IJARSCT
ISSN (Online) 2581-9429
International Journal of Advanced Research in Science, Communication and Technology (IJARSCT)
Volume 2, Issue 3, December 2022
Impact Factor: 6.252

Study of Mycotoxin from Infected Jawar and Bajra by using Fungus *Aspergillus niger*

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Abstract: *Natural toxins in food are an important part of our lives, as is the oxygen we breathe. It is very important to naturally neutralize these toxins from our diet in order to consume food safely. This can only happen if we are exposed to small, non-toxic amounts that our bodies can easily handle. Not only would it be impractical, but it would also be impossible to completely eliminate natural toxins from our diets. Fungi and bacteria are the main causes of food poisoning. Symptoms of diarrhea, stomach cramps, and fever will develop in about 3 days. Fungi release mycotoxins; also known as secondary metabolites for fungi. Aflatoxin belongs to the group of mycotoxins. Aflatoxin biosynthesis was induced by compounds in filtrate (EF) obtained from cultures consisting of ground corn kernels invaded by Aspergillus flavus.*

Keywords: Natural Poison, Mycotoxins, Aspergillus, Aflatoxins, Maize

I. INTRODUCTION

Mycotoxins are secondary metabolites produced by fungi Due to their pharmacological activity, some mycotoxins or mycotoxin derivatives have been used as antibiotics, growth promoter's growth and others are considered chemical warfare agents. Some mycotoxins directly inhibit microbial growth. This review focuses on the most important diseases associated with human and veterinary diseases, including aflatoxin, citrinin, ergot akaloids, fumonisins, ochratoxin A, patulin, trichothecenes and zearalenone, etc.The most important mycotoxins are aflatoxin (AFB1, B2, G1, G2), ochratoxin A (OTA), knitthecenes, zearalenone and fumonisins (B1, B2). Trithecenes mainly include deoxynivalenol (DON or vomiting), nivalenol, and T2 toxin. Some other examples are HT2, 3-Ac-DON, DAS, Fusarenon-X toxins. MAS, Neosolaniol and 15-Ac-DON are also included in the scope of the analysis. Other mycotoxins mentioned are patulin, sterigmatocystin and cytochalasin E.

They are often named after the fungus that produces them. For example, "Aflatoxin" uses the letter A for Aspergillus and fla for the flavus species with the word toxin. There are three main mycotoxin-producing fungi: Aspergillus, Fusarium and Penicillium. Mycotoxin-producing fungi grow on a variety of foods including grains, peanuts, beans, and peas.

II. AFLATOXINS

Aflatoxins are toxic metabolites produced by certain fungi in/on food and animal feed. They are probably the best known and most studied mycotoxins in the world. The presence of aflatoxin is influenced by a number of environmental factors; Therefore, the degree of contamination will vary depending on the geographical location, agricultural and agronomic practices as well as the susceptibility of the product to fungal invasion during the pre-harvest, storage and handling stages and processing.

Aflatoxin is produced by many species of Aspergillus niger, especially flavus and parasiticus, as well as by members of the genera Penicillium and Rhizopus. Aflatoxin can contaminate corn, cereals, sorghum, peanuts and other oilseeds. Aspergillus, which produces aflatoxin, is one of the most common cereal molds.

However, aflatoxin's reputation as a potent poison may explain why it has been used in bioterrorism. Aflatoxin has been isolated and characterized following the death of more than 100,000 birds (turkey X disease) attributed to the consumption of peanut meal contaminated with mold (the four major aflatoxins are referred to as B1, B2, G1 and G2 as a function of their fluorescence under UV or green light) and relative chromatographic mobility during thin layer chromatography.

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Aflatoxins are associated with a variety of diseases such as aflatoxinosis in livestock, companion animals and humans worldwide. Aflatoxins have received more attention than any other mycotoxin due to their demonstrated potent carcinogenicity in susceptible laboratory animals and acute toxicological effects in humans. Aflatoxins are acutely toxic, immunosuppressive, mutagenic, teratogenic, and carcinogenic compounds. Aflatoxin, like other natural toxins found in food, cannot be completely eliminated from food. Aflatoxin poisoning has been reported in nearly all domestic and non-domestic animals and other non-human primates from all regions of the world. Additionally, aflatoxin inhalation is associated with disease and injury in both animals and humans, and genetic resistance to aflatoxin production has been identified in more than five major cultures. Recognized as a serious food safety hazard in the country, more than 50 countries have enacted or proposed regulations to control aflatoxin in food and feed. To the best of our knowledge, there is little evidence of the presence of aflatoxins in European grains or in a few other countries. Anything containing more than 20ppm should not be used in human diets or fed to dairy cows. Crop losses due to aflatoxin contamination are estimated to cost US growers an average of more than $100 million annually. Recognizing that absolute safety can never be achieved, many countries have attempted to limit exposure to aflatoxin by imposing regulatory restrictions on commodities intended for food and feed use. It was made.

