°C (Data was collected).

## CHAPTER-5

### RESULT AND DISCUSSION

**5.1 RNA culture on PDA plate:** Growing of *A. flavus* was on the PDA media plates and preparing slants of *A. flavus* for preservation and future use. These media plate cultures are of 3<sup>rd</sup> day and 5<sup>th</sup> day.



**FIGURE 2:** *Aspergillus flavus* plate on PDA media.

**5.2 Harvesting of conidia from infected maize kernel:** Conidia of *A. flavus* was harvested successfully using PBST and the conidia was stored in PBS. And these harvested conidia was used for the infection of maize seed.



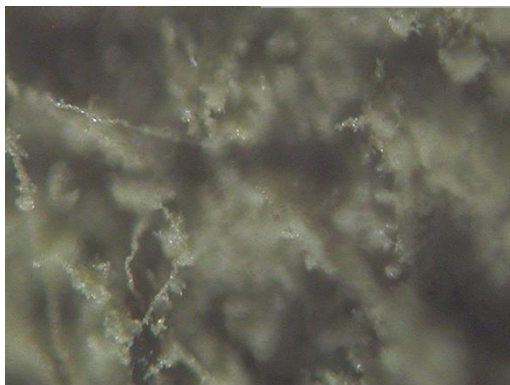
**FIGURE 3:** Conidia of *A. flavus*

**5.3 Conidia Count and dilution:** The cells/ml of  $10^4$  dilutions was  $6.55 \times 10^4$  Spores/ml of suspension was used for infecting the maize seed.

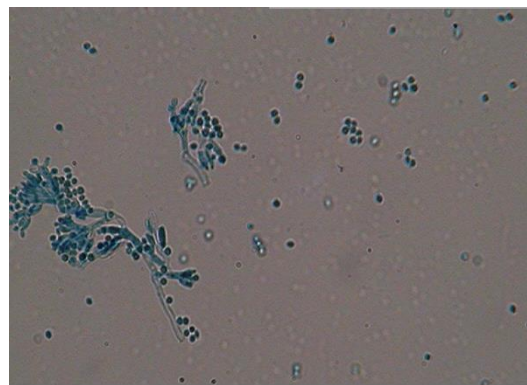


**FIGURE 4:** Diluted cell suspension (Dilution factor 4 times)

**5.4 Fungal Staining and infected maize seed observation:** *A. flavus* was stain with lacto phenol cotton blue stain and the infected seed was observed under microscope. The germinating conidia were seen, branches of *A. flavus* were seen and spore was observed.



**FIGURE 5:** Infected maize seed under microscope (40X)



**FIGURE 6:** *A. flavus* under micro spore (40 X)

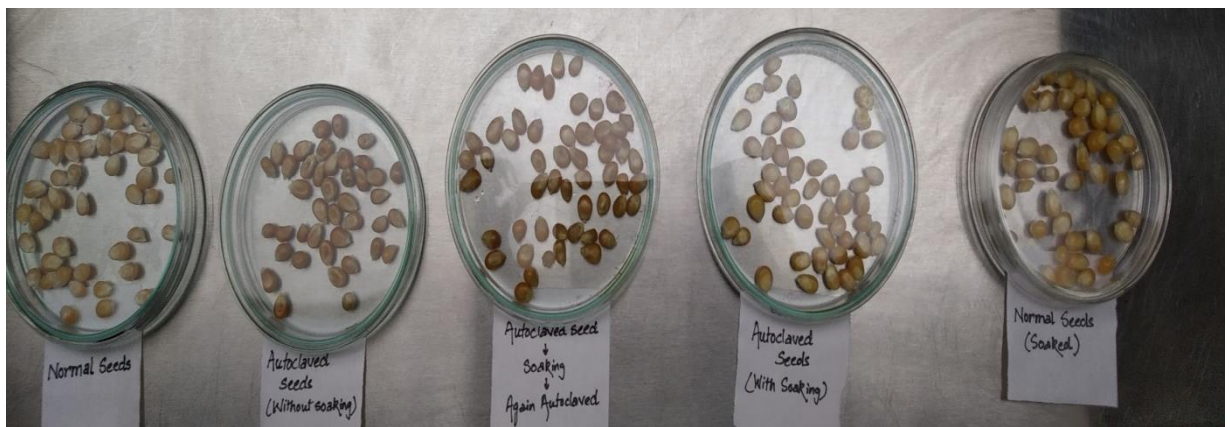
**5.5 Infected seed observation with its conditions:** *A. flavus* was made to grown on maize seed under different condition. The best growth of *A. flavus* was found on the autoclave seeds soaked with autoclave water and then again autoclaved and on normal seed soaked with autoclaved water. Growth of *A. flavus* on autoclave seeds soaked with autoclave water was very less. No growth of *A. flavus* was found on normal seeds and autoclaved seed without soaking. According to the growth of the fungus on seeds the best seed was selected for the

