Expression profile of genes encoding for Heat Shock protein of *Aspergillus flavus* during interaction with maize

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

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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 profile of genes encoding for Heat Shock protein of *Aspergillus flavus* during interaction with maize"** 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.

Kunjan Govil (151839)

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

Dr. Jata Shankar

Associate Professor,

Department of Biotechnology & Bioinformatics

Dated:

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ABSTRACT

Maize is defenceless against sullying in association with the destructive infectious agent *Aspergillus flavus*. Illness follows the decline in the level of grains and contamination of a little part, alongside an extraordinarily malignant growth originating the formation of harmful mycotoxin known as aflatoxin. The contamination caused by aflatoxin in maize or similar host originated by *Aspergillus flavus* tends to an outrageous money related mishap and threat human prosperity. Thusly, this examination was finished to appreciate the establishment of the colony of *Aspergillus flavus* on maize kernel. Seeds of maize were taken for the following project work. *Aspergillus flavus* strain BT05 was spoiled with the parts of maize (i.e., seeds) under different time conditions and autoclaved treatments and after the infection of *A. flavus* on the maize parcel, RNA was extracted to examine the value of verbalization of the given fungal strain i.e., *A. flavus* similarly as the aflatoxin quality of being dependable will be inspected. To fathom the instrument of *A. flavus* related with maize parcel, quality articulation investigation was finished by using RT-PCR for picked characteristics of two heat shock protein quality, HSP70, HSP90 and aflatoxin reliable ability will definitely be in frail in maize. Our results provide cover as a foundation for clear understanding of the generation of aflatoxin in maize and also provide a new snippet of the data to administer aflatoxin contamination. And at last it was observed that the gene expression of both the genes HSP70 and HSP90 are higher on the third day of the fungal infection but on the 5^{th} day of the fungal infection, decrement in the gene expression of both the genes was observed. On both observed days of fungal infection gene expression of HSP90 was much higher than HSP70, also the decrement rate of gene expression of HSP70 was higher than HSP90.

1. INTRODUCTION:

A. flavus is a spearheading exploitative pathogen which is able to pollutes growing crops, attacking vegetation that are crippled because of characteristic weights, low moisture season and the heat. Parasitic establishment of colony of the part diminishes quality of the grains and results in colonization of aflatoxin, a harmful and malignant growth. Screening of quality will be done of generation and maize parts [1]. Tainting parts were breaking down histologically using an infectious express stain. We found *A. flavus* vaccinated parts to respond to infectious agent strike by increased enunciated characteristics related to the opposition response. Aflatoxins are deadly, transcendently malignancy which causes the formation non- compulsory metabolites of *A. flavus*.

The tendency to separate, depiction and some part of the characteristics of all four immovably connected pieces of mycotoxin, the mix of deadly metabolism products conveyed by the explicit strains of *A. flavus*, were already delineated. Those portions were known as aflatoxin B1, B2, G1 and G2. Formulae were created for aflatoxin B1 and G1[2]. The associations of these blended and B2 and G2 were build up, the bond which is two-fold in dihydrofuran ring at the terminal end in the underlying two section having advanced toward forming the hydrogen bonds with other.

A. flavus is sly irresistible specialists of grains. It is an imperative because of this aflatoxin act as an assistant metabolism product in a considerable lot of yields seed both at some point as of late and as a result. Aflatoxin might be with ground-breaking cancer-causing that is exceedingly controlled in real countries. Inside the ground, aflatoxin is identified with dry season dry oilseed yields, cotton seed shelled nut plants nuts and tallying maize.

Underneath the appropriate state, aflatoxin will create and deliver by a living organism into almost any secured alter seed. A considerable lot of the field control re-inforce advancement of *A. flavus*. A considerable lot of the field controls worth are being utilized and examined, checked. Altering with sharpens, improving the methods of safe harvests crops crosswise over Real time-PCR technique: focused evasion using fungal strains which cannot convey aflatoxin and advancements with field medicine which would minimize the generation aflatoxin.

Aspergillus flavus contains wide have broadened deft microorganism. *A. flavus* is an incredibly happening microorganism it results in the real worry of this creature. The fundamental issue with this living being in agribusiness is that it can delivers significantly carcinogenic toxins named aflatoxin which is a prosperity animals hazard. Inside the dirt, *A. flavus* is immensely an issue inside the cottonseed, oilseed crops maize, tree nuts and peanuts. Underneath unseemly limit conditions *A. flavus* is equipped for creating and also forming aflatoxin in about any harvest seed. It besides might be irresistible operator of animals and of frightening little creatures. Individuals as it is fantastically a deft hurtful specialist of resistant stifled patients.

Barely any creatures have had as wide a monetary influence in view of *A. flavus*. It might be poisonous on yields, animals or dreadful little creatures cause limit rots in different developed fauna and it cause the overly increased coordinated aflatoxin B1. Organism of Aspergillus species wound up dynamically basic since immunosuppressed people uncovered astoundingly powerless against infection by these life forms. Of the aspergilla causing mycoses in individuals, figuratively speaking A. treats is progressively important that *Aspergillus flavus*. *Aspergillus flavus* is also an allergen triggered ominously powerless bronchopulmonary aspergillosis. As a dreadful little creature irresistible operator, it impacts a many number of animal varieties, tallying bumble bees, which incite a disease shout out stone brood [20]. This isn't one of a kind inside a particular in any plant and frightening little creature. Chief incredible typically moulded cancer-causing agent is aflatoxin B1.