III. MATERIALS AND METHODS

Part I: Preparation of Potato Dextrose Agar Medium

Requirements

Potato tubers	100g
Dextrose	10g
Agar	7.5g
Peptone	1g
Distilled water	500ml
HCl	1N
NaOH	1N
Knife	
Muslin cloth	
Heater	

Beaker (500ml capacity)
Borosil flask (500ml capacity)

Procedure

1. Take a potato, peel it and weigh 100 g. Use a knife to cut the tubers into small pieces.
2. Transfer the chopped potatoes to a cup containing about 200ml of distilled water. Etc Boil the contents using a heater for about 20 minutes.
3. Decant the supernatant, filter through four layers of cheesecloth, and collect the filtrate in a beaker. This filtrate is called potato extract. Because. Transfer the dextrose (10 g), agar (7.5 g) and peptone (1 g) to the extract, heat and gently swirl to dissolve the components. .
4. Finally, transfer the medium to a 500 ml graduated cylinder and make up to 500 ml by adding distilled water.
5. Measure the pH of the medium and adjust to 5.6 with 1N HCl or NaOH.
6. Pour the medium into the Borosil bottle, plug the cotton stopper and cover the stopper with aluminum foil/aluminum foil, autoclave at 121 ° C for 20 min.
7. Once the temperature has cooled, remove the vial and use as needed or store at room temperature.

Part II: Preparation of PDA Plates

Requirements

PDA medium
Culture tubes (smooth mouth)
Test tube stand

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Heater
Aluminum foil or paper

Procedure:

1. Before starting steaming, place a few Petri dishes in the oven and sterilize them at 200°C for about half an hour.
2. When the temperature cools, transfer them to a laminar airflow cabin or inoculation chamber built for inoculation. However, these must be sterilized with a UV lamp 30 minutes before starting work.
3. Take the vial containing the PDA medium and pour about 15-20ml of the medium aseptically into the bottom half of the petri dish when the temperature is around 40°C (be careful not to pour the medium too hot or condensation will be generated in the tank). water droplets and help pollution). Etc.
4. Place the plates in cascade and wait about 20 to 30 minutes for the medium to solidify.
5. Plates containing this solidifying medium are called PDA plates. Because. Use the mushroom growing dish immediately or save it for later use.

Part III: Streak Plate Method to get Pure Culture
The colonies on mixed plates are separated by spreading on a plate with good spacing each other using streak method.

Requirements:
Tripod and wire gauze Burner
Beaker of water Wire loop PDA pour plates
Mixed culture of fungi

Procedure:

1. Streak the plates following quadrant radiant or T-streak or continuous streak as shown in the Fig.
2. Keep the streaked plates in inverted position at 25℃ for 24 – 48 hours.
3. Place the Petri dishes upside down to solve the problem of water condensation because if it drops down on the colonies, the organisms of one colony can spread on the other colony.

Results
The isolated colony of desired fungi (at the site of last streak) on the plate will beObserved.

Part IV: Preparation of Czapek's Dox Broth
Aflatoxin produced by fungi in culture media can be easily extracted in a chloroform and their toxicity can be assayed using bioassays.

Material:
Aspergillus.niger or A.flavus grown for 10 days on Czapek's broth supplemented with casein to give 0.5g nitrogen
/ L at pH 4.5 as stationary culture. Bucher funnel
Whatman No . 1 filter paper Chloroform
For Czapek (Dox) Broth (CDB) -
Sodium Nitrate : 1gm
Potassium dihydrogen phosphate : 0.5gm
Magnesium Sulphate : 0.25gm
Ferrous Sulphate : pinch
Sucrose : 15.0 gm
Distilled water : 500ml

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Method:
To prepare Czapek broth, mix all ingredients required for preparation, adjust pH to 5.6 and sterilize for 15 minutes in an autoclave at 1.05 kg f/cm2.
Filter the medium through a Buchner funnel using Whatman filter paper No.1. Extract in triplicate 100 ml of the culture filtrate with equal volumes of chloroform. Cool the chloroform extract and evaporate to dryness on a rotary evaporator. Dissolve the residue in a minimum amount of distilled water. If direct biological assay or in chloroform for chromatographic separation is required, its toxicity to plant growth can be tested without further purification.

