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Full Length Research Paper

Incidence of mycotoxigenic penicillia in feeds of Andhra Pradesh, India

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Incidence of different species of *Penicillium* in poultry feeds (starter, breeder, boiler and layer) and cattle feeds was analyzed. In all twenty three species of *Penicillium*, *Penicillium aethiopicum, Penicillium alli, Penicillium aurantiogriseum, Penicillium brevicampactum, Penicillium camemberti, Penicillium caseifulvum, Pchrysogenum, Penicillium citrinum, Penicillium commune, Penicillium crustosum, Penicillium digitatum, Penicillium dipodomyis, Penicillium discolor, Penicillium expansum, Penicillium flavigenum, Penicillium griseofulvum, Penicillium italicum, Penicillium nalgiovense, Penicillium nordicum, Penicillium olsonii, Penicillium roqueforti, Penicillium rubrum, Penicillium tricolor and Penicillium verrucosum* could be recorded in 400 samples of feed samples, poultry feed (280) and cattle feed (120) was analyzed for fungus isolation by dilution plate method, screening of 483 *Penicillium* species by thin layer chromatography (TLC) and spray reagents for mycotoxin production, 299 strains were positive to be mycotoxigenic which elaborates a variety of mycotoxins such as Citrinin, Cyclopiazonic acid, Mycophenolic acid, Ochratoxin A, Patulin, Penitrems, PR toxin, Roquefortine C,Rubratoxin and Terric acid etc.

Key words: *Penicillium* species, *mycotoxins*, cattle feed, poultry feed.

INTRODUCTION

In recent times poultry and dairy farming became an important agro-business, millions of small and marginal farmers use crop residues and natural herbage to feed their live stock. The availability and type of feed depends on local resources, climatic and the socioeconomic condition of the people, lack of scientific knowledge on improper processing during harvest, unseasonal rains and high moisture content provides an ideal condition for the proliferation of moulds and mycotoxins production in foods and feeds (Frisvad, 1995; Fazekas et al., 1996; Trucksess, 2001; Ana et al., 2009). These mycotoxins can be very stable to food processing (Molinie et al., 2005) can be present in fungal product. P*enicillia* from moldy feeds may cause infections, provoke allergic responses in sensitized objects or poison with toxic metabolites (Answorth and Austick, 1973; Lacey, 1975; Abramson, 1997). Hence, the present investigation was aimed to undertake an extensive and intensive survey of different feeds and feed ingredients for the incidence of *Penicillium* species.

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MATERIALS AND METHODS

An extensive survey of different feeds (cattle and poultry) of different geographical regions of Andhra Pradesh State (A.P.), India was undertaken. The samples were collected randomly, and analyzed for the presence of *Penicillium* species by dilution plate technique (Waksmann, 1922). Specific medium such as Czapek Yeast Autolysate (CYA) agar (Pitt, 1979) medium was employed for isolation of *Penicillium* species. In addition macro morphology of structure and branching of the conidiophores, the shape and ornamentation of conidia, colony characters that including growth rate, conidium color and reverse color of the colony, diffusing pigment characteristics for few species were observed and documented. Most of the *Penicillium* isolates inoculated on four enriched media such CYA agar (Pitt, 1979) Blakeslee Malt extract Autolysate (MEA) agar (Raper and Thom, 1949) Yeast extract sucrose (YES) agar (Frisvad et al., 1992) and Creatine sucrose(CREA) agar (Frisvad, 1985) for their identification, and these media gave characteristics aereal and reverse colour on type of media. The sub genus *Penicillium* species were identified by with the help of standard manuals and protocols. (Hyde, 1990; Filtenberg et al., 1992; Svendsen and Frisvad, 1994; Pitt et al., 2000; Samson et al., 2002; Frisvad and Samson, 2004).

The percentage of incidence, frequency and abundance of each fungus with special emphasis on *Penicillium* was calculated by the following formulae:

% of incidence = (No. of colonies of a species in all plates / Total no. of colonies of the all the species in all plates) × 100

% of frequency = (No of observations in which a species appeared / Total no. of observations) × 100

% of abundance = (No. of colonies of species in all observations / Total no. of colonies in all observations) × 100

