Functional analysis of transcriptional factor *crz1*(Calcineurin-Responsive Zincfinger 1) during quercetin mediated inhibition of *Aspergillus flavus*

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CERTIFICATE

This is to certify that the work entitled, "**Functional analysis of transcriptional factor *CRZ1* (Calcineurin-Responsive Zincfinger 1) during quercetin mediated inhibition of *Aspergillus flavus*".**

Submitted by **Parul Mittal (131559), Puja Kumari (131561) & Passang Lhamo (131574)** impartial fulfillment for the award of degree of **Bachelor of Technology in Biotechnology** of **Jaypee University of Information Technology, Waknaghat, Solan** has been carried out under my supervision.

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List of acronyms and abbreviations

µL	Microliter
Mb	Megabytes
mm	Millimeter
B1, B2	Blue1, Blue2
G1,G2	Green1,Green2
Crz1	Calcineurin responsive zinc finger 1
A. flavus	*Aspergillus flavus*
A. oryzae	*Aspergillus oryzae*
^{0}C	Degree Celsius
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
Conc.	Concentration
AFB1	Aflatoxin Blue 1
CN	Calcineurin

INTRODUCTION

Aspergillus flavus

Aspergillus flavus is a saprophytic, disease causing fungus, which is distributed worldwide. It colonizes on cereal grains, legumes and tree nuts. It is considered as a major food contaminant. It is said to produce harmful toxins known as Aflatoxins. Aflatoxin produced by *Aspergillus flavus* is a secondary metabolite and found in the seeds of various crops. Aflatoxin is highly regulated in foodstuff in most of the countries due to its carcinogenic property. (Ref.1)

KINGDOM: Fungi

DIVISION: Ascomycota

CLASS: Eurotiomycetes

ORDER: Eurotiales

FAMILY: Trichocomaceae

GENUS: *Aspergillus*

SPECIES: *A. flavus*

STRAIN: *AF67*

Infection by *Aspergillus flavus* is said to cause diseases such as ear rot (in corn) and yellow mold (in peanuts) either before or after gathering the crops. The contamination can be seen in the field, before-harvest, and post harvest and also during storage/transit. *Aspergillus flavus* aflatoxin production is generally increased by uncontrollable moisture conditions and high temperature during storage of legumes and grains. The pathogen can cause liver cancer in mammals through ingestion of contaminated food or infection by fungus through invasive growth.

The yellow-green spores containing powdery masses on the upper and reddish-gold on the lower surface develop a colony for *A.flavus.* (Ref.2&3).

Toxins

Aspergillus flavus is considered to be the major producer of the most toxic compound i.e. AFLATOXINS. The four major aflatoxins produced are B1, B2, G1 and G2. Among these four compounds Aflatoxin B1 is evaluated as the most toxic and dominant hepatocarcinogenic natural compound identified. It also produces different toxic compounds, namely, sterigmatocystin, aspergillic acid, kojic acid, cyclopiazonic acid, B-nitropropionic acid, gliotoxin, aflatrem and aspertoxin. Aflatoxins enter into the common food supply through processing of contaminated food and it is found in pet and human foods, as well as in feedstock for domesticated animals. The

products modified by aflatoxin can be transferred into eggs, milk products and meat when animals are fed with contaminated food. Especially children are affected by aflatoxin exposure leading to developmental delay, slow growth, liver impairment and hepatic cancer. Adults have chances of getting infected through aflatoxin exposure though they have higher tolerance. There isn't any animal species, which is unsusceptible to this exposure. Generally aflatoxins enter into the body of host via ingestion of contaminated food but aflatoxin, B1; the most toxic type can penetrate through the skin. (Ref.5&6).

A literature review of these toxic substances depicts that there are possible environment friendly ways to inhibit their production. These involves the use of certain plant secondary metabolites i.e. phytochemicals.

Phytochemicals

There has been a rich history of the use of plant by-products as anti-microbial, anti-inflammatory, and anti-fungal agents. As plants are a rich source of various useful secondary metabolites, the experiments conducted on quercetin shows that it could be used as a potent anti-fungal agent.(Ref.9)

Quercetin

Quercetin belongs to a group of plant pigments called flavonoids that provide many flowers, fruits and vegetables with color. Flavonoids are present everywhere in photosynthesizing cells and are found in wine, nuts, honey, fruits, stem, tea, seeds, flowers, vegetables etc. With the progress, the flavonoids are getting its attention in the area of medical research. Many important properties were observed in flavonoids specifically anti-inflammatory, estrogenic, antimicrobial, antiallergic, antioxidant, vascular and cytotoxic antitumor activities and enzyme inhibition. The spore germination of plant pathogens is inhibited by flavonoids. They are used as anti-fungal agent against fungus pathogen present in humans. (Ref.9&10)

The inhibition of DNA gyrase partly contributes to the antimicrobial activity of quercetin. Through its direct antioxidant action quercetin can exhibit anti-inflammatory activity. The basophils and mast cells induce congestion by obstructing the histamine release.

It is said to have a direct impact on one of the main signaling pathways of fungi. The target here is to study the impact of quercetin on the calcium-calcineurin signaling pathway of the fungi under observation. (Ref.7)

crz1

Calcineurin-Responsive Zinc finger 1.

crz1 is one of the most important transcription factor, which is responsible for germination of conidia/morphogenesis in fungi. The arrangement of stress response genes is triggered by *crz1*. The dephosphorylation mediated through calcineurin regulates this nuclear localization positively. Under the stress of blue light illumination, *crz1* gets confined to the nucleus. It has been found that various environmental changes can induce calcineurin activation, which is a calcium-calmodulin dependent phosphatase, this helps in controlling gene transcription by causing dephosphorylation of the transcription factor *crz1*.(Ref.2&4)

Zinc finger is said to be a structural motif of a small protein whose characterization is done by the coordination, which occurs between one or more zinc ions in order to stabilize the fold.

Calcium-calcineurin signaling pathway in fungi

Calcium ion is one of the very important ubiquitous intracellular second messenger molecule involved in signal transduction pathway in mammals and fungus. It is necessary for survival of cells and takes a direct responsibility in controlling the expression patterns of its signaling systems. Furthermore, some of the components including calcium, predominantly calcineurin mediate fungal resistance to antifungal drugs. The signaling pathway of calcium-calcineurin comprise of different channels, pumps, transporters and other proteins or enzymes.(Ref.2)

Calcineurin (CN), one of the regulators of calcium homeostasis, has been recognized as a virulence factor in filamentous fungi. In these pathogenic fungi, filamentation is organized by some calcium channel protein. Most of the researches specify that different components of the calcium-signaling pathway take part in fungal physiological processes, mediate stress responses, and develop virulence.(Ref.2)

Description of calcium-calcineurin signaling pathway

The plasma membrane Ca^{2+} influx systems are activated (HACS & LACS), when encountered with the surface stresses; as a consequence there will be a rapid influx of Ca^{2+}. Due to secretions from internal compartments the intracellular calcium concentrations may increase transiently. Three calcium ions bind to CaM as calcium concentration increases and are sensed by calmodulin. Then, Ca^{2+}-calmodulin particularly binds to subunit A of calcineurin (CN) and concurrently, Ca^{2+}bind to the high affinity Ca^{2+}-binding sites on the B subunit of CN, as a result it is activated. Activated CN move towards downstream targets *crz1 & prz1,* activating their dephosphorylation and translocation from cytoplasm to nucleus. Calcineurin-*prz1/crz1* signaling promotes the expression of a set of Ca^{2+}/CN-dependent target genes, including *pmc1, pmr1 & pmr2.* Then, the intracellular calcium concentration gets decreased to basal levels, owing to the uptake of calcium by organelles.