Part V: Bioassay of Aflatoxins

1. Extraction of Toxins
2. Preparation of TLC Plates
3. Qualitative Analysis of Aflatoxins
4. Seed Germination
5. Detection of Nucleic Acids (DNA and RNA)

IV. RESULTS AND DISCUSSION

4.1 Sterilization of Maize Seeds
Take the seeds in a conical flask. Wash properly with tap water and then with diluted Savlon or Dettol. Rinse twice with tap water. Wash with alcohol 70 % for half a minute. Transfer the seeds to a sterile conical flask containing 0.1% HgCL2 solution. Cap the bottle and shake gently for 5-7 minutes. Remove the HgCL2 solution from the vial. Wash seeds thoroughly 3-4 times with sterile DW. Transfer the seeds to a Petri dish. Seeds germinate in the dark, Calculate germination rate. Formed roots are kept in the light for further growth.

Sterilization of Soil: The garden soil is been taken and placed in a vessel. The vessel had been placed in a cookerso the micro-organisms or any of the contamination will not be there because of high temperature.

Extraction of Aflatoxins from Culture Media: Aflatoxin produced by fungi in culture media can be easilyextracted in a chloroform and their toxicity can be assayed using bioassays.

Material
Aspergillus niger or *A. flavus* grown for 10 days on Czapek's broth supplemented with casein to give 0.5g nitrogen/ L at pH 4.5 as stationaryculture.
Bucher funnelWhatman No. 1 filter paper Chloroform
For Czapek (Dox) Broth (CDB) -
Sodium Nitrate: 1gm
Potassium dihydrogen phosphate: 0.5gm
Magnesium Sulphate: 0.25gm
Ferrous Sulphate: pinch
Sucrose: 15.0 gm
Distilled water: 500ml

Method
To prepare the Czapek broth, mix all the ingredients required for its preparation, adjust the pH to 5.6, and sterilize for 15 minutes in an autoclave at 1.05 kg f/cm2. Filter the medium through a Buchner funnel using Whatman filter paper No.1. Extract three times 100 ml of the culture filtrate with equal volumes of chloroform. Cool the chloroform extract and evaporate to dryness on a rotary evaporator. Dissolve the residue in a minimum amount of distilled water. If direct biological assay or in chloroform for chromatographic separation is required, its toxicity to plant growth can be tested without further purification.

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4.2 Bioassay of Aflatoxins

The toxicity of aflatoxin extracts obtained from culture filtrate to plant growth can be tested using seed germination and seedling growth tests.

Materials

Aflatoxin extract
Maize seeds
Test tubes
Pipettes, 10 ml
Petri dishes

Methods

Inhibition of Seed Germination

Soak the surface-sterilized beads in 20 ml of culture filtrate at appropriate dilutions of the toxin extract for 24 h. in vitro. Spread 100 treated seeds on a damp filter paper placed in a petri dish. Annealed at room temperature.
Maintain proper control with seeds soaked in distilled water. Count the number of seeds germinated after 7 days.
Calculate the percentage of germinated seeds.

Reduction in Seedling Vigour

Sprouting seeds soaked in water in dishes. Select 12 germinated seeds with a uniform bud length of about 5 mm and place them in a dish lined with filter paper impregnated with 5 ml of culture filtrate or toxin extract. Maintain control with distilled water. After 6 days, measure the whole shoot growth of 10 seedlings avoiding the shortest and longest plants and measure the elongation over the 5-day incubation period. When aflatoxin extract was applied to corn kernels and growth of a specific fungus was observed. All 40% of the seeds germinated, of which only 15% showed seedling development. Corn plants grow closed and slow compared to standard plants.

