

Isolation and identification of fungal isolates from contaminated meadow grass fodder

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Abstract

The worldwide contamination of food and feed with mycotoxins is a significant problem. In one of the disease investigation process, it was observed that, a total of 17 cattle and buffaloes were exhibiting the maladies such as anorexia, rumen impaction, adduction of hock, posterior paralysis, downer cow syndrome etc. It was noticed that all the affected animals were stall fed and dried meadow grass (*Poa pratensis*) fodder was the major feed served to the animals, with low concentrate feed. Fungal contaminated dry meadow grass were collected from suspected areas, cultured in PDA agar, isolated and identified based on their microscopic and macroscopic appearances. The fungal species identified were *Trichoderma harzianum*, *Penicillium citrinum*, *Aspergillus versicolor* which were capable of producing mycotoxins. Hence, it could be concluded that mycotoxicosis caused by *Trichoderma*, *penicillium* and *Aspergillus spp* were suspected to be the most probable reason of these ailments as per the signs exhibited by the animals.

Key words: *T. harzianum*, *P. citrinum*, *A. versicolor*, Meadow grass, Mycotoxicosis

Introduction

The word mycotoxin was derived from mycotoxicosis which was first used in 1955 to describe diseases of animals caused by fungal toxins. Mycotoxicosis is difficult to diagnose, because few clinical signs overlapping with the clinical signs of other poisonings or diseases. The impact of mycotoxins upon animals extends beyond their obvious effect in producing death in a wide variety of animals. The economic impact of lowered productivity, reduced weight gain, reduced feed efficiency, damage to body organs, and interference in reproduction is many times greater than that of immediate mortality and morbidity (Wu et al., 2004).

In one of the disease investigation processes at Dhannalli and Nilkund villages, Siddapur Taluk of Uttara Kannada District, it was observed that, a total of 17 animals (eight cattle and nine buffaloes) were exhibiting the clinical signs such as anorexia, rumen impaction, adduction of hock (Figure 1), posterior paralysis, downer cow syndrome etc., and among those, three buffalo calves died. It was noticed that all the affected animals were stall fed and dried meadow grass (Figure 2) fodder was the major feed served to the animals, with low concentrate feed. *Poa pratensis*, commonly known as, smooth meadow-grass, common meadow-grass or Kentucky bluegrass. It is a perennial species of grass forms a valuable pasture plant. In serum biochemistry of affected animals, there was an increase in the concentration of serum AST and ALT. The post mortem revealed that there was severe liver damage, haemorrhages and congestion. And also there were severe congestions of liver, kidney and haemorrhages on visceral organs. Further investigations revealed that the meadow grass fodder was containing various fungi *spp*. After withdrawing the feeding of fungal contaminated meadow grass and with symptomatic treatment, the clinical signs got subsided. This gave out the relevance of mycotoxicosis from fungal infected meadow grass. The current research work was aimed to isolate and identify the various fungi present in the infected meadow grass.



Fig 1: Abduction of hock joint



Fig 2: Infected meadow grass

Materials and methods

Collection of fungal contaminated meadow grass

Fungal contaminated dry meadow grass were collected from Dhannalli and Nilkund villages, Siddapur taluk, Uttara Kannada district where the cattle and buffaloes were exhibiting clinical signs of posterior paralysis, downer cow syndrome, rumen impaction, keratomalacia of tail, etc. Mortality of buffalo calves was also reported in places where the grass was being served as major roughage. The fungal contaminated meadow grass that had caused the illness was collected from the above said suspected area and used for further study.

General laboratory procedures

The present investigation of isolation of the fungi from the fungal contaminated meadow grass was done in the Mycology Laboratory of the Department of Veterinary Pharmacology and Toxicology, Veterinary College, Hebbal, Bangalore.

Media used for isolation

Potato Dextrose Agar (PDA) (Himedia, India) was used for the cultivation of fungi as it is the universally recommended media for the isolation and enumeration of yeasts and moulds (Hildebrand, 1938).

Preparation of PDA Petri-plates

Before conducting mycological work, the inoculation laminar flow chamber was sterilized with UV. The PDA media was prepared as per the standard requirements mentioned in the product (39g PDA in 1000 ml water) and was sterilized by autoclaving. To avoid bacterial contamination, 25 ppm of streptomycin was added to the PDA containing flask and mixed well before pouring into Petri-plates. The PDA (10 ml) was poured into the sterilized

Petri-plate in front of a Bunsen burner in the UV sterilized inoculation chamber. The Petri- plates were then allowed to cool for 30 min or until the media solidifies.