Since, *crz1* gene is playing an important role as a transcription factor in this signaling pathway. Therefore, a methodology to inhibit the expression of this gene can be explored.(Ref.2)

OBJECTIVE

The rationale behind this project lies in the fact that there is a need for development of an environment friendly methodology to eradicate the infection caused by harmful pathogen of human beings i.e. *Aspergillus flavus* and to hinder aflatoxin production. A number of beneficial secondary metabolites were derived from plants. Use of plant products in the form of plant extracts and essential oils provide an opportunity to avoid synthetic chemical preservatives and fungicide risks. Here, the literature review of quercetin shows that it is a promising candidate to be tested as an anti-fungal agent. The transcription factor *crz1* is consequently under analysis because it is responsible for the germination of conidia in fungi, which is here the focus of inhibition.

Thus, the objective of this project is to study the functional analysis of transcriptional factor *crz1* during quercetin mediated inhibition of *Aspergillus flavus.*

REVIEW OF LITERATURE

Aspergillus flavus is an exploitative pathogen of crops. The assembly sequence of *A. flavus* shows that the genome is 36.3 Mb in size and it consists of about eight chromosomes and 13,071 predicted genes. *A. flavus* is almost identical to *A. oryzae* genetically. *A. flavus* is a common environmental organism so its comparative genomics studies are quite interesting although the sequence strain of *A. oryzae* is a domesticated fungus and it has been used in soy fermentation for thousands of years in which it rarely caused any disease. (Machida et al.,2005; Galagan et al.,2005).

A. flavus is known for velvety, yellow to green or brown moulds with a red-brown color to its reverse. Its conidiophores are heavily walled, uncolored, coarsely roughened and are usually less than 1 mm in length. Its vesicles are quite elongated when young, later they become subglobose or globose, and then it varies from 10 to 65 mm in diameter. Phialides, projections arising from vesicles in the shape of a flask, were found to be uniseriate or biseriate. The primary branches were up to 10 mm in length, and the secondary branches were upto 5 mm in length. Conidias were found to be commonly globose to subglobose and were varying from 3.5 mm to 4.5 mm in diameter. (Link, 1809)

It had been found to have a large distribution in the environment, because of production of the numerous airborne conidia, which could be easily dispersed by the movement of air and possibly by insects as their carriers. *A. flavus* has got the ability to survive in harsh conditions which allows it to out-compete other organisms for various substrates in the soil or in the plant very easily. It had got the potential to survive at a temperature which might range from 12°C to 48°C, but the ideal development temperature is found to be ranging from 28°C to 37°C. It is also said to produce a secondary metabolite in various crops, which is known as aflatoxin, the production is found to be both before and after harvest. Because of the discovered aflatoxin production in the species it had become the most widely reported food-borne fungus. Aflatoxin is said to be a carcinogenic substance and thereby it is highly regulated in most of the countries. *A. flavus* is found to be capable of growing and forming aflatoxin in probably all the crop seeds. It is also known as a pathogen of animals and insects. In humans it might predominantly be an opportunistic pathogen in the immunosuppressed patients. Contaminated foodstuffs by these mycotoxins had also been associated with high incidence of liver cancer in human system. (Maren A. Klich et al., 2007)

It had been first reported in samples of peanuts and it was observed that the toxic properties of certain samples were due to the metabolic products of the fungus *A. flavus*. The growth of *A. flavus*

in peanuts and in other agricultural commodities is said to be governed by temperature and moisture. *A. flavus*, which grows at 30^0C and a relative humidity of about 80%-85% produces appreciably different amounts of toxin in . The amount of toxin produced by different species of *A. flavus* differs appreciably. (C.W. Hesseltine et al.)

A secondary metabolite is produced by it called aflatoxin. Aflatoxins produce colourless to pale-yellow in color crystals. Production of intense fluorescent in ultraviolet light emitting blue or green and green-blue fluorescence occurs. Water solubility is very slight and there is insolubility in non-polar solvents. Moderately polar organic solvents are quite good for the solubility such as chloroform, methanol and especially dimethyl sulphoxide. The four major Aflatoxins were found to be B1, B2, G1 and G2, which could be distinguished by the color of their fluorescence under ultraviolet light. B1, B2, G1 and G2 had been clarified as potential human carcinogen in the group I by The International Agency for Research on Cancer (IARC). Aflatoxin B1 is said to be the most toxic and is also known for being one of the most potent genotoxic agent and hepatocarcinogen. Other toxic compounds produced by it are: cyclopiazonic acid, β-nitropropionic acid and aspergillic acid. (ZohraMohammedi*et al*., 2013)

The toxic and carcinogenic effects of the various aflatoxins got intimately linked to its biotransformation. Accordingly, differences among aflatoxin biotransformation pathways were critical determinant underlying variations in species sensitivities towards aflatoxin B1-induced carcinogenesis. AFB1 required microsomal oxidation to reactive AFB1-8, 9-epioxide in order to exert its hepatocarcinogenic effects, thereby extent of covalent binding of AFBO to cellular DNA was highly correlated to the carcinogenic potency of AFB1. (David L. Eaton & Evan P. Gallager, 2014)

Azoles are used as anti-fungal drugs. The disadvantages of Azoles, which includes rashes, anaphylaxis, Stevens-Johnson syndrome, resistance against azole containing pesticides is most probably adding major problem to the farmers. (Sanjay G. Revankar et al.)

The expression patterns of its signaling systems are controlled by calcium and it had been found to have important roles in the cell survival process of the fungi. In the fungal calcium-signaling pathway, it was found that calcium and some other components mainly Calcineurin are responsible for mediating fungal resistance towards antifungal drugs. This pathway consisted of various channels, pumps, various proteins or enzymes and some transporters. Most of the transcriptional profiles had been indicating that mutant strains were lacking some of these components and were therefore sensitized to fluconazole or some other antifungal drugs. Many researchers had identified

various efficient compounds that showed potential antifungal activity either by themselves or in combination with other potential antifungal drugs that was carried out by targeting some of the components of the fungal calcium-calcineurin signaling pathway. This targeting might have disrupted Ca^{2+}homeostasis, which suggested that this pathway contained many potential targets for the synthesis of new antifungal drugs. (Gerald R. Crabtree et al., 2000)

Plants contained a variety of secondary metabolites, which were namely, tannins, alkaloids, terpenoids and flavonoids. Flavonoids were reported to have had an in-vitro anti-fungal property. In vitro studies showed that, Quercetin, a bioflavnoid, is an anti-aspergillus agent. (Shraddha et al., unpublished)