Penicillium mycotoxins (Extrolites) were analyzed by employing thin layer chromatography (TLC) (Frisvad and Filtenberg, 1989; Filtenberg et al., 1983, 1992; Lund, 1995). The TLC plates (Silica Gel GF 254) were impregnated in 10% solution of oxalic acid in methanol solution for 10 min, after heating at 110°C for two minutes the plates were kept for cooling and immediately the mycotoxin extract (20 µl) was spotted on activated and cooled TLC plates (Smedsgaard, 1997). The spotted plates were developed in suitable solvents system (Samson and Pitt, 2000) by ascending chromatography. The compounds thus separated were identified either by the color of the fluoresce under (U.V.333 nm) or by the Rf value, they were further confirmed by chemical tests using different spray reagents (Pitt and Hocking, 1996, and Frisvad et al., 2004), and U.V Spectrum (U.V-10 VIS). The R_f value was calculated by the following formulae:

R_f = Distance traveled by the compound / Distance traveled by the solvent.

RESULTS AND DISCUSSION

The identification of subgenus *Penicillium* species is difficult (Thom, 1930; Raper and Thom, 1949; Smith 1960; Ciegler et al., 1969; Frisvad, 1981, Samson and Pitt, 1990; Larsen and Frisvad, 1995) because the micro morphology of the strains is very similar. In total twenty three *Penicillium* species were associated with both poultry and cattle feed (Table 1) collected from different geographical region of Andhra Pradesh with the employment of CYA agar media. *Penicillium rubrum, Penicillium citrinum* and *Penicillium olsonii* occurred with highest percentage of incidence followed by *Penicillium chrysogenum, Penicillium aethiopicum Penicillium alli* and *Penicillium aurantiogriseum* could be isolated only feed collected from Adilabad Districts. The incidence of *Penicillium* species was dominated and followed by *Aspergillus* species. *Penicillium brevicampactum* was associated with all the samples except in Nalgonda and Guntur. *Penicillium flavigerum was* associated with all the samples except the sample collected from Khammam, Warangal, and Nalgonda Districts. *Penicillium nalgiovense* could be detected in all the samples except those collected from Adilabad. *Penicillium roqueforti* and *P. rubrum* were associated with the samples of both poultry and cattle feed. *Penicillium verrrucosum* was detected in all poultry feed samples of Warangal, similarly. *Penicillium caseifulvum* could not be detected in poultry feed samples of Adilabad. On the other hand, *P.chrysogenum, P.citrinum* and *Penicillium commune* were common to all samples. The incidence of *Penicillium crustosum* could not be recorded in cattle feed samples of Warangal, Khammam, Nalgonda, Krishna, Guntur and Adilabad .The incidence of *Penicillium* species more in poultry feeds than in cattle feeds.

Screening for toxigenic potential of different species of *Penicillium* revealed (Table 3) that, large numbers of *Penicillium* isolates were mycotoxigenic. However, the percentage of mycotoxigenic strains varied with the species and place of collection. All the isolates of *Penicillium* sub genus were mycotoxigenic and many cases more than one mycotoxin was detected thus so called OSMAC (one strains many compounds), (Bode et al., 2002).

Table 2 reveled that out of 483 strains of *Penicillium* species isolated from cattle and poultry feed 299 strains were mycotoxigenic and 65 toxigenic out of 95 strains of *P. citrinum, P. expansum, P. nordicum and P. verrucosum* were screened for citrinin production. More strains of *P. citrinium* were toxigenic from the cattle feed samples collected from Khammam and poultry feed samples of Warangal, Nalgonda and Krishna Districts. Out of 25 strains of *P. camemberti* and *P. commune* were screened, 17 strains produced Cyclopiazonic acid. Out of 40 strains of *P. verrucosum* and *P. nordicum,* 28 strains produced Ochratoxin A. Comparatively more numbers of strains were toxigenic in cattle feeds of Khammam, Guntur, Adilabad and poultry feed of Krishna districts. Out of 23 strains of *P.expansum* and *P.dipodomyis* screened, 7 strains were positive for Patulin elaboration. The incidence of mycotoxigenic strains were more in cattle feed samples collected from all districts of Khammam and Nalgonda.Out of 16 strains of *P. flavigenum*, 10 strains produced penitrems (penitrems A). Contamination of penitrems was comparatively more in poultry feeds of Guntur and Adilabad District. When 19 strains of *P.aurantiogriseum* and *P.alli* from Adilabad districts were screened, 8 strains were positive for Penicillic acid, Similarly 93 strains of *P. alli, P. chrysogenum, P. crustosum, P. flavigenum, P. expansum* and *P. roqueforti* when screened for their mycotoxigenic potential, 57 strains were found to produce roquefortin C toxin, However, *P. alli* failed to produce the toxin. When 50 strains of *P. rubrum* were screened, 36 strains were positive for rubratoxin B.The incidence of this mycotoxin was comparatively more in poultry feeds of Khammam, and cattle feed of Adilabad. When 18 strains of *P. crustosum* and 53 strains of *P. chrysogenum* and *P. roqueforti* were screened, 5 strains were terric acid positive and 30 produced PR toxin. Similarly out of 41 strains of *P. brevicampactum* and *P. roqueforti,* 33 strains elaborated mycophenolic acid.