A.flavus may prove itself very infective operators of rice, grain crop, maize, peanuts and so forth. *Aspergillus flavus* is also capable in corn as an ear decimate [1]. In corns and peanuts *A. flavus* in charge of tree disease known as yellow type of seedlings or "alumroot". Most of the symptom shows necrosis wounds, chlorosis in high-ground consolidate and there is a need in progress for helper roots, 'alumroot' [6]. The root effect can be a result of aflatoxin destructiveness since it's been showed up with impede improvement of plant organ internal tobacco. *Aspergillus flavus* may something awful to rot too, to create shelled nut inside the ground. In cotton, *A. flavus* impacts cotton reasonableness past making boll ruin.

Secondary metabolites having high carcinogenic properties are produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Although there are some management strategies designed to minimize the growth and contamination of this mycotoxin in corn fields, but the leading or preeminent way to eliminate the contamination by aflatoxin is to produce a pre harvest host which is resistance to aflatoxin interactions. This method of aflatoxin elimination gained a stature because of the recent findings related to the resistance in corn plant, which can be used for plant breeding strategies. The capability to find out the resistant genotypes in corn plants has been improved and enhanced due to the advancements of in-vitro kernel screening assay with the helping fungal strain of *A. flavus.*

Fibre defilement is remarkably named as "yellow spot sickness". This 'yellow' escapes bright greenish yellow (BGY) brilliance shown at the cotton fibres underneath long –wavelength high intensity force light.

A. flavus is a comprehensive dispersal filamentous, saprophytic development that once in a while pollutes oil inexhaustible seeds of various yield species in the midst of pre just as post-reap assemble, with subsequent formation results in arrangement of aflatoxin, for instance, cyclopiazonic destructive, excited, and the eminent aflatoxin [1,2]. All this noxious impact on prosperities are major indisputable in making countries lacking advancements to search and analyse and diminish aflatoxin sum in harvests and was poor storing state as often as possible arrangement is progressively conspicuous aflatoxin assembling in the sustenance supply. Other than the prosperity recommendation in individuals and animals. *A. flavus* colonization in yields causing basic money related disasters by virtue of crushed/set apart low quality use and cheaper cost of aflatoxin-spoiled grains [5, 6]. Utilization of RNA sequence have essentially enlivened, appreciation of the unpredictability at quality enunciation, rule or frameworks of animal under various acquired spaces-brief situation or trait explanation could be all the more correctly assessed using RNA sequence moved toward, after that the customary transcriptomics examination [22].

Biotic or abiotic stress is an ominous arrangement for an organism which includes growth. To conquer pressure, Organism communicates heat shock proteins (HSPs) or escorts to perform organic capacity, to conquer pressure.

Heat shock proteins are associated with different standard organic procedures, for example, interpretation, interpretation and post-translational alterations, protein collapsing, and total and disaggregation of proteins. Accordingly, it is imperative to comprehend all-encompassing job of Hsps in light of pressure and other natural conditions in parasites [37].

Heat shock proteins, Hsp90 has been anticipated as a most prospective antifungal focus because of the morphogenesis capability, out of all other heat shock proteins. Hsp90 and Hsp70 together or separately conduct a noteworthy job in morphogenesis and dimorphism.

2. LITERATURE REVIEW:

2.1 Maize Seed Contamination

From 1994 to 1995, about 8 to 60 maize swards were overviewed, only to screen Aspergillus ailment and degree of aflatoxin polluting influence of foregather maize in Benin. Species include in Aspergillus genera, a total of three, were withdrawn from different sectors, with an extensive abundance of *A. flavus*. In the states of Guinea Savannah, toxic substance obsession showed a specific zonal accumulation, with genuinely peculiar forms in the aforementioned. Consequently, the topographical perspective found in case of *A. flavus* and aflatoxin may be related to higher rate [22].

In spite of the manner that *Aspergillus flavus* is portrayed as a point of confinement assembly, it has been thoroughly perceived that disease of maize at this improvement happened at the field. For a long time, in any case, it was imagined that pre-gather maize was without aflatoxin. [24] show-cased, on the maize, the familiarity at the aflatoxin B1 before collects. Subsequently these inspections were composed expansive work to evaluate the vitality of the pre-collect sullying of the maize has been performed so as to pick the feature affecting the ailment method by dangerous constituent passing on living things, particularly in the United States.

Interaction of fungal pathogens in plants, results in different type of defence mechanisms. The traits of a necrotrophic pathogens is shown by the toxicogenic strains of *Aspergillus flavus*. Maize hybrids which are commercially acquired may susceptible to *Aspergillus flavus.* In some maize germplasm lines, it is found that the maize host could be tolerant to infection by *Aspergillus flavus* and accumulation of aflatoxin.

A couple of examinations in the African tropical, have been done to pin point the implication of *A. flavus* in pre-reap maize. In Benin, maize develops a chief substance to the general population and organic situations are reasonable of the parasitic enhancement. Pre-reap polluting of maize by *A. flavus* and following aflatoxin formation may comprise an inconvenient issue [26,] unequivocal that maize in Benin were truly contaminated at a beginning time of point of confinement. This might have been a post effect of significantly large pre-collect polluting. The aforementioned inspection was tried to clear up the suspicion. This goal was to pick the dispersing and measurement of the maize partition disease by parasite in the Benin. Likewise,

relationship concerning the *A. flavus* sickness and the extent of aflatoxin made at pre-collect maize were created and fuse of freighting little animal naughtiness to the ear in the midst of the time spent aflatoxin debasing in pre-reap maize was researched. Bugs harming maize grains have been had every one of the reserves of being by and large compared to the rate and estimation of the aflatoxin dirtying in pre-reap maize. Strategies using insilico tools are being also applied to understand mechanism on how phytochemicls or other inhibitors can resitrict aflatoxin contamination [37].

