III. MATERIAL AND METHODS

The present in vitro study was conducted at Department of Avian Production and Management, Karnataka Veterinary, Animal and Fisheries Sciences University, Hebbal, Bangalore. The main objectives of the study were production, extraction, isolation, purification and standardization of aflatoxin B₁ and T-2 toxin as reference standards for the estimation of mycotoxin and mycotoxin residues in food and feeds.

3.1 Maintenance of parent culture

Parent culture of A. parasiticus (MTTC* 411) and F. sporotrichoides (MTCC*-1894) were maintained on potato dextrose agar and oatmeal agar, respectively at refrigeration temperature (5-8 °C).

3.1.1 Production of subculture

For the production of subculture, potato dextrose agar and oat meal agar were prepared (Annexure I) and 10 to 20 ml of media were taken into each test tube, autoclaved at 121 °C (15 psi) for 20 minutes and tubes were kept in slanting position till the medium solidified.

* Procured from Microbial Type Culture Collection & Gene Bank (MTCC), Institute of Microbial Technology, Sector 39-A, Chandigarh - 160036
Slants were inoculated under aseptic conditions in laminar flow with the spores collected from the parent culture and left them in a dark place for two weeks at room temperature to allow the fungus to sporulate and these subcultures were used for production of aflatoxin B₁ and T-2 toxin culture material.

3.2 Production of aflatoxin B₁ culture material

Culture material of aflatoxin B₁ was produced on broken rice, using the above produced subcultures as outlined by Shotwell et al. (1966) with minor modifications.

Thirty gram of broken rice was taken in 250 ml conical flasks. Cold tap water was added so as to get the total moisture of 35%. The flasks were sealed with non-absorbent cotton plug and aluminum foil, left over night for soaking.

The flasks were autoclaved at 121°C (15 psi) for 20 minutes and allowed to cool. After cooling the flasks were shaken to prevent the clump formation.

Fungal spores were collected in sterile distilled water from the subcultured slants of *A. parasiticus* by adding 5 ml of sterile distilled water with Triton-X @ 0.05%, to aid in greater recovery of spores. Then 0.5 to 1.0 ml of spore suspension was inoculated to the autoclaved flasks containing
broken rice under aseptic condition in laminar flow. The flasks were gently mixed to aid in uniform distribution of spores.

Flasks were incubated in the biological oxygen demand (BOD) incubator at 30 °C for seven days. They were vigoursly shaken twice a day from 3rd day onwards, inoculated rice shows the white mycelial growth by 2nd day, which gradually turn to green and finally to dark green by 7th day.

After seven days of incubation, the flasks were autoclaved at 121 °C (15 psi) for 20 minutes and culture material was collected on aluminium foil and dried in a forced draft hot air oven at 80 °C overnight (8-12 hours).

Dried culture materials was ground to fine powder and packed in a closed plastic container, stored in a dark place, and analyzed for aflatoxin B1 concentration by TLC as per AOAC, (1995) (Annexure II).

3.2.1 Effect of various substrates on Aflatoxin B1 production

In the same way, broken rice, broken wheat and broken maize substrate were inoculated to study the effect of variation in substrates on aflatoxin B1 production. The total moisture of the substrates was maintained at 35 % and the flasks were incubated in BOD incubator at 30 °C. They were harvested on 7th day and analyzed for aflatoxin B1 concentration by TLC as per AOAC, (1995) (Annexure II).
3.2.2 Effect of incubation time on aflatoxin B<sub>1</sub> production

To study the effect of incubation time on aflatoxin B<sub>1</sub> production on broken rice, 250 ml conical flasks containing 30 gm of rice were inoculated with 0.5 to 1.0 ml of spore suspension and incubated in BOD incubator at 30°C. The total moisture of the substrate was maintained at 35 % by adding cold tap water. They were harvested on 3<sup>rd</sup>, 7<sup>th</sup> and 9<sup>th</sup> day and analyzed for aflatoxin B<sub>1</sub> concentration by TLC as per AOAC, (1995) (Annexure II).