Quercetin was found be present in onions, apples, broccoli and berries. Quercetin, which was the most abundant flavonoid, consisted of 3 rings and 5 hydroxyl groups. It did not possess any carbohydrate moiety in its structure. It is said to have a direct impact on one of the main signaling pathways of fungi. The target here is to study the impact of quercetin on the calcium-calcineurin signaling pathway of the fungi under observation. It was found that when the cytosolic calcium concentration had increased, calmodulin activated calcineurin, which in turn dephosphorylated *Crz1*. *Crz1* was then imported into the nucleus where it induced or repressed expression of its target genes. After phosphorylation had occurred, *Crz1* was exported from the nucleus. Several regulators responsible of this pathway had been identified. (S. Thewes, 2014)

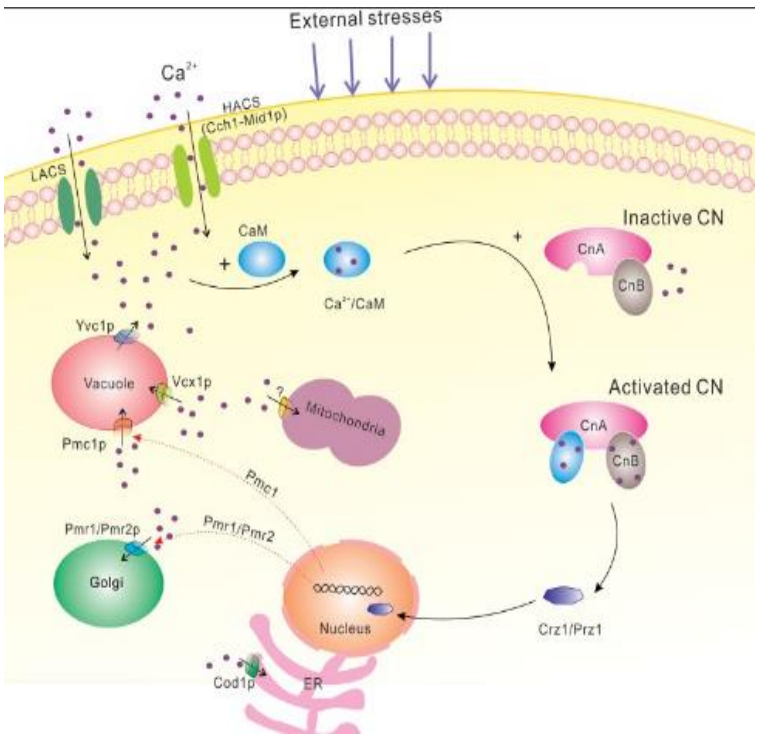

Figure 1: The pathway of calcium-calcineurin signal occurrence in fungal cells.

The fungal cells, calcium concentration might have increased in response to external or internal stresses, which lead to a variety of intracellular responses, including the opening of calcium channels and exchangers which were present on the plasma membrane or endomembrane system. These calcium channels or exchangers and their genes were said to contribute significantly towards the fluctuations in cytosolic calcium concentration. It was observed that the deletion of some calcium signaling components could be detrimental to fungal cell survival system. Thus, the influx or uptake of calcium could be interfered through the channels or transporters in order to disturb the calcium homeostasis, which might be beneficial in the fungicidal activity. (Shuyuan Liu, 2016)

The transduction system of calcium signaling included enzymes and proteins, which included calmodulin, calcineurin, and the various transcription factors encoded by *CRZ1/TNC1* and *PRZ1*, that were nonessential for normal growth but were very critical in response to stresses in order to mediate cell survival. In the yeast *S.cerevisiae, crz1* was firstly identified as a calcineurin target. Since then, the orthologues of *crz1* had been identified in various lower eukaryotes. C_2H_2 zinc finger DNA binding motifs were harbored by *crz1* and all of its orthologues. The number of zinc fingers was found to be ranging from one to four. *crz1* and its orthologues were found to share involvement in metal ion resistance, cell wall integrity and its development. The role of *Crz1* and its orthologues was investigated by many studies in its resistance towards antifungal agents. (YinglongHou, 2016)

It was observed that a calcium cell survival pathway might be involved in the survival of cells, which were subjected to a variety of cellular stresses. The activation of a variety of calcium ion channels, calcineurin, calmodulin and other factors were found to be necessary for the long-term survival of cells which were undergoing ER stress, and the genes found to be involved in this pathway were known to be absolutely necessary in many cellular biological processes. It was observed that these essential components would make beneficial targets for the study of antifungal therapies. (Shuyuan Liu, 2016)

MATERIALS & METHODS

1. 10 slants containing the media PDA (Potato Dextrose Agar) were prepared.
    - 3.9 grams of PDA was dissolved in 100 ml of distilled water and the media was then autoclaved. It was then put in the test tubes to make the media slants.
2. Primary inoculation was done on 5 slants and they were then incubated at 37^0C for about 48 hrs.
    - 2 slants were found contaminated while 3 slants were found to be pure.
3. Secondary inoculation was carried out using the 3 pure slants onto the remaining 5 media slants, to obtain pure culture of *Aspergillus flavus.* 3 slants out of these were kept at about 27^0C so that the growth of the fungi is slow. This is done in order to obtain the hyphal part of the fungi.
4. Lactophenol cotton blue test was carried out by taking colonies from the inner part of the slant underneath the conidia's to visualize the structure of fungi.
5. 100 ml of PDA was prepared and the media was then poured into petri plates. After the agar solidified, the pure culture of *A. flavus* was streaked onto it.
6. Haemocytometer count for *A. flavus* was carried out.
    - Prepared a spore suspension in 1mL Phosphate Buffer Saline.
    - Cleaned all surfaces of the hemocytometer and cover slip.
    - Pipetted approximately 10 µL of the cell suspension into one of the two counting chambers.
    - Counted the conidia.
7. Calculation of CFU (colony forming units) was done using the formula:

CFU= (No. of Colonies* dilution factor) / Volume of inoculum taken.

8. 2 flasks, each containing 50 ml of PDB (Potato Dextrose Broth) were prepared.
    - 1.2 grams of PDB was added to 50 ml of distilled water to prepare broth and both the flasks were autoclaved.
    - Both the flasks were inoculated with pure cultures of *A. flavus* and were kept for incubation at 37^0C in the shaker for about 24 hrs.
9. Various buffers were prepared to carry out the process of DNA isolation.
    - Composition of 0.5M EDTA solution: Added 9.08g of $NA_2EDTA.2H_2O$ to about 45ml of distilled water. Adjusted the pH to 8.0 by adding NAOH pellets (~1g needed) to it while constantly stirring it. Made up the volume to 50 ml.
    - Composition of 100ml lysis buffer: 10ml 1mM/L Tris-HCL

1ml of 0.5M/L EDTA (pH:8)

2ml of 1% w/v SDS

Made up the volume to 100ml. Adjusted the pH to 7.0 and then autoclaved the buffer.