2.2 Aflatoxin

In the 1960s, that the early era of the decade, Aflatoxin were in the first place found and portrayed after the in excess of 1000000 turkey mopes in Britain passed on to clear hurting from form tainting shelled nuts dinner. The disease has two rudimentary states caused by the introduction to Aflatoxin. Extreme aflatoxicosis occurs in transitory. The disease is caused by Ingrained aflatoxicosis, in liver and as it the fundamental bull's eye organ, safe disguise, the teratogenicity and the opposite reactions [3, 4]. There is in addition a couple demonstrate that inhalator prologue to aflatoxin augments the occasion of inhalator and other the malignant growths. Artificially, aflatoxin is a Difuran coumarin backup made by the “Polyketide Pathway”. Aflatoxin B1, B2, G1 and G2 are the four noteworthy aflatoxins, with their letters insinuating on the fluorescence shading underneath splendid light (green or blue) and the numbers showing a Relative movement detached on the Chromatographic plates’ little layer [4].

There are some additional tools exists which are particularly made to counter the danger or threats which are possessed by aflatoxin to the safety of food and security, this also give enhances the knowledge of interaction between two different kingdoms also known as cross kingdom interaction which might lead to some other mycotoxin generating infectious agent in various fields of agriculture.

For the Aflatoxin, the characteristics which intake the biosynthesis, similar to those of the various assistant metabolites, are bunches [3]. The sequencing of the complete group has been done and the same has been clarified [11]. The quality group, in terms of length, is 82 kb, and contains characteristics that total up to 25. The subject of true consideration is the Bearing of the pathway [10]. It corresponds to the development and enhancement of control system and gives an understanding of the how’s and why’s

related to the aflatoxin progression, largely, the genome of the *A. flavus* have been sequenced [26]. The information in the same will provide gainful tools for grasp of both life form and aflatoxin. In the scarcest ninety-nine countries, the Prohibitive sum are put in for the aflatoxin [10, 11]. A managerial level move from one country to the next country. The USA FDA movement levels are set at 20 sections for every billion (p.p.b., µg/kg) for the general population sustenance, 0.5 p.p.b for dribble and up to 300 p.p.b for the corn and cotton seed in animal support. Inside the EU, 4p.p.b are the set levels while in India the authoritative levels are the set at 30 p.p.b for every one of the nourishments. In the combined the state alone, the merciless money related incident related to the mycotoxins is assessed to be the $932 million (CAST 2003). Aflatoxin might be the real supporter to this issue. Practical expense of aflatoxin is irksome to evaluate since such signify measures should consolidate incident of empathy animals to aflatoxisis. Assessing the expense of the Aflatoxicsis in individuals around the globe is in reality progressively inconvenient. In term of trim adversity alone, measures of yearly expenses inside the US creating locales powerless to aflatoxin were $ 36,700,000 for California pecans $23,000,000 to $ 47,000,000 for the California almonds [34]. Furthermore, morphotypes of Aspergillus species including *A. fumigatus, A. niger, A. terreus* delivering mycotoxin require additional examination to their job in nourishment crop defilement or their job in the pathogenesis in wellbeing host or insusceptible traded off patients or giving obstruction against antifungal drugs [35]

2.3 Heat Shock Proteins

Some stress proteins have been accounted for the confer pressure resilience and stress tolerance yet in additional upgrade ailment opposition or disease resistance. The expression analysis of Heat shock proteins, particularly the smaller ones under stress and pressure has been broadly considered and appeared to show a molecular activity upon the chaperons. Beside from Heat stress, HSPs are additionally incited by different burdens, for example low temperature, dry environment or saline alterations [38,39].

A connection between adjustments of transcripts levels and the proteins which corresponds to that was recognized for half of the differentially directed or regulated proteins. As the matter of interest, some beforehand undescribed putative target sites for the Heat shock controller Hsf1 were recognized.

One of the essential impacts of Heat shock in life forms is refolding and unfolding of misfolded proteins which is interceded by heat shock proteins (HSPs, cheprones) [40].

Heat shock proteins (HSPs) are a pervasive group of proteins associated with reactions to numerous abiotic stresses, including warmth, saline value, and oxidation burdens. They are named by their sub-atomic load or molecular weight in kilodaltons and frequently fill in as molecular chaperones, helping denatured proteins and peptides overlay appropriately or be labeled for corruption. Aspergillus HSP90, an antigen in hypersensitive bronchopulmonary aspergillosis by Aspergillus fumigatus, advances sedate obstruction and conidiation and is associated with sign transduction. Hsp70 encourages protein collapsing and assumes a job in protection from antifungal medications [42].

Cells which are affected with high thermal stress, generally shows the heat-shock proteins response. Enhancement in the production of reactive oxygen species in cells due to thermal stress may acts as activation source for heat shock proteins. Hsp70 and Hsp90 proteins have been implicated in drug resistance mechanism against antifungals agents in aspergillus species, summarised in recent review [36]

The expression of heat shock proteins is shown under different stress situation, these expressions of HSPs might be particular according to the conditions, such as osmotic pressure, antifungals, temperature, pH and oxidative stress. During the morphogenesis phase HSP79 and HSP90 are found in abundance. HSP70 and HSp90 could work individually or also form a complex of hsp90-hsp70, the also plays a key role in nascent folding of aggregated peptides. Expression of HSP90 and HSP60 are induced by the heats stress, whereas the upregulation of HSP12 was found in freeze resistance fungi. At very high hydrostatic pressure the expression of HSP104 was shown. Various heat shock proteins like HSP30, HS70 and HSP90 are mostly upregulated in non-alkaline pH [38, 40].