further work i.e. autoclave seeds soaked with autoclave water and then again autoclaved and on normal seed soaked with autoclaved water.

- From These infected seeds three conditions was taken i.e., zero day of infection, 3<sup>rd</sup> day of infection and 5<sup>th</sup> day of infection.
- From these three conditions the RNA was isolated for rest of the work.



**FIGURE 7:** Seed was infected with *A. flavus* & RNA was extracted for zero hour



**FIGURE 8:** Infected seed with *A. flavus* (Day-1)



**FIGURE 9:** Infected maize seed with *A. flavus* (Day-3)



**FIGURE 10:** Infected maize seed with *A. flavus* (Day-5)

- Autoclave seed soaked with autoclave water and again autoclaved condition seeds were selected for RNA extraction for zero days, 3<sup>rd</sup> day and 5<sup>th</sup> day.



**FIGURE 11:** Zero hour of infected seed with *A. flavus*.

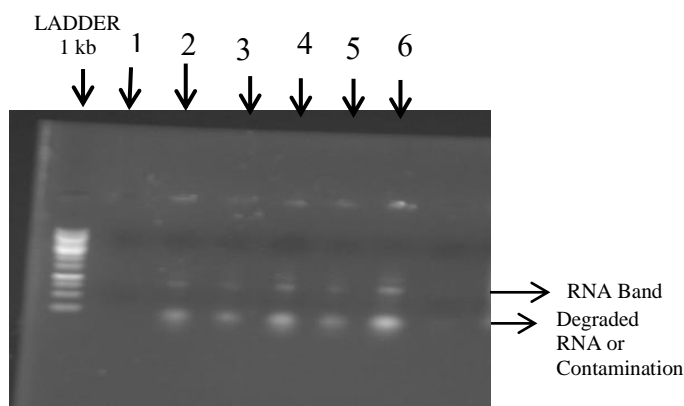


**FIGURE 12:** 3<sup>rd</sup> day after the infected seed with *A. flavus*.



**FIGURE 13:** 5<sup>th</sup> days after the infected seed with *A. flavus*.

**5.6 RNA isolation:** The isolated RNA of these three conditions was loaded on 1.5 % agarose gel with corresponding ladder of the size 1 kb. The gel was run with the altering voltage of 100-150V. The RNA sample was mixed with loading dye. The RNA band was observed in lanes mention below with RNA contamination or no RNA band.



**FIGURE 14:** Gel Doc image of RNA band of zero days.

Zero hour (conidia uninfected) RNA band

Lane 1- 1kb ladder.

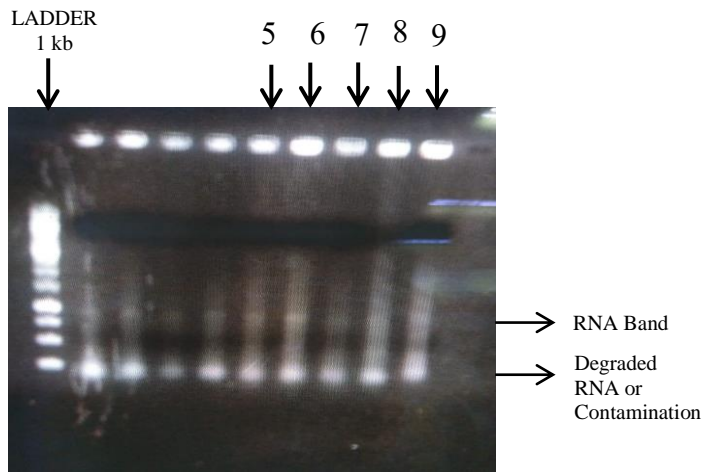
All lane contain sample

Lane 2, 4 & 6 contain RNA band.