- Composition of 100ml TE buffer: 1ml of 1M Tris-Cl

  0.2ml of 0.5M EDTA solution

  Made up the volume to 100ml and then pH was adjusted to 8.0, the buffer was then autoclaved.
- Composition of 100ml of 50X TAE buffer:

  24.2g of Tris base dissolved in 10ml of distilled water.

  To this, 5.71ml of glacial acetic acid and 10ml of 0.5M EDTA were added.

  Volume was made up to 100 ml and adjustment of pH tool place to 8.3 and then the autoclaving of buffer took place.
- Composition of 5M/L NaCl: Dissolved 14.61g of NaCl to 45ml of distilled water. After dissolving it, the volume was made upto 50ml.

10. DNA was isolated from the 24 hr culture of *A. flavus* using the following protocol.
    - Autoclaved mortar pestle was kept at -80^{0}C for about 10 min.
    - Using sterilized muslin cloth, the culture was poured in a beaker in order to filterate the mycelia and remove the liquid culture from it.
    - After the mycelium was completely dried off of liquid medium, it was put in the pre-cooled mortar pestle.
    - The mycelium was then grounded to fine powder using liquid nitrogen.
    - Lysis buffer was added to it at 1ml buffer/ 500mg culture.
    - This was then added to 4 eppendorfs.
    - 0.165ml of 5M/L NaCl solution was added to each eppendorf.
    - It was mixed by inverting it several times.
    - Centrifugation was carried out at 12,000 rpm for 20 min at 4^{0}C.
    - Transferred the supernatant to the fresh tubes.
    - 400 µL phenol and 400 µL chloroform were added in 1:1 ratio.
    - Mixed by inverting until the solution becomes milky.
    - Centrifuged at 12,000 rpm for 20 min at 4^{0}C was carried out.
    - The transfer of aqueous supernatant to fresh tubes took place and using 2 volumes of chilled absolute ethanol precipitated the DNA in aqueous phase.
    - Centrifuged at 12,000 rpm for 10 min at 4^{0}C.

- The pellet obtained was then washed using ice-chilled 70% ethanol and mixed well.
- Centrifuged at 10,000 rpm for 5 min at 4^0C.
- Discarded supernatant and again added chilled 70% ethanol.
- Centrifuged at 10,000 rpm for 3 min.
- Discarded supernatant when was pellet settled to tube bottom.
- Dried DNA pellet at room temperature and was then dissolved in 50 µL of TE buffer and stored at -20^0C.

11. The concentration of DNA was calculated by checking its OD in the nanodrop spectrophotometer.
12. Gel electrophoresis of the DNA sample was done.
    - 50 ml of 0.8% agarose gel was prepared by dissolving 0.4g of agarose in 50 ml of 1X TAE buffer.
    - To this, 3 µL of ethidium bromide was added and gel was poured on the casting tray containing wells forming comb.
    - After the gel had solidified, casting tray was put in electrophoresis tank containing 1X TAE buffer and the comb was removed.
    - DNA sample was added to wells along with the dye in the ratio of 5:1.
    - The DNA was then made to run from negative to positive pole for about 50 mins at 100W.
    - The gel was observed in the gel documentation system.
13. A flask containing 50 ml of PDB was prepared, autoclaved and inoculated with pure culture of *A. flavus* and was kept at 37^0C in the shaker for about 24 hrs.
14. The process of RNA isolation was carried out using the 24 hr old culture of *A. flavus*.
    - Fungal culture was taken and freeze dried using liquid nitrogen.
    - This was homogenized with pre-cooled mortar pestle.
    - Added trizol reagent at about 1ml reagent/100mg culture.
    - Incubated at room temperature for 5 mins to permit complete dissociation of nucleo-protein complex.
    - Transferred this to eppendorf tubes.
    - Added 0.2ml of chloroform per 1ml of trizol reagent.
    - Shaken tubes vigorously by hand for 15 sec and then incubated it at RT for 2-3 min.
    - Centrifuged the sample at 12,000 rpm for 15 min at 4^0C
    - Transferred the upper aqueous phase to the fresh tube.
    - Aqueous phase was used to precipitate RNA by mixing with 500µL of isopropyl

alcohol.

- Incubated the sample at RT for 10 min.
- Centrifuged at 12,000 rpm for 10 min at 4^0C.
- Supernatant discarded.
- Washed the RNA pellet with 75% ethanol and mixed well.
- Centrifuged at 7500rpm for 5min at 4^0C.
- Discarded the supernatant.
- Washed again with 75% ethanol and centrifuged for 2min at 7500rpm.
- Discarded the supernatant and the pellet was found at the tube bottom.
- The RNA pellet was dried at room temperature.
- 50 µL of TE buffer was added to dissolve the pellet and then the RNA sample was kept at -20^0C.

15. Gel electrophoresis of RNA sample was done.
    - 50ml of 1.2% agarose gel was prepared by mixing 0.6g agarose in 50ml of TAE buffer.
    - To this, 3 µL of ethidium bromide was added and gel was poured on the casting tray containing wells forming comb.
    - After the gel had solidified, casting tray was put in electrophoresis tank containing 1X TAE buffer and the comb was removed.
    - RNA sample was added to wells along with the dye in the ratio of 5:1.
    - The RNA was then made to run from negative to positive pole for about 50 min at 100W.
    - The gel was observed in the gel documentation system.
16. PCR reaction was carried out on the DNA sample.
    - The primers of the gene *CRZ1* were used.

PRIMER SEQUENCES:

crz1 FP: 5'- CCA CCA TCC ATT AAC GTG G -3'

crz1 RP: 5'- CGG ATC AGA TTT GCT ACG C -3'

- 15 µL of total reaction mixture was prepared.
- 2 separate samples were prepared. In one mixture genomic DNA was first diluted before addition while in the 2nd mixture, undiluted sample of DNA was taken.
- Composition of reaction mixture was:

crz1 FP: 0.5 µL

crz1 RP: 0.5 µL

gDNA: 2 µL(diluted sample) and 1 µL(undiluted sample)

Master mix: 7.5 µL

Nuclease free water: 4.5 µL (diluted sample) & 5.5µL (undiluted sample).

- PCR vials were put in the thermal cycler and reaction was set according to the following time-temperature profile.

  Initial denaturation: 95^0C for 5min.

  Denaturation: 95^0C for 30 sec.

  Annealing: 53.6^0C for 45 sec.

  Extension: 72^0C for 30 sec.

  Final extension: 70^0C for 7min.

  Hold at 4^0C
- 35 cycles of PCR were carried out.
- The PCR product was then stored at -20^0C.

17. Gel electrophoresis of PCR sample was carried out.
    - 50ml of 1.0% agarose gel was prepared by mixing 0.5g agarose in 50ml of TAE buffer.
    - To this, 3 µL of ethidium bromide was added and gel was poured on the casting tray containing wells forming comb.
    - After the gel had solidified, casting tray was put in electrophoresis tank containing 1X TAE buffer and the comb was removed.
    - PCR sample was loaded in the wells along with a 1Kb DNA ladder, without using loading dye as the sample was pre-mixed with the dye.
    - The ladder used here is #SM0313 and is a 1Kb ladder.
    - The samples were made to run from negative to positive pole at 100W for about 40 min.
    - Gel was observed on the gel documentation system.
18. 150ml of PDA was prepared by dissolving 3.6g of PDB and 2.25g of agar in 150ml of distilled water, which was sterilized by autoclaving. This was done to make 6 agar plates.
    - 90% methanol was prepared by dissolving 90ml of 100% methanol to 10ml of distilled water.
    - 1mg/ml stock of quercetin was prepared by dissolving 2mg of quercetin powder in 2 ml of 90% methanol.