2.4 Metabolism in Aflatoxin

Aflatoxin are risky, fundamentally infection instigating optional metabolites of *Aspergillus flavus* and *A. parasictius*, which when prepared amidst irresistible disorder of a vulnerable yield in the field or after obtain contaminate sustenance and feed and undermine human and creature thriving [3]. Despite the route that there are a few association structures that may lessen aflatoxin corruption of corn, the amazing way of thinking for termination of aflatoxin is to make pre-gather have confirmation from aflatoxin accumulation. The mentioned methodology has extended fundamentally dynamically obvious noticeable quality of ceaseless introductions of standard impediment in the corn that can be mauled in plant impersonating systems. The capacity to perceive safe corn genotypes has been updated by the development of a lab part screening portion and by strain of *A. flavus* hereditarily planned to make B-glucuronidase, an intensify whose improvement can be believed to audit the component of irresistible disease in bits [4]. Scrutiny of safe corn genotypes have correlated piece pericarp wax attributes with opposition, perceived piece proteins linked with security from and confinements of irresistible headway aflatoxin biosynthesis, and saw chromosome domains associatedto the confirmation from Aspergillus ear ruin and aflatoxin creation. Such research advances could lead as quickly as time permits to monetarily open, ergonomically adequate corn lines with different pre-reap protections from aflatoxin corruption [3]. Infirmity reduces the quality of grain and tarnishes the portion with mycotoxin aflatoxin which causes harm. The improvement of secure maize lines has shown inconvenient paying little mind to the route there is confirmation for wellsprings of block. The non-participation of solid obstruction phenotyping marks the irregularity of sickness betterment reliably, and coming up short on a view of host opposition portions all have chosen the choice of limitation irksome. Advances being developed for example miniaturized scale exhibits cause it conceivable to research the possibility to test interpretation on a genome level estimation and gave superior comprehension of how creatures react to their condition on amount of cell [6]. This report asking about arrangement quality clarification amidst the strike of pathogen have been discovered the shield reaction leaves PR-proteins behind and consolidate elucidation alteration in both fundamental and optional plant pathways of metabolic and detoxification. Phytohormones, for example, salicylic dangerous, damaging, for quite a while ethylene held the title to be known as a fundamental piece of the resistance

reaction. At any rate starch preparing pathways in any case not normally connected with limitation maize a basic bit of the plant guard reaction intertwining in maize [3-5]. Higher levels of maize stalks starch have been related with expanded protection from the living creatures which cause stalk sullying, a huge package of which are progressively arranged for contaminating the piece and ear.

2.5 Effect on maize by *A. flavus*

Transcriptional alteration of maize parts amidst sullying by *A. flavus* has been broken down with the assistance of qPCR and microarrays. Kelly et al looked like it was invulnerable to gathering of aflatoxin. 16 attributes significant in the protected gathering were discovered and 15 in the exposed change and accepted in different systems and possibly associated with protection given by aflatoxin hiding away. [15]. revealed progressively lifted proportions of significant worth clarification in pushed associated attributes at secure lines of the maize [18]. Discovered that large maize qualities were impelled by *A. flavus* in vulnerable components separated and secure portions. According to these tests, ensure associated and definitive attributes were related with the reactions to *A. flavus*. To provide an all progressively clear energy about maize partition protection from *A. flavus* it was verified that the transcriptional response of harvest i.e., maize parts amidst disease with *A. flavus* in area with the assistance of a custom-made DNA miniaturized scale exhibit. We archived contrast in articulation of all around delineated guard consisting pathways and confirmation which is connected qualities correspondingly as the great alteration in clarification at attributes respected to retention of starch. There are a couple of levels in disease method have obstruction could limit irresistible headway and contamination of aflatoxin. Bit ailment with *A. flavus* beginning through silk establishment of colonization [18, 15]. Conidia develop and make on senescing silks going down the channel of silk to developing bits and the same procedure is done by pit-bull in 8 days. Coming about steps in the debasement system are less depicted at any rate information prescribed that *A. flavus* can strike bits and pieces amidst their 6 phases of their betterment sulking, trouble, milk, mix, physiological and scratch headway, According to late Reese et al, vaccinated disconnected pieces at levels R2-R5 in laboratory and it was discovered that parcels at this 4 phases is feeble against contamination in light of *A. flavus* Parasitic disease have been found in harmed pieces as youth in milk mastermind. These youthful pieces will

accumulate high mixes of aflatoxin as a result of deferred establishment of colonization by thebe pathogen. Contamination in pieces which are not hurt in field happens last, amidst scratch formative level just before physiological betterment [16]. Once inside, *A. flavus* seldom authority over the rich germ tissue which is rich in oil, Parasitic betterment inside endosperm, has been seen in any case there are anomalies in the organization concerning the degree of establishment of colonization. Our tests focused on modification reaction of building segments that were illuminated with, *A. flavus* by harm, we understand that this procedure could slight some limitation devices yet it gave dynamically steady illness progress as outcome [18]. Security from corrupting of hurt pieces is in like way significant as it copies alarming minimal creature hurt, which is imperative in the examination of illness conveyance of the friendship, moreover to achieve the reaction of various times of the part betterment; we overviewed, *A. flavus* disease of 4 the field levels R2 and R5. We increasingly gave priority to particular time of the 4 days after the vaccination to analyze quality articulation subject to past histological tests by [29] who display that within 4 days after the inoculations *A. flavus* mycelium achieved the endosperm, fleuron, germ tissue.