3.2.3 Effect of variation in moisture on Aflatoxin B<sub>1</sub> production

Three levels of moisture, 25 %, 35 % and 45 % was maintained in broken rice and transferred to 250 ml conical flasks. The flasks were inoculated with 0.5 to 1.0 ml of spore suspension and incubated in BOD incubator at 30°C for 7 days. After 7 days the culture material was analyzed for aflatoxin B<sub>1</sub> by TLC as per AOAC, (1995) (Annexure II).

3.3 Extraction of Aflatoxin B<sub>1</sub>

Aflatoxin B<sub>1</sub> was extracted from the culture material as per Romer (1975) and was quantified by TLC as outlined in AOAC, (1995) (Annexure II).

3.3 Isolation and purification of Aflatoxin B<sub>1</sub>
Aflatoxin B₁ was isolated and purified by the preparative thin layer chromatographic method. After the extraction of aflatoxin B₁, the extract was applied to the preparative TLC plates. 200 µl of extract was applied in each spot and spots were placed 2 cm apart from each other and plates were developed in chloroform: acetone (85:15) in an unequilibrated chamber.

After development, plates were dried and viewed under long way UV-light (365 nm). The different Aflatoxins were separated based on the R_f value and molecular weight.

The aflatoxin B₁, showing bluish fluorescence at R_f value of 0.31 was scrapped out carefully from the plates along with silica gel.

The scrapped silica gel containing toxin was dissolved in chloroform solvent, at the rate of 10 ml per 2 g of silica gel and for duration of 30 minutes.

Dissolved silica gel was filtered using sintered glass filters and filtrate was again spotted on preparative TLC plates as described above for purification and the same steps were repeated four times to get the purity to maximum extent (>98%).

3.3.1 Preparation of preparative TLC plates
The plates were prepared as outlined in AOAC, (1995) with minor modifications.

The plates were prepared by adding 50 ml of distilled water to 25 gm silica gel powder and mixed uniformly to get homogenous mixture (slurry) and then drawn on glass plates of 20 x 20 cm using TLC applicator with thickness of two mm and allowed to dry.

3.3.2 The activation of TLC plates

The coated plates were activated by drying in forced draft hot air oven at 110°C for one to two hours.

3.3.3 Quantification of aflatoxin B₁

The concentration of purified aflatoxin B₁ was tested by TLC method, with visual comparison to the authentic aflatoxin B₁ standard (Sigma Standard).

The known standard was spotted on commercially available TLC plates (Merck®) at different volumes, 5 µl, 10 µl, 15 µl ... 40 µl of known concentration and on the same plates the purified aflatoxin B₁ solution (unknown concentration) was spotted at same volumes and plate was developed using chloroform: acetone (85:15) and toxin was quantified by visual analysis as outlined in AOAC, (1995).
3.3.4 Preparation of developing solution

Developing solution was prepared by mixing chloroform : acetone in the ratio of 85:15 and preserved in a closed container for further use.

3.4 Standardization

The produced aflatoxin B$_1$ solution was standardized to appropriate concentration by thin layer chromatographic and spectrophotometric method.

3.5 Determination of the purity by Chromatographic method

As described by Rodricks and Stoloff (1970), on a commercially available TLC plate (Merck®) 5 µl resolution reference standard (Sigma), 5 µl produced aflatoxin B$_1$ solution and 5 µl produced aflatoxin B$_1$ solution + 5 µl resolution reference standard were spotted successively at 2 cm intervals. After development, spot of individual aflatoxin B$_1$ standard should reveal no other aflatoxins and at most faint fluorescence spots near origin.