- 2 agar plates were prepared containing quercetin mixed with agar, onto which 10 µL of pure fungal culture was dispensed on filter paper discs.
- 2 agar plates were prepared in which first the fungal culture was spread and later wells were created in the agar in which quercetin was put.
- 2 agar plates were prepared as controls in which fungal culture was spread without pouring quercetin.
- Out of 6 plates, one plate of each type was incubated at 37^0C while one plate of each type was incubated at 30^0C.
- This was done to observe the production of aflatoxin, which occurs at 30^0C and not at 37^0C.

19. A flask containing 50 ml of PDB was prepared, autoclaved and inoculated with pure culture of *A. flavus.* To this, 0.113mg/ml quercetin was added from the stock of 1mg/ml and was kept at 37^0C in the shaker for about 24 hrs.
20. The process for isolation of DNA of *A. flavus* culture containing quercetin in the media was carried out.
    - Autoclaved mortar pestle was kept at -80^0C for about 10 min.
    - Using sterilized muslin cloth, the culture containing quercetin was poured in a beaker in order to filtrate the mycelia and remove the liquid culture from it.
    - After the mycelium was completely dried off of liquid medium, it was put in the pre-cooled mortar pestle.
    - The mycelium was then grounded to fine powder using liquid nitrogen.
    - Lysis buffer was added to it at 1ml buffer/ 500mg culture.
    - This was then added to 4 eppendorfs.
    - 0.165ml of 5M/L NaCl solution was added to each eppendorf.
    - Inverting it several times mixed it.
    - Centrifugation was done at 12,000 rpm for 20 min at 4^0C.
    - Transferred the supernatant to the fresh tubes.
    - 400 µL phenol and 400 µL chloroform were added in 1:1 ratio.
    - Mixed by inverting until the solution becomes milky.
    - Centrifuged at 12,000 rpm for 20 min at 4^0C.
    - The aqueous supernatant was transferred to fresh tubes and using 2 volumes of chilled absolute ethanol precipitated the DNA in aqueous phase.
    - Centrifuged at 12,000 rpm for 10 min at 4^0C.
    - The pellet obtained was then washed using ice-chilled 70% ethanol and mixed well.

- Centrifuged at 10,000 rpm for 5 min at 4^0C.
- Discarded supernatant and again added chilled 70% ethanol.
- Centrifuged at 10,000 rpm for 3 min.
- Discarded supernatant when was pellet settled to tube bottom.
- Dried DNA pellet at room temperature and was then dissolved in 50 µL of TE buffer, stored in an eppendorf labeled DNA with Quercetin and was stored at -20^0C.

21. Gel electrophoresis of the DNA sample derived from *A. flavus* conataining quercetin was done.
    - 50 ml of 0.8% agarose gel was prepared by dissolving 0.4g of agarose in 50 ml of 1X TAE buffer.
    - To this, 3 µL of ethidium bromide was added and gel was poured on the casting tray containing wells forming comb.
    - After the gel had solidified, casting tray was put in electrophoresis tank containing 1X TAE buffer and the comb was removed.
    - DNA sample was added to wells along with the dye in the ratio of 5:1.
    - The DNA was then made to run from negative to positive pole for about 50 min at 100W.
    - The gel was observed in the gel documentation system.
22. A flask containing 50 ml of PDB was prepared, autoclaved and inoculated with pure culture of *A. flavus*. To this, 0.113mg/ml quercetin was added from the stock of 1mg/ml and was kept at 37^0C in the shaker for about 24 hrs.
23. The process of RNA isolation was carried out using the 24 hr old culture of *A. flavus* containing quercetin in the media.
    - Fungal culture containing quercetin was freeze-dried using liquid nitrogen.
    - This was homogenized with pre-cooled mortar pestle.
    - Added trizol reagent at about 1ml reagent/100mg culture.
    - Incubated at room temperature for 5 min to permit complete dissociation of nucleo-protein complex.
    - Transferred this to eppendorf tubes.
    - Added 0.2ml of chloroform per 1ml of trizol reagent.
    - Shaken tubes vigorously by hand for 15 sec and then incubated it at RT for 2-3 min.
    - Centrifuged the sample at 12,000 rpm for 15 min at 4^0C
    - Transferred the upper aqueous phase to the fresh tube.
    - Precipitated RNA from aqueous phase by mixing with 500µL of isopropyl alcohol.

- Incubated the sample at RT for 10 min.
- Centrifuged at 12,000 rpm for 10 min at 4^0C.
- Supernatant discarded.
- Washed the RNA pellet with 75% ethanol and mixed well.
- Centrifuged at 7500rpm for 5min at 4^0C.
- Discarded the supernatant.
- Washed again with 75% ethanol and centrifuged for 2min at 7500rpm.
- Discarded the supernatant and the pellet was found at the tube bottom.
- The RNA pellet was dried at room temperature.
- 50 µL of TE buffer was added to dissolve the pellet and then the RNA sample was kept at -20^0C.

24. Gel electrophoresis of RNA obtained from fungal culture containing quercetin was carried out.
    - 50ml of 1.2% agarose gel was prepared by mixing 0.6g agarose in 50ml of TAE buffer.
    - To this, 3 µL of ethidium bromide was added and gel was poured on the casting tray containing wells forming comb.
    - After the gel had solidified, casting tray was put in electrophoresis tank containing 1X TAE buffer and the comb was removed.
    - RNA sample was added to wells along with the dye in the ratio of 5:1.
    - The RNA was then made to run from negative to positive pole for about 50 min at 100W.
    - The gel was observed in the gel documentation system.
25. cDNA was prepared for both RNA samples (obtained with and without quercetin.
    - TAKARA BIO INC. cDNA synthesis kit was used.
    - A total of 20 µL of sample was prepared.
    - 2 separate samples of cDNA were prepared. One of the two samples contained RNA derived from fungal culture containing quercetin, while the other sample contained RNA derived from fungal culture which did not contain any quercetin.
    - In order to take similar concentration of RNA for cDNA preparation for both the samples, which was 1000ng/µL, the nanodrop spectrophotometer reading was taken into consideration to obtain a volume, which would contain equal concentration of RNA in both the samples.
    - Initially a total of 10 µL of mixtures were prepared for both the samples. The

reaction mixture components for the preparation of cDNA are as follows.