3.OBJECTIVES:

- To studythe interaction between host and pathogen, in maize kernel infection under in-vitro conditions.
- Analysis of gene expressions of the genes encoding for heat shock proteins of *Aspergillus flavus*,during interaction between*A. flavus* (BT05) and maize seed.

4.MATERIAL REQUIRED AND METHODOLOGIES:

4.1. Materials:

4.1.1 Devices and Instrumentations:Maize kernel, Incubators, Gel Documentation setup, Bunsen burner, RT-PCR, Marker,PCR, Petri plate, Matchsticks, Fungal strain*A. flavus* (BT05),Eppendorf tubes, Incubators, Tips, micro-pipette,Centrifuge, Laminar air flow, Flask,Autoclave, Beaker, Measuring cylinders, Gel electrophoresis setup,

4.1.2 Chemicals and Reagents: 70% ethanol,Phenol, Phosphate-Buffered Saline-twin-20 (PBST), 1x TAE Buffer, Primers, Agarose,PBS (phosphate-buffered Saline), 2-mercaptoethanol, RNAiso plus,DPEC (Diethyl pyro carbonate), Isopropanol, Chloroform,extraction buffer,LCB (lacto phenol cotton blue).

4.2. Methodologies:

4.2.1. Preparing of Media and Sub-Culturing of Fungus:

- 19.5gPDA (potato dextrose agar)was measured and added to 500ml of distilled water.
- It was dissolved by mixing and boiling.
- Autoclaved of prepared media was done.
- Pouring of media into petri plates was done and kept isolated until the solidification of media.
- Culturing of *A. flavus* fungal strain over prepared PDA plates was done and marked accordingly.

4.2. 2 Harvesting conidia from the fungal culture plate

- Prepared cultured plates of *A. flavus* (BT05) was taken.
- 4ml-6ml of PBST was spread all over the plate.
- Approximately 2ml of conidia and PBST solution was obtained in the Eppendorf.
- Centrifugation was done at the desired settings i.e., 10000rpm for 5min. at 4ºC and discard the supernatant

- 2ml PBS was appended to the remaining pellet.
- Again, centrifugation was done.
- Again, supernatant was discarded and PBS was added to the pellet.
- Conidia suspension was store at 4ºC.

4.2. 3. Staining of fungal culture and microscope observations

- A small drop of LCB was poured on clean and dry slide.
- Culture of the fungal strain was gently blend with LCB using a toothpick or a micro tip.
- Avoiding the formation of air bubble, cover slip was placed over the LCB carefully.
- Prepared slide was observed under the different magnifications of microscope.

4.2. 4. Spore count

- Conidia suspension was taken, and dilution was done for example 900µl of PBS and 100µl of the sample.
- Number of dilutions was 4 times i.e. 10^4.
- 10µl ofthe dilutedsample was loaded in haemocytometer and the spore count calculation was done under the different magnifications of the microscope.

4.2. 5. Infection of maize kernel:

1.) Various conditions for the maize seeds were taken:
    - Autoclave seeds without soaking.
    - Normal seeds.
    - Normal seeds soaked with autoclaved water.
    - Autoclaved seeds soaked with autoclave water.
    - Autoclaved seeds soaked with autoclaved water and again autoclaved.

2.) All the seeds were infected with *A. flavus*(BT05) (conidial culture of 10^4 cells/mL was used).

4.) Maize seeds were incubated for several days. RNA was isolated from uninfected maize seed, 3rd day of infection and 5th day of infection.

4.2. 6. RNA-EXTRACTION PROCEDURE:

4.2. 6.1 Homogenization of the sample:

1.) *A. flavus* was separated from seeds tests for homogenizing in 1 ml of RNAiso reagent per 50 to 100mg of tissue applying a glass-bead.
2.) The sample volume was not permitted to increment from 10% of the volume of RNAiso reagent which was utilized in homogenization of the considerable number of tests.
3.) Homogenized sample was incubated for 5 min. at R.T for the total disengagement of nucleoprotein edifices.
4.) After centrifugation the cell debris was expelled out.
5.) The supernatant was transferred into new vials.

4.2. 6.2 Phase separation method:

1) Chloroform was added for each 1ml of RNAiso reagent.
2) Samples were vortexed for 15 sec approx. then incubated for 5min.
3) The samples were centrifuged at 4,000 rpm for 30 min at 4°C.
4) After centrifugation the samples were isolated into three layers.
5) The RNA wasfound in upper aqueous phase which is the desired product.
6) Upper aqueous phase was mindfully movedin new eppendorf without aggravating the other two phases.

4.2. 6.3 RNA precipitation and washing:

1.) RNA was precipitated using isopropyl alcohol for per ml of RNAiso reagent required for appropriate blending.
2.) Prepared samples were incubated at 15 to 30ºC for 10 minutes.
3.) Centrifugation was done for 30 min. at less than 400 rpm.
4.) Formation of white pellet after centrifugation is considered to be RNA.
5.) Supernatant was discarded.
6.) Washing of RNA pellet was done twice using 70% ethanol.
7.) Centrifugation was done for 2minutes at less than 5000rpm.

4.2.7. Synthesis of cDNA from the extracted RNA:

4.2.7.1 Reagents requirements for 1x reaction;

- 5× cDNA synthesis buffer(4µl)
- dNTPS mix(2µl)
- RT-enhancer(1µl)
- Template(RNA) (1-5µl)
- Nuclease Free water(14µl)

4.2.7.2Procedure for cDNA synthesis:

- 1mg RNA was used per 20µl of reaction volume
- During the whole addition of compounds, the reaction tubes were placed on ice.
- Nuclease free water was added to makeup the final reaction volume.
- In 20µl reaction volume, 4µl of 5× iScript Reaction was added.
- The reaction was incubated under several time and temperature conditions:
    a) 5 min at 25ºC.
    b) 30min at 42ºC.
    c) 5 min at 85ºC.
- The samples were stored at -20ºC.
- To check where RNA was used to synthesise first cDNA strand,cDNA sample was checked with PCR reaction with housekeeping gene (tubulin).