Calculation:

Percentage of germination of: -
Percentage of germination = no. of germinated seeds× 100 Total no of seeds
Percentage of germination of the standard seed of maize is = 70× 100 = 0.70100
Percentage of germination of the infected seed of maize by *Aspergillus niger* = 25 × 100 = 0.22
Percentage of germination of the infected seed of maize by *Aspergillus flavus* = = 45 × 100 = 0.45

Result:

1. Percentage of germination of the standard seed of maize is = 0.70
2. Percentage of germination of the infected seed of maize byAspergillus niger = 0.22
3. Percentage of germination of the infected seed of maize by Aspergillus flavus = 0.45

IV. THIN LAYER CHROMATOGRAPHY

The separation and identification of organic compounds is routine work in many service laboratories. This layer chromatography (tlc) is an easy technique to apply for this purpose. It is useful in research laboratories to separate, identify and characterize unknown compounds. A wide range of small molecules such as amino acids, sugars, organic acids, organic acids, lipids, etc. separated by TLC technique. The biggest advantage of TLC is the degree of separation achieved. When using volatile solvents, the time required to complete separation is only about 30 minutes, and with non-volatile solvents it is rarely longer than 90 minutes.

PRINCIPLE

The general principle regarding TLC is similar to that of column chromatography i.e. adsorption chromatography. During adsorption, the solute competes with the solvent for the surface position of the adsorbent. Depending on the

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distribution coefficient, the compounds are distributed on the surface of the adsorbent. Of course, in tlc, the partitioning effect during the split is also not excluded. The commonly used adsorbent contains a binding agent such as calcium sulfate which facilitates the retention of the adsorbent to the glass plate.

Materials

- Thin Layer Chromatography (tlc) Kit
- Ultra – Violet (UV) Chamber
- Mechanical Shaker
- Toluene
- Ethyl Acetate
- Formic Acid
- Chloroform
- Silica Gel G (tlc Grade)

Procedure:

Extraction of Toxins

1. Weigh exactly 50g of the ground sample and transfer to a 250ml conical flask.
2. Moisten the ingredients evenly by adding 10-15ml of distilled water and add about 200ml of chloroform. Cover the mouth with a cotton swab in aluminum foil.
3. Shake the bottle mechanically for one hour. (It is important that materials containing oil be degreased prior to extraction.)
4. Filter the suspension through a Buchner funnel under gentle suction. An equal amount of a filter aid such as chili peppers can be added prior to filtration to facilitate filtration. Rinse the flask thoroughly and suspend with additional chloroform (25 mL) and collect the filtrate.
5. Transfer the filtrate quantitatively to the separating funnel and shake with half volume of chloroform water. After the phases have separated, withdraw the lower phase (chloroform) into a vessel containing about 10 g of sodium sulfate (anhydrous) to absorb the water.
6. Concentrate the clear chloroform extract on a warm water bath. Add the concentrate to known volume with chloroform and store in an amber vial, refrigerate until analysis.

Preparation of the Plates

1. Put 30g silica gel G into a sealed vial, divide vigorously with 60-65ml distilled water for about 1 minute, transfer to applicator and spread evenly on a glass dish (20×20cm). Precise amount of chloroform to get a delicious porridge. The layer thickness should generally be 0.25 mm.
2. Let the plates dry for 1 hour. In dust-free conditions.
3. Divide the gel into several lanes by drawing lines on the gel with a sharp needle.
4. Carefully mark different volumes of the sample extract in different lanes with a capillary on an imaginary line 2.5 cm from one end of the plate.
5. Deploy the chromatographic identity in the solvent system toluene: ethyl acetate: formic acid (6:3:1) in a chromatographic bath for about 50 minutes. At this point, the solvent front may have moved up to 20 mm below the top end of the plate.
6. Dry the plate at room temperature to remove solvent. Visualization of the fluorescein toxin spot under UV light; otherwise, vision will be affected.
7. Identify each fluorescent dot in the sample extract. Determine the Rf value of each point.

Calculation:

Rf = Distance (cm) moved by the solute from the origin

Distance (cm) moved by the solvent from the origin

For a fluoresce green (G) G1 & G2

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Rf of Aspergillus niger = 14.6

20 = 0.73

Rf of Aspergillus flavus = 15.3

20 = 0.77

Result:

1. Rf of Aspergillus niger = 0.73
2. Rf of Aspergillus flavus = 0.77

V. EXTRACTION AND DETECTION OF NULEIC ACIDS

The nucleic acid consists of Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA).