RNA contamination was found.

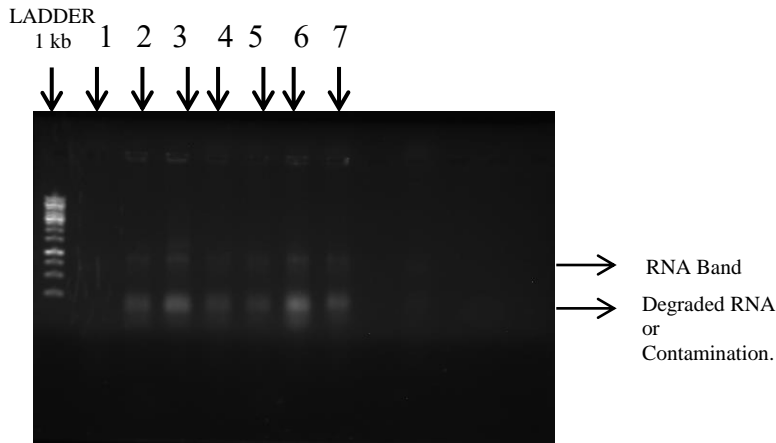
Rest lane don't contain RNA band.

Lane 3 & 5 contain light band of RNA.



3<sup>rd</sup> day RNA band  
 Lane 1- 1kb ladder.  
 Lane 5-9 contain sample.  
 Lane 5 & 6 contain RNA band.  
 Lane 7 have light RNA band.  
 RNA contamination was found.  
 Rest lane contain very light RNA band.

**FIGURE 15:** Gel doc image of RNA band of 3<sup>rd</sup> day.



5<sup>th</sup> day RNA band  
 1kb ladder was used.  
 Lane 1-7 contains samples.  
 Lane 2, 3, 6 & 7 contain RNA band.  
 RNA contamination was found.  
 Lane 1 & 4 don't can't RNA band.  
 Lane 5 have light RNA band.

**FIGURE 16:** Gel doc image of RNA band of 5<sup>th</sup> day.

- Before making cDNA with isolated RNA the RNA concentration was measure to quantify the RNA using Nano drop of all the samples i.e., zero day, 3<sup>rd</sup> day and 5<sup>th</sup> day. The concentration of RNA was as followed:

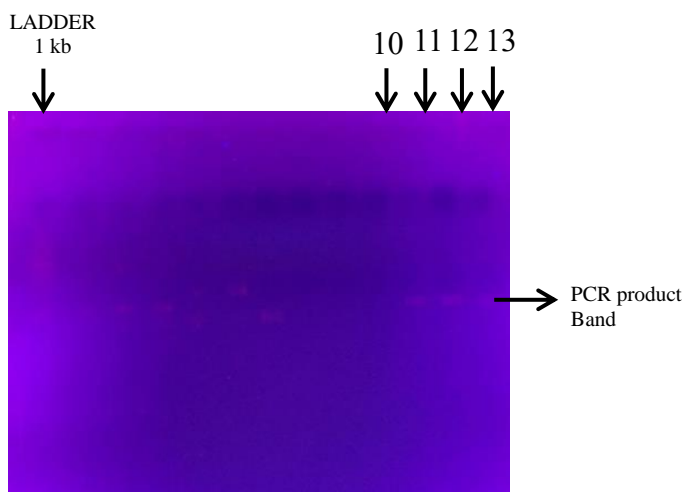
**TABLE 2:** Quantification of RNA

Time (hour)	Concentration of RNA (ng/μl)
Zero hour	301
3 <sup>rd</sup> day	270
5 <sup>th</sup> day	290



**5.7 cDNA synthesis :** cDNA was made from RNA which was isolated from the maize kernel.

**5.8 PCR:** PCR was done to check the cDNA. The formation of band on gel confirms the presence of cDNA.



**FIGURE 17:** Zero hour PCR gel

Zero hour (conidia uninfected)  
PCR product band

Lane 1- 1kb ladder.