Fungal isolation

Fungal contaminated meadow grass bits of 4-5 mm size were undergone surface sterilization with 0.1% Mercuric chloride solution for 1-2 min followed by 3 succeeded washings in distilled water for 1 min each. Then the samples were inoculated into PDA Petri-plates after drying. The inoculated plates were incubated at 28⁰C for 3-5 days. The fungal growth on the sample was evident in about 5 days. The different fungal colonies were transferred to another plate containing PDA medium and incubated at room temperature for 5 days(Narayanaswamy, 2011).

Upon sporulation, after ruling out any contamination, the fungi colony characters were observed and pure culture of each fungus was raised by hyphal tip culture from the apparently pure culture colonies. The pure isolates of the fungi thus obtained were maintained on PDA slants and were periodically used for preparing mass culture.

Identification of the fungi

Initial identification of the fungi was done in Dept. of Plant Pathology in University of Agricultural Sciences, Bangalore. Further it was confirmed by Fungal Identification Service, Agharkar Research Institute (ARI), Pune.

Results

The fungal species identified were:*Trichoderma harzianum*, *Penicillium citrinum* and *Aspergillus versicolor*.

1. *Trichoderma harzianum*

Scientific classification:

Kingdom	: Fungi
Division	: Ascomycota
Subdivision	: Pezizomycotina
Class	: Sordariomycetes
Order	: Hypocreales
Family	: Hypocreaceae
Genus	: <i>Trichoderma</i>
Species	: <i>T. harzianum</i>

Macroscopic characters: *Trichoderma* species had a rapid growth rate. Optimum temperature for linear growth on PDA was 30⁰C. In many colonies conidia were first off-white or yellow, then becoming yellow-green. Conidia formed densely over the center and in undulating concentric rings toward the edge. Colony texture was wooly (Figure 3).

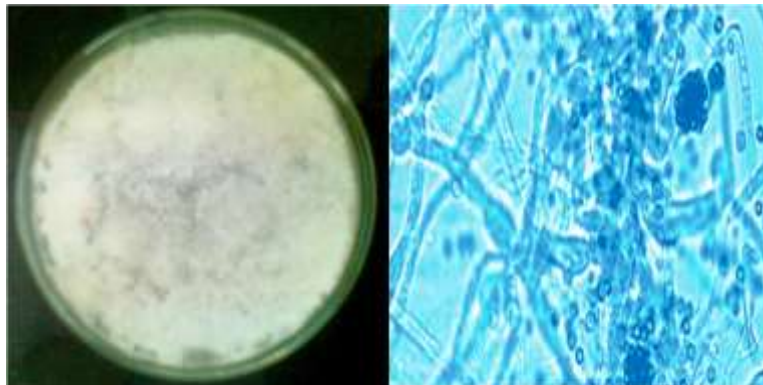


Fig 3. Macroscopic and microscopic appearance of *T. harzianum*

Microscopic characters: The conidiophores were branched and hyaline. Conidia are generally green, smooth or roughened, range in shape from globose to ellipsoidal, and are produced in slimy heads. Phialides were divergent and those in whorls typically flask shaped, enlarged in the middle, sharply constricted below the tip to form a narrow neck and slightly constricted at the base. Within these systems branches tend to be paired with the longest branches forming near the base of the system and nearest the main axis.

2. *Penicillium citrinum*:

Scientific classification:

Kingdom	: Fungi
Phylum	: Ascomycota

Class	: Eurotiomycetes
Subclass	: Eurotiomycetidae
Order	: Eurotiales
Family	: Trichocomaceae
Genus	: <i>Penicillium</i>
Species	: <i>P. citrinum</i>

Macroscopic characters: Petri dish containing PDA media showed rapid growth, attaining a diameter of 05-09mm after 05 days, dark green color, granular powdery colony and the back side of colony was pale yellow in color (Figure 4).