COMPONENT	VOLUME IN SAMPLE CONTAINING QUERCETIN	VOLUME IN SAMPLE NOT CONTAINING QUERCETIN
dNTP mixture	1µL	1µL
OligodT primer	1µL	1µL
Template RNA	4.3µL	5µL
RNase free water	3.7µL	3µL
Total	10 µL	10 µL

- The PCR vials were incubated at 65^0C in the water bath for about 5 min.
- The reaction mixture in a total volume of 20µL was prepared for both the samples by adding the following components.
  Template RNA primer mixture: 10µL (from previous step)
  5X PrimeScript Buffer: 4µL
  RNase inhibitor: 0.5µL
  PrimeSrciptRTase: 1µL
  RNase free water: 4.5µL
- These vials were then mixed gently and placed in the thermal cycler to carry out a single cycle for the synthesis of cDNA by using the following time-temperature profile.
  30^0C for 10 min.
  42^0C for 1 hour.
  95^0C for 5 min.
  cool on ice
- At the end of this cycle, we obtained cDNA as the product whose amplification had to be carried out.

26. Amplification of the cDNA product was carried out.

- PCR reaction was prepared for both the cDNA samples.
- The PCR reactions were prepared using *crz1* gene and 2 housekeeping genes ie. *GAPDH* gene and *TUBULIN* gene.
- In order to take similar concentration of cDNA for its amplification in both the samples, which was 1000ng/μL, the nanodrop spectrophotometer reading was taken into consideration to obtain a volume, which would contain equal concentration of cDNA in both the samples.
- A total of 12X reaction was prepared.
- The reaction mixture of a gene fragment amplification using of cDNA were as follows:

COMPONENTS	VOLUME IN SAMPLE CONTAINING QUERCETIN	VOLUME IN SAMPLE NOT CONTAINING QUERCETIN
cDNA product	1 μL	1.3 μL
Reverse primer	0.5 μL	0.5 μL
Forward primer	0.5 μL	0.5 μL
Master mix	6 μL	6 μL
Nuclease free water	4 μL	3.7 μL
Total	12 μL	12 μL

- The vials were kept in the thermal cycler and the PCR was set to the following time-temperature profile.

  Initial denaturation: 95^0C for 5min.

  Denaturation: 95^0C for 48 sec.

  Annealing: 61.3^0C for 30 sec.

  Extension: 72^0C for 45 sec.

  Final extension: 72^0C for 7min.

  Hold at 4^0C

27. The Preparation of cDNA was confirmed by performing gel electrophoresis on the amplified cDNA sample.

- To carry out the gel electrophoresis for cDNA sample, 1.5% agarose gel was used.
- 0.75g of agarose was weighed and added to 50 ml of 1X TAE buffer.
- To this, 3 μL of ethidium bromide was added and gel was poured on the casting tray containing wells forming comb.
- After the gel had solidified, casting tray was put in electrophoresis tank containing 1X TAE buffer and the comb was removed.
- To the wells, 3.5 μL of each amplified cDNA sample was added along with one well containing 1 μL of 1Kb DNA ladder in order to check the results of amplification.
- The gel was made to run at 100W for about 45 min.
- The gel was then observed in the gel documentation system.

RESULTS

FIG.2: *A. flavus* cultures

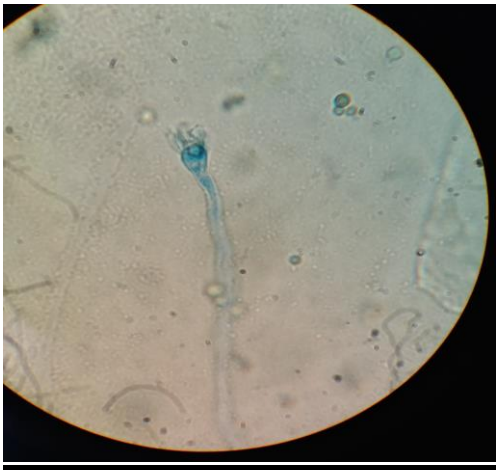

FIG.3: *Aspergillus flavus* under microscope (40X)

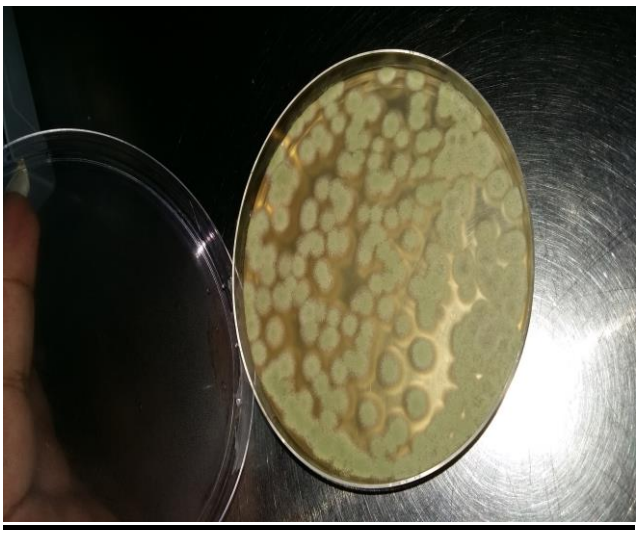

FIG.4: The plate used to count colonies of *Aspergillus flavus*

Haemocytometer count= 654cells/ml Cell density= $6.54*10^6$ cells/ml

No. of cells in stock= $6.54*10^{11}$ cells/ml CFU count= $1.4*10^9$cfu/ml

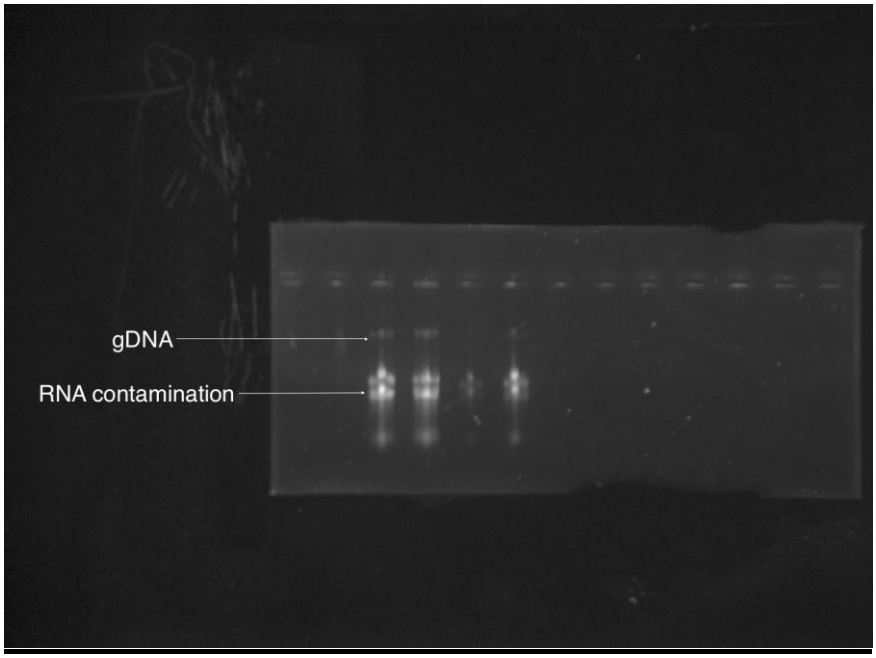

FIG.5:Gel electrophoresis results of DNA sample

The concentration of DNA was observed to be:

Sample1: 283 ng/µL Sample2: 185 ng/µL

Sample 3: 55.6ng/µL Sample4: 371ng/µL

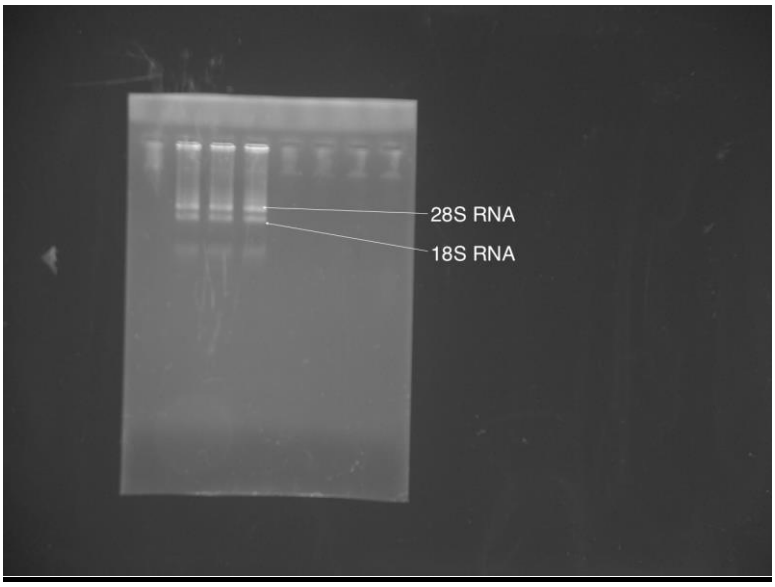

FIG.6:Gel electrophoresis result of RNA sample containing 2 bands; 28S&18S.

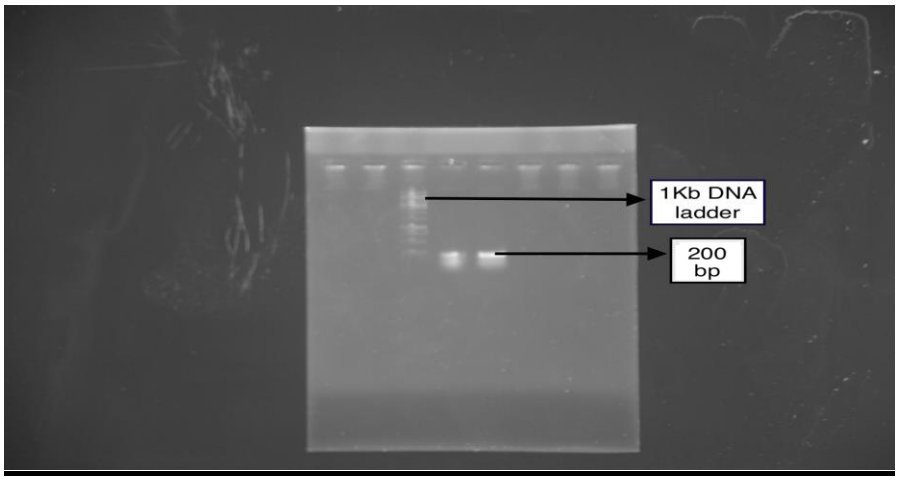

FIG.7:Gel electrophoresis result of PCR sample.

The ladder used was of 1Kb. (#SM0313)

The DNA bands were observed at 200bp.

FIG.8: PDA agar plate not containing quercetin in the wells

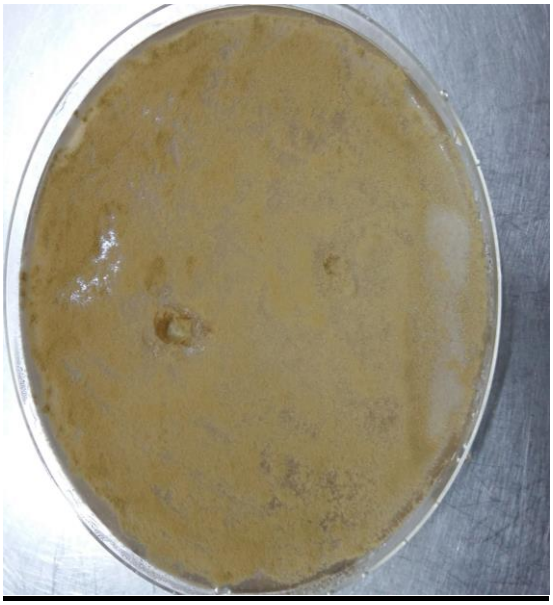

FIG.9: Quercetin mixed in agar plate and fungal culture spread onto it

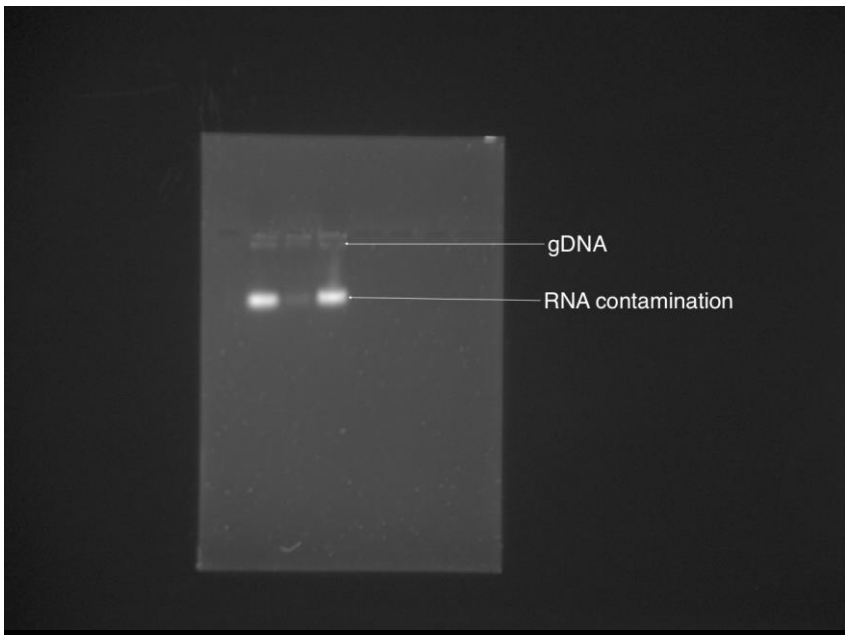

FIG.10: Gel picture of the DNA for the fungal culture obtained from fungal culture grown in presence of quercetin.