A) EXTRACTION AND DETECTION OF DNA FROM INFECTED SEEDS

Requirements:

Radicals of germinating seeds
15 % TCA (Trichloro acetic acid)
10 % NaCl (Sodium chloride)
Chilled ethanol
EDTA (Ethylene diamine tetra acetic acid) powder
Grinder (motor and pestle)
Centrifuge
Test tubes.

Extraction of DNA

Weigh 1 g of root sprouts and cut into small pieces. Add a pinch of EDTA powder and grind with a blender or motorized pestle. Then add 5 ml of TCA (remove fat and carbohydrates). Collect the contents in the centrifuge tube and centrifuge the solution at 5000 rpm. Remove the supernatant and ppt and 10 mL of 10% NaCl (to dissolve the DNA solution). Boil the ingredients in bain-marie for 30 minutes. Cool the solution and centrifuge again at 5000 rpm for 5 min. Take the supernatant and refrigerate it. Add an equal amount of chilled ethanol. DNA occurs at the junction of two liquids. Shake the tube and store in the refrigerator for 30 minutes. After 30 min, centrifuge to separate ppt DNA. Remove the floating top and collect the ppt. Put a drop of Acetocarmine dye. The DNA ring turns purple-pink.

Chemical Preparation:

1. 15%TCA:1.5gm of TCA in 30ml of distilled water.
2. 10%NaCl: 10gms of NaCl in 100ml of distilled water.

Detection of nucleic acid

The nucleic acids absorb strongly in the ultra violet region of the spectrum (due to conjugate double bond system). They show characteristic maxima at 260 nm and minimum at 230nm.

Result of OD:

1. Standard material: 0.56
2. *Aspergillus flavus*: 0.66
3. *Aspergillus niger*: 0.45

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B) Extraction and Detection of RNA from plant material

Requirement:

Maize seeds, saline EDTA (Ethylene diamine tetra acetic acid), Phenol 80%, chilled ethyl alcohol, centrifuge, pestle and mortar.

Procedure:

Weigh 1 g of corn and cut it into small pieces. Crush the ground corn kernels in 5 ml of EDTA saline or distilled water with a pestle and mortar. Add 10 ml of this 80% phenol. Shake the mixture and centrifuge it. Take the supernatant and 2 volumes of cold ethanol. Keep it in the refrigerator overnight. Centrifuge the next day and collect RNA ppt.

Detection of RNA

Dissolve the RNA crystals from the previous experiment in 1 ml of water. Also dissolve 0.5 g of RNA crystals. Use these two materials for the reduction chromatography on Whatman PAPER No. 1. After running the chromatography, allow to air dry for 4 h. And locate the bases under ultraviolet light. Both will be identical.

Preparation: 80% Phenol. Take 80ml of Phenol and 20ml of distilled water.

Calculation:

Rf = Distance (cm) moved by the solute from the origin Distance (cm) moved by the solvent from the origin
Rf of Pure maize = 8.1/8.9 = 0.91
Rf of Aspergillus niger = 7.2/8.4 = 0.84
Rf of Aspergillus flavus = 7.3/8.2 = 0.89

Result:

Rf of Pure maize = 0.91
Rf of *Aspergillus niger* = 0.84
Rf of *Aspergillus flavus* = 0.89

VI. DISCUSSION

The toxicity of the aflatoxin extract of the culture filtrate used for seed incubation and germination for the non-fungal standard corn kernels was 0.70%, while the growth viability of A. niger and A. flavus, respectively. Are 0.22 and 0.45. Rf values of A. niger and A. flavus were 0.73 and 0.77, respectively. Fungal contamination also showed an impact on nucleic acids related to the number of DNA and RNA components. Here we find that the DNA composition of standard uninfected corn kernels is 0.56 mg, while the DNA of A. niger and A. flavus is 0.66 and 0.45 mg, respectively. The RNA composition for the non-fungal standard corn kernels was 0.91 mg, while for the growth potential of A. niger and A. flavus it was 0.84 and 0.89 mg, respectively.

VII. CONCLUSION

If seeds are infected with a fungal disease, the germination rate will decrease. Besides the germination rate, the nucleic acid composition in terms of DNA and RNA will be synthesized in less quantity, affecting the overall growth and development of the plant. Therefore, in order to sow a seed, care must be taken to ensure that it is free of any kind of infection. As a precaution, seed sterilization can be used with mercuric chloride or a fungicide.

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