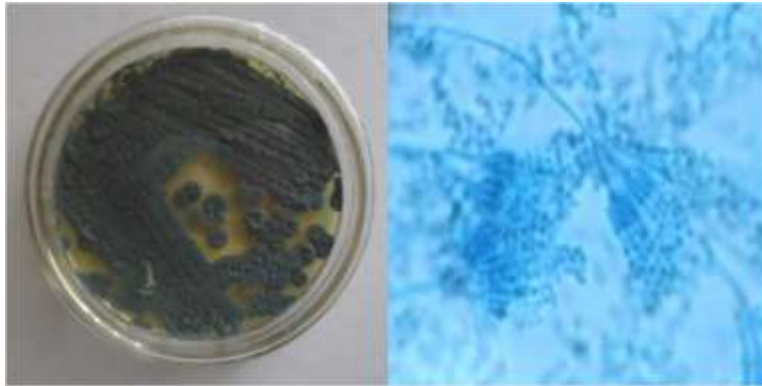


Fig 4. Macroscopic and microscopic appearance of *P. citrinum*

Microscopic characters: Microscopic feature of *Penicillium citrinum* showed septate hyphae, onidiophores was attached to the septate hyphae and conidia were spheroidal to subspheroidal. The fungal thallus typically consists of microscopic threads or filaments which branches in all direction.

3. *Aspergillus versicolor*

Scientific classification:

Kingdom	: Fungi
Phylum	: Ascomycota
Subphylum	: Pezizomycotina
Class	: Eurotiomycetes
Order	: Eurotiales
Family	: Trichocomaceae
Genus	: <i>Aspergillus</i>
Species	: <i>A. versicolor</i>

Macroscopic characters: Growth rate was slow to moderate when compared with *Trichoderma harzianum* and *Penicillium citrinum*. Colonies were generally raised in the central region, close textured, velvety rather floccose, and with regular margins. The variation in color was as follows: white at first, later becoming grayish yellow to blue green with age; margins white, radially sulcate; exudates absent and reverse pale yellowish (Figure 5).



Fig 5. Macroscopic and microscopic appearance of *A. versicolor*

Microscopic characters: Conidiophores from substrate and aerial hyphae were colorless, smooth, hyaline and thick-walled. Conidial heads were radiated. Phialides were flask-shaped. Some phialides directly bore on mycelia. It was interesting to find that a large number of heads were fragmentary and like *Penicillium spp.*

Discussion

In the present study, the species of fungi isolated and identified from the fungal contaminated dry meadow grass were *Trichoderma harzianum*, *Penicillium citrinum* and *Aspergillus versicolor*. There was a long history of acute or chronic intoxication and damage associated with *Aspergillus* and *Penicillium spp* by both humans and animals after ingestion of contaminated food and feed (Marasas and Nelson, 1987). Coulombe (1993) stated that *Aspergillus*, *Fusarium*, and *Penicillium* were the fungi that affect the stored feedstuffs and forages due to faulty storage practices. Sivasithamparam and Ghisalberti(1998) reported that *Trichoderma spp.* were free-living fungi that were highly interactive in root, soil and foliar environments. Trichothecenes are mycotoxins produced by *Trichoderma*, *Fusarium*, and at least four other genera in the fungal order Hypocreales (Cardoza *et al.*, 2011). Trichothecene production by *Trichoderma brevicompactum* and the *T. harzianum* strain from which *harzianum* A was originally isolated were belonging to *T. brevicompactum* (Nielsen *et al.*, 2005). It was confirmed that *P. citrinum* (25 isolates) consistently produced citrinin and tanzawaic acid A (Smedsgaard and Frisvad, 1996). Isolates of *P. citrinum*, the mold most frequently found in connection with the 'yellow rice', produced copious quantities of this yellow mycotoxin. It has also been isolated from cereals causing porcine nephropathy in Brazil (Rosa *et al.*, 1985). *A. versicolor* is known to be the major producer of the hepatotoxic and carcinogenic mycotoxin sterigmatocystin (Cole and Cox, 1981 and Barnes *et al.*, 1994). Hsieh *et al.* (1973) reported that sterigmatocystin isolated from cultures of *A. versicolor* supplemented with acetate (1-14⁰C) was shown to be efficiently converted to aflatoxin B₁. Hence from this investigation, it could be concluded that mycotoxicosis was suspected to be the most probable cause of these ailments as per the signs exhibited by the animals.

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