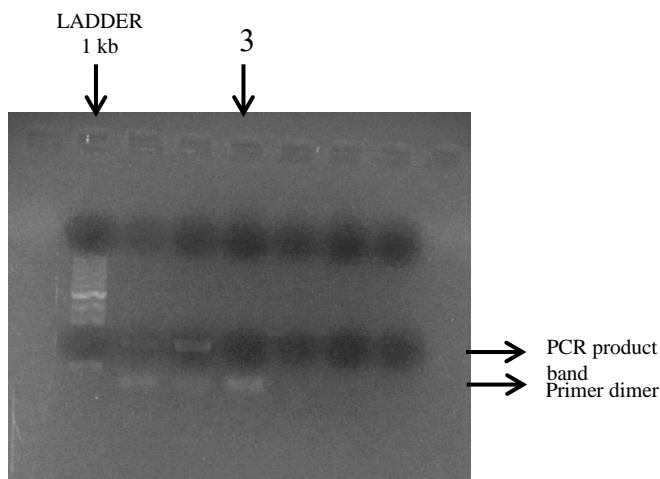
Lane 11-13 contain sample

Lane 10, 11, 12 and 13 contain  
band.

No contamination was found.

In lane 11, 12 & 13 have band.

Lane 10 has no RNA band.



**FIGURE 18:** 3<sup>rd</sup> day PCR gel

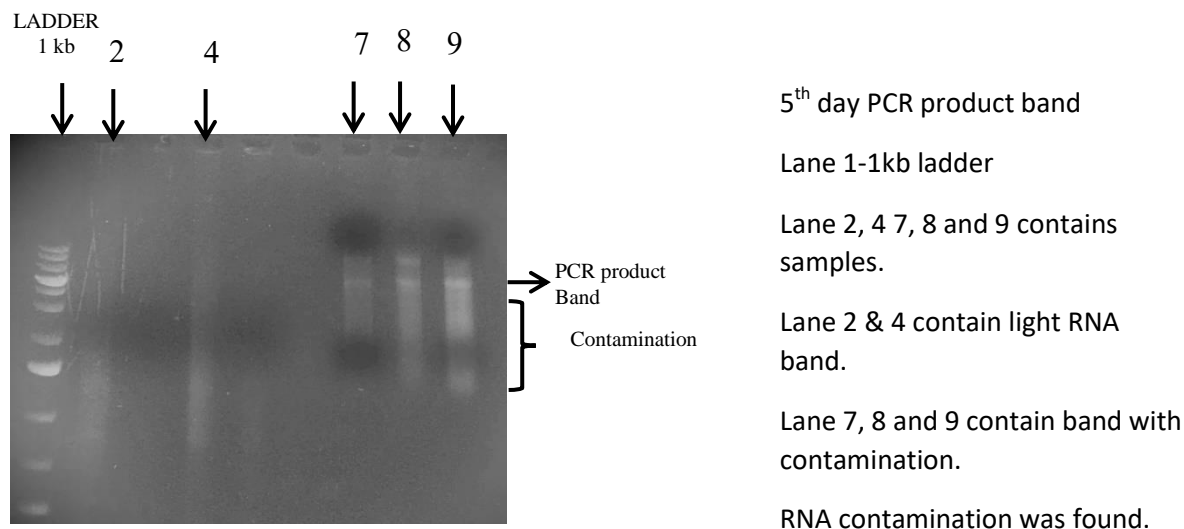
3<sup>rd</sup> day PCR product band

Lane 1- 1kb ladder.