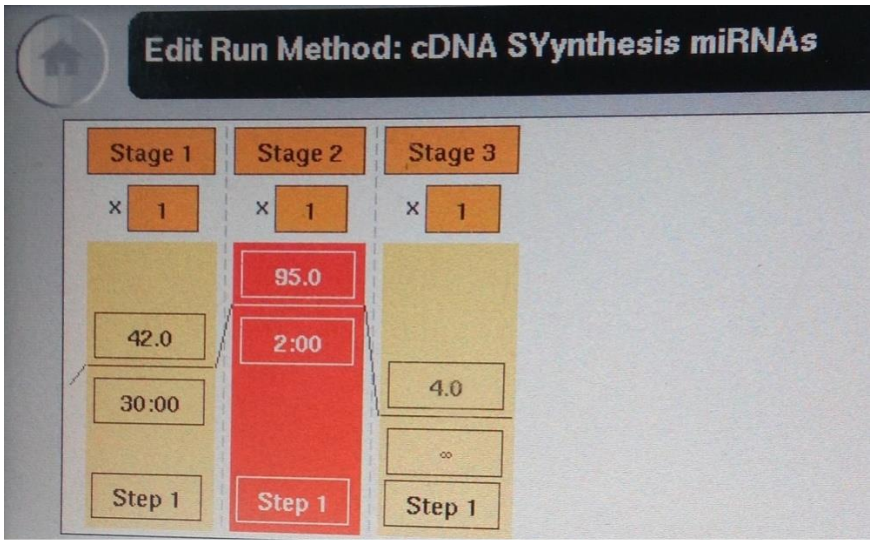

Figure 1: cDNA synthesis cycle from REAL Time PCR machine in the genomic laboratory

4.2.8Polymerase chain reaction (PCR)

4.2.8.1 METHODS OF PCR:

Requirements:

1) Thermal cycler (thermo cycler).
2) PCR amplification mix.
3) cDNA samples.
4) Forward and reverse primer for desired gene (encoding for Hsp70 and Hsp90).
5) dNTPs.
6) 10× Reaction buffer.

4.2.8.2 Procedure:

1.) Based on total number of samples two master mix for 5x of reaction were prepared for both the genes. The given calculation is based on the 1× of reaction:
   - i-taq polymerase (0.25µl)
   - Buffer(2µl)
   - Forward primer(0.5µl)
   - Reverse primer (0.5µl)
   - DNTPs (0.4µl)
   - Distilled water(15.35µl)
   - RNA (1µl)

2.) Excluding RNA, all the reagents were added in master mix for 5x reaction, i.e., 5 times the volume of all the reagents.

3.) The last reagent to be added in the master mix should be Taq polymerase.

4.) 1µl of RNA was added to separate PCR vials, after RNA addition 11.5µl of master mix was added.

5.) PCR vials with the RNA samples and master mix were gently mixed and placed in the thermal cycler.

6.) The various temperature and time condition for amplification were:
   - 94ºC for 3 minutes.
   - 94ºC for 30 seconds.

- 44ºC for 30 second.
- 72ºC for 45 second.
- 72ºC for 7 minutes.

7.) Total number of PCR cycle was 35.

8.) Amplified samples were taken for gel electrophoresis, or kept at -20°C.

4.2.9 GEL ELECTROPHORESIS

A direct system for agarose gel electrophoresis allowing the synchronous parcel of fifteen tests in under sixty minutes is delineated. The methodology is altogether sensible for clinical furrow examination of proteins in plasma and other body liquid since a basic objective is get with models which are definitely not's difficult to change. It is furthermore useful in lipoprotein examinations in a to some degree balanced structure.

Other than the typical use the technique could fill in as a productive explanatory gadget in protein science. The methodology is successfully balanced for preparative work on 5-100 mg scale. The usage of sterilized agarose for segment of proteins not disconnected in regular agarose is moreover depicted.

4.2.9.1Preparation of agarose gel and pouring:

1) Concentration of gel varies from 0.7% to 2% which depends on the size of the RNA band which is desired.
2) 1.2g of agarose was mixed with 100mL of TAE buffer for the preparation of 1.2% gel concentration.
3) The mixture of agarose and TAE buffer was heated for proper dissolving.
4) 5µL of Etbr was added to the agarose solution after it reaches the room temperature. Etbr was added according to volume of agarose solution.
5) Agarose solution was poured into the casting tray with comb.
6) Agarose gel was kept undisturbed to solidify.

4.2.9.1 Samples loading and running of agarose gel:

1) 4µl of sample was properly mixed with 3ul of loading dye.
2) The sample mixed with loading dye was loaded in the wells (compartments in solidified gel).

3) The gel was completely drowned with 1x TAE buffer.
4) Ladder was added according to the base pair size of the PCR products or samples.
5) The gel was permitted to run at 100 to 150 volt for approximately 20min.
6) After proper running of the gel power was disconnected and gel was removed carefully from casting tray.
7) To observe the RNA bands under ultra violet light, gel documentation system was used.