3.5.1 By Spectrophotometric method

As described by Rodricks et al. (1970), the spectrophotometric method of purity was tested in acetonitrile: benzene (98:2) solvent system at wavelength of 333 nm. The produced aflatoxin B$_1$ reference standard solution was diluted to different concentrations, absorbance values were recorded in
spectrophotometer and molar absorptivity values were calculated at different concentrations using the following formula.

\[
\text{OD value} \times \text{Mw} \times 1000 \\
\text{Molar absorptivity} = \frac{\text{----------------------}}{\text{Concentration (µg/ml)}}
\]

OD- Optical density
Mw – Molecular weight (for aflatoxin B₁ - 312)

The molar absorptivity value of developed reference standard was compared with that of the molar absorptivity values of Sigma aflatoxin B₁ reference standard.

3.6 Production of T-2 toxin culture material

Culture material of T-2 toxin was produced on whole wheat, using the above produced subcultures as outlined by Burmeister (1971) with minor modifications.

Thirty gram of whole wheat was taken in a 250 ml conical flask. Cold tap water was added so as to get the total moisture of 35 %. The flasks were sealed with non-absorbent cotton plug and aluminum foil, left over night for soaking.
The flasks were autoclaved at 121°C (15 psi) for 20 minutes and allowed to cool. After cooling the flasks were shaken to prevent the clump formation.

Fungal spores were collected in sterile distilled water from the sub cultured slants of *F. Sporotrichoides*, by adding 5 ml of sterile distilled water with Triton-X @ 0.05%, to aid in greater recovery of spores. Then 0.5 to 1.0 ml of spore suspension was inoculated to the autoclaved flasks containing whole wheat under aseptic condition in laminar flow. The flasks were gently mixed to aid in uniform distribution of spores.

Flasks were incubated in the BOD incubator at 20°C for 21 days. They were vigoursly shaken twice a day from 3rd day onwards. Inoculated wheat shows the white mycelial growth by 2nd day, which gradually turn to red and finally to dark red by 21st day.

After the 21 days of incubation, the flasks were autoclaved at 121°C (15 psi) for 20 minutes and culture material was collected on aluminum foil and dried in a forced draft hot air oven at 80°C overnight (8-12 hours).

Dried culture materials was ground to fine powder and packed in a closed plastic container, stored in a dark place, and analyzed for T-2 toxin by TLC as suggested by Rukmini and Bhat (1978) (Annexure II).

3.6.1 Effect of various substrates on T-2 toxin production
In the same way, wheat, maize and rice substrate were inoculated, to study the effect of variation in substrates on T-2 toxin production. The total moisture of the substrate was maintained at 35% and the flasks were incubated in BOD incubator at 20\(^0\)C. They were harvested on 14\(^{th}\), 21\(^{st}\) and 28\(^{th}\) day and analyzed for T-2 toxin by TLC as suggested by Rukmini and Bhat (1978) (Annexure II).

3.6.2 Effect of incubation time on T-2 toxin production

To study the effect of incubation time on T-2 toxin production in whole wheat, 30 flasks containing 30 gm of whole wheat were inoculated and maintained as above and ten flasks were harvested at 14\(^{th}\), 21\(^{st}\) and 28\(^{th}\) days, dried and analyzed for T-2 toxin by TLC as suggested by Rukmini and Bhat (1978) (Annexure II).

3.6.3 Effect of variation in moisture on T-2 toxin production

Three levels of moisture, 25 per cent, 35 per cent and 45 per cent was maintained and transferred to 250ml conical flasks. The flasks were inoculated with 0.5 to 1.0 ml of spore suspension and incubated in BOD incubator at 20\(^0\)C for 21 days. After 21 days the culture material was analyzed for T-2 toxin by TLC as suggested by Rukmini and Bhat (1978) (Annexure II).

3.7 Extraction of T-2 toxin
T-2 toxin was extracted from the culture material as per the method of Romer *et al.*, (1978) and was quantified by TLC as suggested by Rukmini and Bhat (1978) (Annexure II).

### 3.8 Isolation and purification of T-2 toxin

T-2 toxin was isolated and purified by the preparative thin layer chromatographic method. After the extraction of T-2 toxin, the extract was applied to the preparative TLC plates. 200 μl of extract was applied in each spot and spots were placed 2 cm apart from each other and plates were developed in toluene: ethyl acetate: formic acid (6: 3: 1 v/v) in an unequilibrated chamber.