Lane 3 contain sample.

Lane 3 contain band.

RNA contamination was not  
found.



**FIGURE 19:** 5<sup>th</sup> day of PCR gel

### 5.9 RT-PCR for *aflR* and *aflD* Gene:

- *aflR* and *aflD* are the selected genes which take part in the aflatoxin path.

**TABLE 3:** Selected gene Ct (cycle threshold) value

Days	Genes		
	<i>Tubulin</i>	<i>aflR</i>	<i>aflD</i>
Zero hour	25.32	28.19	29.15
3 <sup>rd</sup> day	23.95	28.3	32.06
5 <sup>th</sup> day	24.08	28.07	35.06

**TABLE 4:** Normalizing of CT value with tubulin gene (reference gene)

Days	<i>aflR</i>	<i>aflD</i>
3 <sup>rd</sup> day	4.35	8.11
5 <sup>th</sup> day	3.99	10.98

**Note:**

- Low Ct value represents high gene expression.
- High Ct value represents low gene expression.

- From the normalizing Ct value table we suppose that at initial stage there was more expression of gene (*aflR* and *aflD*) but after the days of infection was increased the expression of gene is low.
- In 3<sup>rd</sup> day of treatment it represents the high gene expression of gene *aflR*. Where as in *aflD* there was low gene expression.
- After 5<sup>th</sup> day of treatment there was low expression of the *aflD* genes but it *aflR* expression was high.
- We assume that in these days i.e., 3<sup>rd</sup> day and 5<sup>th</sup> day the expression of *aflR* gene is high and the expression of *aflD* gene is low.

## CHAPTER-6

### CONCLUSION

Our study demonstrated that the *A. flavus* can infect the crop at high rate and it play key role in the formation of aflatoxin. Aflatoxin is naturally occurring mycotoxin produced by fungi in the soil and on plants. They can cause significant crop loss by rejection of produce secondary to regulations against contamination in foods. These toxins have a global distribution with the greatest number of health problems occurring in developing countries where exposure is not adequately controlled. In addition to crop damage, inhalation of *Aspergillus* spores can cause lung damage and ingestion of aflatoxin in contaminated foods can cause serious health consequences in both humans and animals.

Control of aflatoxin production and food contamination can be considered a One Health issue because of the interaction of environmental conditions and human practices that determine impacts on human and animal health.

Certain crops are more susceptible to aflatoxin formation than others; maize (corn) and peanuts are two key staples that are prone to extensive contamination. Other staples such as rice and cassava may also be contaminated. Contamination can occur at all stages of crop production: harvest, storage and processing. Farming and storage practices which affect temperature, humidity, and insect damage to crops can significantly impact aflatoxin levels.

Aflatoxin exposure can be prevented or decreased by improving and enforcing safety regulations, changes in farming and crop storage practices, detoxification and other preventive measures. Both pre- and post-harvest interventions can be used to mitigate the health impacts of aflatoxin in humans and animals.

The main idea of the project was to look the gene expression. *Aspergillus flavus* was infected with maize seed under various conditions to look upon best growth of *A. flavus* on seed. We observed that the autoclave seeds soaked with autoclave water and these seed were again autoclaved and normal seed shows the best growth of *A. flavus* whereas the rest seeds do not show the growth or less growth was found. From this well growth *A. flavus* (BT05) the RNA was isolated to look

upon the gene expression with selected gene. The study done till now shows that the *A. flavus* (BT05) infect the maize-kernel with high frequency at 25-27°C and at optimal condition. From the isolated RNA the cDNA was made look upon the aflatoxin pathway i.e., cDNA synthesis will be carried out from the RNA. Before making cDNA the concentration of RNA was checked so the cDNA could be made by the best RNA. To check the cDNA, PCR is done. Finally the gene expression analysis using RT-PCR for selected genes such as *aflR* and *aflD* was done and using the Ct value of all the condition RT-PCR the regulation of gene was studied, to understand the role key genes from aflatoxin pathway during colonization of *A. flavus* on natural host.

## CHAPTER-7

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