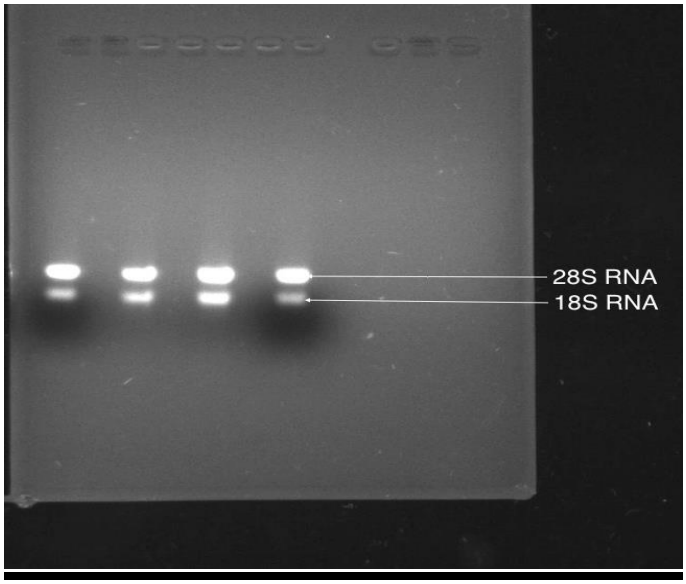

FIG.11: Gel picture of RNA obtained from fungal culture grown in presence of quercetin.

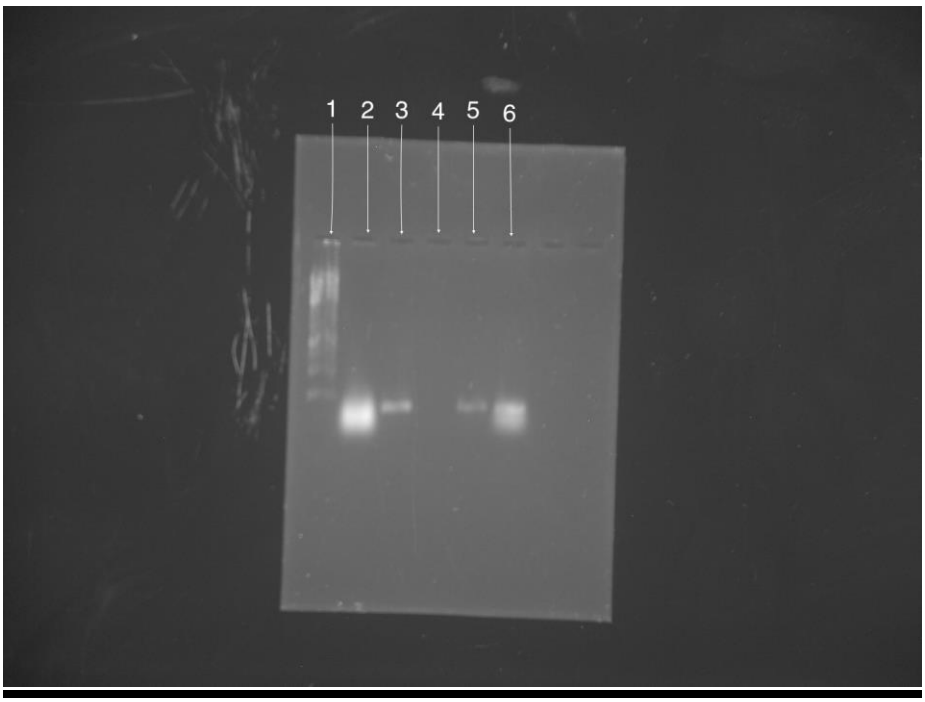

FIG.12: Gel picture of amplified cDNA product

Lane 1: 1 Kb DNA ladder.

Lane 2: amplification of *crz1* gene from cDNA obtained from *A. flavus* treated with quercetin.

Lane 3: amplification of *crz1* gene from cDNA obtained from *A. flavus* alone.

Lane 5: amplification of tubulin gene from cDNA obtained from *A. flavus* alone.

Lane 6: amplification of tubulin gene from cDNA obtained from *A. flavus* treated with quercetin.

This result suggested that though the cDNA product obtained from fungal culture alone is getting amplified while using *crz*1 gene primers and tubulin, the housekeeping gene primers, but the cDNA obtained from fungal culture treated with quercetin is not showing appropriate amplification results.

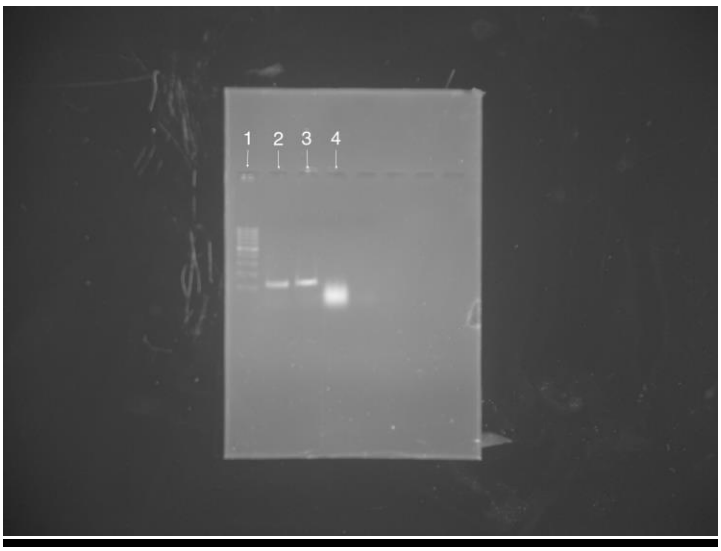

FIG.14: Gel picture of amplified cDNA product

Lane 1: 1Kb DNA ladder

Lane 2: PCR product for GAPDH from cDNA obtained from *A. flavus* treated with quercetin.

Lane 3: PCR product for GAPDH from cDNA obtained from *A. flavus* alone.

Lane 4: PCR product for *crz1* from cDNA obtained from *A. flavus* treated with quercetin.

The result suggested that cDNA product obtained from both the quercetin treated and untreated fungal culture amplified with GAPDH, the housekeeping gene, is showing bands at almost the same position.

However, the slight difference in their positions can be attributed to somewhat more concentration of cDNA in the quercetin treated sample, due to which the band has formed a little smearing.

While the result in the 4th lane shows that the transcription factor *crz*1, in case of cDNA product obtained from quercetin treated fungal culture is down regulated or is not expressed.

However, in case of cDNA product of *A. flavus* alone, the expression of the transcription factor *crz1* has been seen.

CONCLUSION

The idea behind the whole project was based on the development of environment friendly techniques to eradicate the harmful effects caused by pathogenic fungi i.e. *Aspergillus flavus.* The use of the plant by-product i.e. quercetin which is a secondary metabolite is being explored to ensure that there are least side effects of the alternative produced thereafter.

The major focus was to study the role of *crz1* gene in quercetin mediated inhibition, which plays a major role in the morphogenesis of fungi and germination of conidiophore.

Hence, the calcium-calcineurin pathway is one of the mandatory pathways during the morphogenesis *of Aspergillus flavus.*

After carrying out the various experiments, we had come to the conclusion that the expression of the transcription factor *crz1* was seen in the fungi *Aspergillus flavus* while its expression was down regulated in *Aspergillus flavus* treated with quercetin.

Therefore, there is a need to carry out more experimentation in order to study the exact role of the transcription *factor crz1* during the quercetin mediated inhibition or maybe other transcription factor needs to be explored to study the mechanism of quercetin mediated inhibition.

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