4.2.10 Real Time-PCR:

Real time PCR is indistinct from a fundamental PCR however that the development of the reaction in checked by a camera or discoverer in "Real time". There are various systems that are used to allow the advancement of a PCR to be checked. Every system livelihoods a couple of sort of fluorescent marker which ties to the DNA. Thusly as the quantity of value copies increase in the midst of the reaction so fluorescence increases. Normally priceless since the effectiveness and the response can be seen. There is furthermore no should run the PCR thing out on a gel after the response. Strategies for watching DNA increase "progressively". Fluorescent colors intercalating fluorescent hues (for example SYBER green) are the best and least expensive approach to screen PCR progressively. These hues fluoresce in a manner of speaking when bound to two overlay strand DNA. So as the quantity of duplicates of DNA increase in the midst of the reaction so the fluorescence increase.

4.2.10.1 Reagents required:

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

4.2.10.2 PROTOCOL:

1.) After the mixing of all the reagents and sample the PCR vials was transferred into the RT- thermal cycle.

2.) For RT-PCR the following temperature time arrangement was followed:

- 10min at 95ºC(taq DNA polymerase attack host) – 1 cycle.
- 15sec at 95ºC(data was stored throughout this step) – 40 cycles.
- 1min at 60ºC.

3.) To analyse the formation of fluorescence curve during the dissociation of samples, SYBER green dye was used.

- 15sec at 95ºC (Denaturation).
- 15sec at 60ºC (data collection).
- Increment in the temperature 60ºC to 95ºC with +/- 2% temperature error rate.
- 15 sec at 95ºC.

5. RESULTS & DISCUSSION

5.1 Fungal culture growth on PDA media: Sub-cultured plates of *A. flavus* (BT05) for preservation and future use.

5.2 Conidial Suspension: Successfully harvested conidia spores of *A. flavus* using PBST. The conidial suspension was kept in PBS. Maize seed was later infected using these conidial suspensions.

5.3 Spore enumeration and conidia dilution: The spore count in conidia suspension was 6.55×10^4 spores/ml in 10^4 dilutions.

5.4 Infected maize seed and LCB staining observation: Lactophenol cotton blue stained *A. flavus* and infected mainze seed with *A. flavus* was observed under different lenses of microscope. Observations:

- Conidia germination were observed,

- Branches of *Aspergillus flavus, and* spores were observed.

5.5 Infected maize seed with the applied conditions: *A. flavus* has the capability to grow on maize seed under different condition. *A. flavus* growth was found best in two conditions i.e., autoclaved seeds which are soaked in autoclaved water and then again autoclaved and Normal seed soaked with autoclaved water. There was very less growth on autoclaved seed soaked with autoclaved water, but no growth was observed on normal seeds and autoclaved seeds without soaking. Autoclaved seeds soaked with autoclaved water and then again autoclaved and normal seeds soaked with autoclaved water was best suited for the further experiments in accordance to their growth.

- Seeds from zero hour, 3rd day and 5th day of infection were taken from which the RNA was isolated for later experiments.
- Autoclave seed soaked with autoclave water and again autoclaved were selected for RNA extraction for zero hour, 3rd day and 5th day.

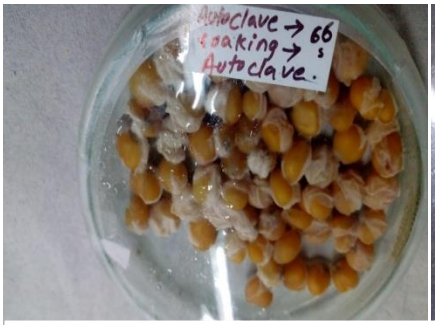

FIGURE 2 Maize seeds

FIGURE 3: Infection of *A flavus* on maize seeds **(5th day).**

5.6 RNA extraction: Extracted RNA from all 3 condition was loaded on 1.2% agarose gel. The size of the corresponding ladder which is loaded was 1kb. The gel was run at the voltage alteration of 100-150V.

RNA band, RNA contamination and no bands were observed.

The RNA from the zero hour, 3rd day, 5th daywas observed with a little RNA contamination.

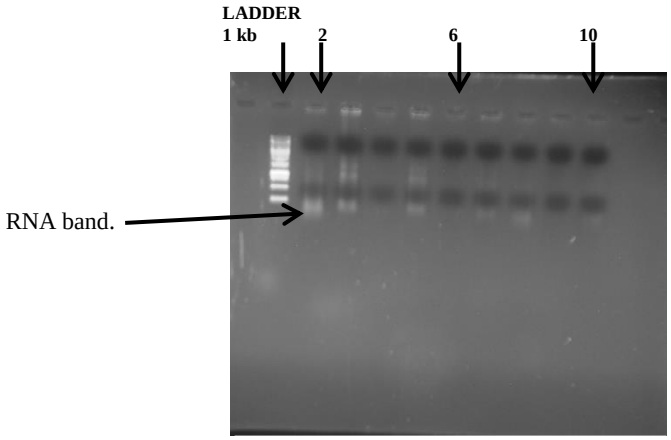

FIGURE 4: Gel doc image of extracted RNA band(zero hour).