After the development, plates were dried and viewed under long way UV-light (366 nm). The different trichothecenes were separated based on the R<sub>f</sub> value and molecular weight.

The T-2 toxin, showing bluish fluorescence at R<sub>f</sub> value of 0.36 was scrapped out carefully from the plates along with silica gel.

The scrapped silica gel containing T-2 toxin was dissolved in chloroform solvent, at the rate of 10 ml per 2 g of silica gel and for duration of 30 minutes.
Dissolved silica gel was filtered using sintered glass filters and filtrate was again spotted on preparative TLC plates as described above for purification and the same steps were repeated four times to get the purity to maximum extent (>98%).

### 3.8.1 Preparation of preparative TLC plates

The plates were prepared as outlined in AOAC (1995) with minor modifications.

The plates were prepared by adding 50 ml of distilled water to 25 g silica gel powder and mixed uniformly to get homogenous mixture (slurry) and then drawn on glass plates of 20 x 20 cm using TLC applicator with thickness of two mm and allowed to dry.

### 3.8.2 The activation of TLC plates

The coated plates were activated by drying in forced draft hot air oven at 110 °C for one to two hours.

### 3.8.3 Quantification of T-2 toxin

The concentration of purified T-2 toxin was tested by TLC method, with visual comparison to the authentic T-2 toxin standard (Sigma standard).
The known standard was spotted on commercially available TLC plates (Merck®) at different volumes, 5 µl, 10 µl, 15 µl … 40 µl of known concentration and on the same plates the purified T-2 toxin, with unknown concentration was spotted at same volumes and plate was developed using toluene: ethyl acetate: formic acid (6:3:1) and toxin was quantified by visual analysis as described by Rukmini and Bhat (1978).

3.8.4 Preparation of developing solution

Developing solution was prepared by mixing toluene: ethyl acetate: formic acid in ratio of 6:3:1 and preserved in a closed container for further use.

3.9 Standardization

The produced T-2 toxin solution was standardized to appropriate concentration by thin layer chromatographic and spectrophotometric method.

3.10 Determination of the purity by chromatographic method

As described by Nesheim et al. (1973), on a commercially available TLC plate (Merck®) 5 µl resolution reference standard (Sigma), 5 µl produced
T-2 toxin solution and 5 µl produced T-2 toxin solution + 5 µl resolution reference standard were spotted successively at 2 cm intervals. After development, spot of individual T-2 toxin standard should reveal no other trichothecenes mycotoxins and at most faint fluorescence spots near origin.

3.10.1 Spectrophotometric method

As described by Garaleviciene et al. (2002), the spectrophotometric method of purity was tested in ethyl acetate solvent system at wavelength of 254 nm. The produced T-2 toxin reference standard solution was diluted to different concentrations, absorbance values were recorded in spectrophotometer and molar absorptivity values were calculated at different concentrations using the following formula.

\[
\text{Molar absorptivity} = \frac{\text{OD value} \times \text{Mw} \times 1000}{\text{Concentration (µg/ml)}}
\]

OD - Optical density

Mw – Molecular weight (T-2 toxin- 466)

The molar absorptivity value of developed reference standard was compared with that of the molar absorptivity values of Sigma T-2 toxin reference standard.

3.9.1 Packing and labelling
The produced aflatoxin B$_1$ and T-2 toxin reference standards were filled in disposable plastic vials of capacity two ml and vials were labeled with indications like concentration, date of production, etc.

Broachers were also supplied along with the standards indicating procedure of application, solvent to be used for the dissolution of toxin and precautions.

**3.9.2 Distribution**

The produced reference standards were distributed to feed manufacturers, state and central government institutions, analytical / quality control laboratories on a complimentary basis and feed back from the same organizations and institutes was received for purity confirmation.
RESULTS AND DISCUSSION