The order of percentage of contamination of different secondary metabolites by *Penicillium* species were

Table 1. Incidence of mycotoxigenic *Penicillia* in feed samples.

	Incidence												Frequency		Abundance	
Name of fungus	**Khammam**		**Warangal**		**Nalgonda**		**Krishna**		**Guntur**		**Adilabad**					
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
P. aethiopicum	--	--	--	--	--	--	--	--	--	--	2.1	1.3	16.6	16.6	0.85	0.46
P. alli	--	--	--	--	--	--	--	--	--	--	0.6	1.4	16.6	16.6	0.24	0.48
P. aurantiogriseum	--	--	--	--	--	--	--	--	--	--	1.3	0.8	16.6	16.6	0.53	0.28
P. brevicampactum	5.2	1.4	2.2	1.6	--	--	--	1.5	2.9	--	1.4	3.7	50	66.6	4.77	2.93
P. camemberti	0.9	2.1	--	3.7	3.1	1.4	2.5	6.7	--	1.3	2.2	--	50	83.3	3.55	5.44
P. caseifulvum	1.5	1.3	2.6	4.4	1.2	6.2	1.6	4.4	2.4	2.8	--	1.1	83.3	100	3.7	7.24
P. chrysogenum	4.6	3.4	3.1	4.8	1.6	2.1	5.1	1.8	1.9	5.3	3.9	4.3	100	100	8.24	7.77
P. citrinum	2.7	5.9	6.2	1.8	5.8	1.8	4.6	5.3	2.3	4.6	4.2	5.1	100	100	10.2	8.78
P. commune	2.2	1.5	1.6	0.6	2.4	1.2	1.8	0.6	3.8	2.3	3.8	1.3	100	100	6.36	2.68
P. crustosum	1.6	--	--	2.8	--	--	--	2.1	7.2	--	1.9	2.7	50	50	4.36	2.72
P. digitatum	--	2.8	--	2.6	--	--	2.6	2.9	--	2.7	2.1	1.6	33.3	83.3	1.91	4.51
P. dipodomyis	--	--	--	--	--	--	0.6	--	--	--	1.9	2.2	33.3	16.3	1.02	0.78
P. discolor	2.1	1.8	--	--	--	1.9	--	1.3	3.1	2.8	1.6	2.7	50	83.3	2.77	3.76
P. expansum	2.3	4.5	2.7	0.8	2.7	5.9	3.8	0.8	--	1.1	3.3	2.3	83.3	100	6.04	5.51
P. flavigenum	0.9	--	1.9	--	1.9	2.5	0.7	2.9	0.5	2.1	4.5	1.8	66.6	100	4.24	3.33
P. italicum	--	1.2	--	1.5	--	4.2	--	1.4	2.6	1.5	0.7	3.1	33.3	100	1.34	4.62
P. nalgiovense	3.2	3.6	4.2	--	4.2	2.1	2.5	3.3	0.2	2.9	--	--	83.3	66.6	5.83	4.26
P. nordicum	1.5	2.6	3.1	3.7	3.1	0.9	5.4	4.7	1.2	2.2	--	0.9	83.3	100	5.83	5.37
P. olsonii	2.4	3.8	2.8	5.4	2.8	2.6	6.5	4.2	1.7	5.4	2.1	2.7	100	100	7.4	8.63
P. roqueforti	2.3	2.8	1.4	4.5	1.4	2.2	2.3	4.9	2.5	2.3	--	0.3	83.3	100	4.04	6.09
P. rubrum	6.4	4.9	2.9	3.2	2.9	3.1	2.9	1.4	1.2	2.4	2.3	4.1	100	100	7.59	6.84
P. tricolor	0.2	0.9	1.2	--	--	--	0.4	1.1	0.5	--	--	--	66.6	33.3	0.93	0.71
P. verrucosum	2.9	4.1	3.4	--	3.9	3.7	2.7	2.2	2.3	4.6	3.7	4.2	100	83.3	7.71	6.73
Otherfungi =	57.1	51.4	60.7	58.6	63.o	58.2	54.1	46.5	63.7	53.7	56.4	52.4	100	100		

Otherfungi: *Aspergillus Fusarium, Mucor Rhizopus, Neurospora, Cladosporium* species.