5.7PCR: To check the presence of cDNA, PCR **was done. Forward and** reverse primer of HSP70 and HSP90 was used. Band formation on agarose gel confirms the presence of cDNA

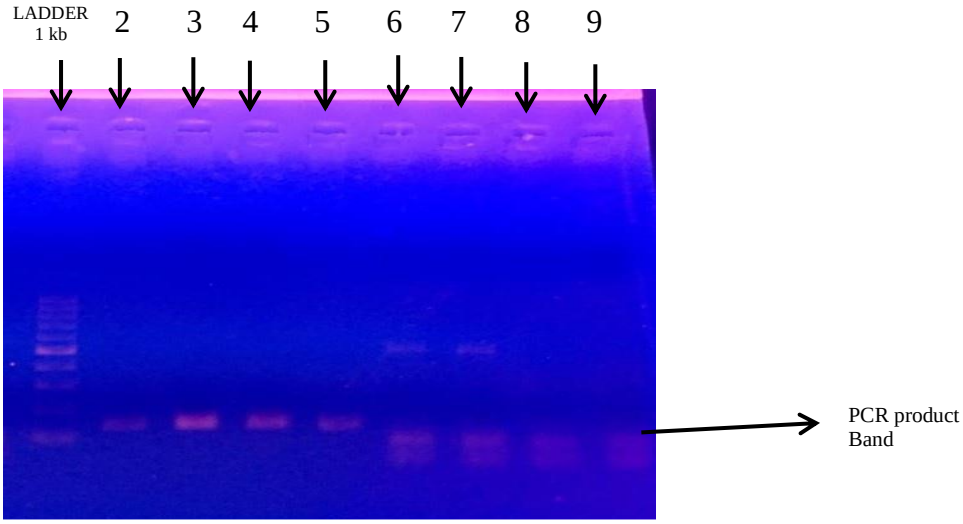

FIGURE 5: Gel doc image of agarose gel containing samples of cDNA with HSP70 & HSP90 primers of 3rd and 5th day

TABLE 1: Position and description of the sample across the gel.

Lane 1	Ladder (1kb)
Lane 2 and 3	cDNA sample of control, 3rd day with HSP70 primers.
Lane 4 and 5	cDNA sample of control, 5th day with HSP70 primers.
Lane 6 and 7	cDNA sample of control, 3rd day with HSP90 primers. HS
Lane 8 and 9	cDNA sample of control, 5th day with P90 primers.

RNA bands was visible in all the samples under UV light in gel doc chamber. Some RNA contamination was also observed.

5.8 Real Time-PCR:

- *Hsp70*& *hsp90* were selected genes which take part during host pathogen interaction.

TABLE 3: Threshold Cycle value of selected genes in mean

Days	Genes		
	Tubulin	***HSP70***	***HSP90***
3rd day	25.18	24.78	20.74
5th day	24.31	28.99	22.08

TABLE 4: Normalized Ct value with tubulin gene (reference gene)

Days	***HSP70***	***HSP90***
3rd day	-0.4	-4.44
5th day	4.68	-2.23

Note:

- Lower Ct value depicts higher gene expression.
- Higher Ct value depicts lower gene expression.
  - As per the normalized Ct value, we consider that in the initial stage of infection the gene (HSP70 and HSP90) expression was more, but after some days when there's increment in the infection the expression is found to be lower.
  - Gene expression of HSP90 was much higher than the HSP70 on the 3rd day of infection.
  - Gene expression on the 5th day of infection was found to lower than third day both the genes (HSP70 and HSP90). Gene expression of HSP90 on 5th day was much higher than HSP70 gene.
  - Gene expression of HSP90 is high and the expression of HSP70 is low on both the days of infection i.e., 3rd and 5th day.

6. CONCLUSION

Above experimentation showed that the *Aspergillus flavus*has the capability to contaminate yield with higher degree of contamination, and it also plays a major key role in the formation of mycotoxin, aflatoxin. Alatoxin is a mycotoxin which grows naturally and produced by the fungi which is present in the soil or in the plants. These aflatoxins can cause a big amount of harvest misfortune by dismissal of generated auxiliary to regulations against sullying in food or edible items. Toxins like these have a global appropriation with the highest number of health issues or medical problems happening in the countries which are under development and have inadequately controlled exposure. Notwithstanding yield harm, respiratory intake of Aspergillus spores, can affect lungs and also cause ingestion of mycotoxin in polluted nourishments may cause genuine wellbeing outcomes in the two people and creatures.

Control of aflatoxin formation and sustenance defilement can be viewed as a One Health issue as a result of the collaboration of ecological conditions and human practices that decide impacts on human and creature wellbeing.

Certain yields are more helpless to aflatoxin arrangement than others; maize (corn) and peanuts are two key staples that are inclined to broad pollution. Different staples, for example, rice and cassava may likewise be tainted. Defilement can happen at all phases of yield creation: reap, stockpiling and preparing. Cultivating and capacity rehearses which influence temperature, stickiness, and creepy crawly harm to harvests can fundamentally affect aflatoxin levels.

Aflatoxin introduction can be forestalled or diminished by improving and upholding security guidelines, changes in cultivating and yield stockpiling practices, detoxification and other preventive measures. Both pre-and post-gather intercessions can be utilized to relieve the wellbeing effects of aflatoxin in people and creatures.

The primary thought of the venture was to linger the quality articulation. *Aspergillus flavus* was tainted with maize seed under different conditions to view best development of *A. flavus* on seed. We saw that the autoclave seeds splashed with autoclave water and these seed were again autoclaved and ordinary seed demonstrates the best development of *A. flavus* though the rest seeds don't demonstrate the development or less development was found. From this well development *A. flavus* (BT05) the RNA was

secluded to view the quality articulation with chose quality. The examination done till now demonstrates that the *A. flavus* (BT05) taint the maize-part with high recurrence at 25-27℃ and at ideal condition. RNA was isolated from zero hour, 3rd day and 5th day after maize infection with *A. flavus* from which RNA from 3rd and 5th day of infection was best suited for the further experiments. cDNA was made from the selected RNA. PCR was performed to analyse the cDNA. To check the gene expression of the selected genes HSP70 and HSP90 real time PCR was performed. Using the normalized Ct value from the result of RT-PCR gene regulation was contemplated, to know the key role of the genes during the maize interaction with *Aspergillus flavus.*

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