Rubratoxin, Citrinin, Ochratoxin Roquefortine followed by Patulin, PR, Mycophenolic acid, Cyclopiazonic acid, Penicillic acid and Terric acid respectively.

The critical perusal of Table 1 reveals that the cattle feed was most ideal substratum for the proliferation of *Penicillia* and mycotoxin elaboration. The mycotoxigenic potential of *Penicillium* species isolated from poultry feed was intermediate. Hyde (1990) has isolated *Cladosporium herbarum, Alternaria tennuissima and Aspergillus fumigatu*s from feeds, which are responsible for various allergic diseases in cattle and farm workers. Fungal growth in feeds may also deplete the nutritive value and can alter the availability of micronutrients (Zohri et al., 1993; Broster, 1998). In all Cyclopiazonic acid followed by Patulin, Citrinin, Ochratoxins

Table 2. Contamination of Mycotoxins produced by *Penicillium* spp.

Name of fungus	TS	PS	Ts (%)	Name of the toxin
P. citrinum	40	28	70	Citrinin
P. verrucosum	30	22	73	
P. nordicum	10	6	60	
P. camemberi	10	5	50	Cyclopiazonic acid
P. expansum	15	9	60	
P. commune	15	12	80	
P. verrucosum	30	22	70	Ochratoxin A
P. nordicum	10	6	60	
P. dipodomyis	8	2	25	Patulin
P. expansum	15	5	45	
P. flavigenum	16	10	62	Penitrem A
P. aurantiogriseum	14	6	42	Penicillic acid
P. alli	5	2	40	
P.chrysogenum	35	24	68	Roquefortine C
P. crustosum	9	4	44	
P. expansum	15	9	60	
P. flavigenum	16	8	50	
P. roqueforti	18	12	66	
P. rubrum	50	36	72	Rubra toxin B
P. crustosum	18	5	27	Terric acid
P. chrysogenum	35	24	68	PRtoxin
P. roqueforti	18	6	33	
P. brevicampactum	33	25	75	Mycophenolic acid
P. roqueforti	18	11	66	
	483	299		

TS= Total strains; PS= Positive strains; %Ts= Percentage of toxigenic strains.

Table 3. Detection Penicillium producing Mycotoxin by different spray reagents.

Name of toxin	TEF	U.V	Spray reagents				
	Rf		$CesO_4$	2.4.DNP	FeCl3	*P.anisaldehyde*	Alcl3
Citrinin	0.52	y	Y	Bry	Br	--	LY
Cyclopiazonic acid	0.52	Y	Br	Y	Br	--	Lb
Ochratoxin A	0.32	B	B	Lo	Pbr	--	Lbr
Patulin	0.22	P	G	Y	--	--	Gr
Penitrem A	0.4	LP	Lo	P	G	--	LY
Penicillic acid	0.16	B	O	--	Lo	--	--
Roquefortine C	0.3	P	--	Gr	--	--	--
Rubra toxin B	0.35	Y	--	--	--	--	--
Terric acid	0.23	B	--	--	--	--	--
PRtoxin	0.19	PB	--	--	Br	--	--
Mycophenolic acid	0.36	Y	--	--	--	--	--

Detection color: Y=Yellow, LP=Light purple, B= blue, Pb= Purple blue, Br= brown, Ybr=Yellow brown, G= green, Lo= Light orange, O= orange, Bry= brown yellow, RBr= Red brown, Gr= grey, vo= violet, LY= Light yellow, Lb= Light brown, Ly= Light yellow, Lbr= Light brown, Pbr=Purple brown.
Spray reagents: 1= $CeSO_4$ 1% IN 6N $H2SO_4$, 2 = 2,4 DNP,3= FeCl3 3% in Ethanol, 4=p-anisaldehyde,5=50% H2SO4, 6 = 1%FeCl3 in Ethanol,Iodine ,AlCl3.Solvent system: TEF= Toluene, Ethyl acetate, Formic acid (6;3;1).

Rubratoxins, Roquefortine, Mycophenolic acid and Penicillin etc. could be spotted in feed samples analyzed from different geographical places of Andhra Pradesh.

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