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Airborne *Penicillium* in the grain shops of Nagpur (India)

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The airborne *Penicillium* spp. and total airborne fungal spore concentration was investigated in the grain shops of Nagpur city, India, using a volumetric Hi-Air sampler system Mark II (Hi Media Laboratories Ltd., India). The mycotoxins were analysed from the *Penicillium* isolates obtained from the seeds by thin layer chromatography.

The mean concentration of the total fungi isolated from different grain shops ranged from 7.8×10^2 to 1.1×10^3 CFU/m³. The mean concentration of *Penicillium* isolated from the air of grain shops ranged from 8.6×10^1 CFU/m³ (10.8%) to 1.7×10^2 CFU/m³ (19.9%). Among the 13 species of *Penicillium* which were isolated, *P. citrinum* Thom was the most prevalent species (24.2%), followed by *P. oxalicum* Currie & Thom (16.5), *P. digitatum* Saccardo (8.9%), *P. janthinellum* Biourge (8.7%), *P. funiculosum* Thom (8.3%), *P. chrysogenum* Thom (6.4%), *P. purpurogenum* Stoll (6.2%), *P. brevicompactum* Dierckx (4.8%), *P. frequentans* Westling (4.2%), *P. italicum* Wehmer (3.8%), *P. rubrum* Stoll (3.4%), *P. expansum* Link (2.9%) and *P. cyclopium* Westling (1.6%).

Penicillium species were also isolated from seeds such as wheat, maize, soybean, and groundnut. The mycotoxins roquefortin C, citrinin, rubratoxin B, cyclopiazonic acid, verrucosidin, mitorubrinic acid and two unknown metabolites were isolated from *Penicillium* isolates.

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Fungi and bacteria, the decomposers of food grains, are generally present on grains in a dormant condition. When climatic conditions are favourable, they grow and deteriorate the food grains (Christensen 1978, Sinha 1984). The species of storage fungi belong particularly to two genera, *Aspergillus* and *Penicillium* (Mehrotra 1983).

Airborne spore load is one of the most important parameters affecting seed grains in warehouses, and in wholesale markets and shops. The quantitative study on the prevalence of storage fungi, *Aspergillus* and *Penicillium*, in air becomes necessary to understand the inoculation of grains by storage pathogens. Aeromycoflora has been studied earlier in India in warehouses where grains are stored from months to years (Zahid et al. 1994, Chitnavis & Khilare 1994). However, the conditions and aeromycoflora of wholesale markets and shops where grains are stored for considerable periods are poorly understood. Storage conditions are not optimal thereby making these sites the most vulnerable stage in the storage of grains before they reach the consumers. Even consumers' store grains together in their homes for months before final consumption, predisposing the grains to deterioration and production of toxins by storage fungi, particularly when grains have been earlier attacked to a higher level by these fungi in markets and shops.

Aspergillus and *Penicillium* cannot be recorded as separate genera by spore trapping, since their spores are similar in size, shape and colour and are indistinguishable from one another. The use of a high volume air sampler for cultivable spores is indispensable to determine the presence of different species of *Penicillium* in air and their concentration. However, this type of data is meagre and practically not

available in India and therefore information on the occurrence of *Penicillium* in the air has always remained incomplete.

The presence of deteriorated and infected grains in shops increases the spore load in the air, which in turn may become a source of inoculation for the newly stored grains and in general antifungal treatment of any kind is lacking in these sites. Once infected with the storage fungi, mycotoxins accumulate in the grains and may be responsible for mycotoxicoses of the humans and animals.

Therefore, with the aim of evaluating the occurrence of *Penicillium* in the air of grain shops, the investigations were undertaken in the present study. The mycotoxins were also analysed from *Penicillium* isolates obtained from the seeds by thin layer chromatography to explore the mycotoxin producing potential.

MATERIAL AND METHODS

Four grain markets were selected for the present investigation in different areas of Nagpur (a city in Central India) namely Kalamana, Itwari, Juni Shukrawari and Mahal. Shops at Kalamana grain market have good ventilation but the market is very close to a large fruit market. The grain shops at other markets are on the ground floor in single or double storied old buildings. All these localities except Kalamana are in old crowded parts of the city with old buildings and very narrow lanes. Sampling at each of these sites was carried out in very poorly ventilated storerooms fully occupied by gunny bags containing grains. The sampler was kept at a height of about 120 cm from the ground level. For the average indoor concentration, air sampling using the Hi-Air sampler in the bedrooms during the same period at four sites including the

residential area close to the grain market of Mahal. The houses sampled were single or double storied buildings. In all, 384 air samples were analysed during two years' study (Sawane and Saoji, unpublished data).

Air samples were taken at two shops in each market for one-year duration from January 2002 to December 2002 fortnightly using Hi-Air sampler system Mark II (Hi Media Laboratories Ltd., India). Samples were taken for five minutes at each site in triplicate. Exposed strips were incubated in an inverted position at $25 \pm 2^\circ\text{C}$. After seven days of incubation, the Colony Forming Units (CFUs) were visually counted by holding the agar strip against a light background and the total fungal count was expressed as colony forming units per cubic metre of air (CFUs/m^3). Afterwards, *Penicillium* colonies were identified with the help of stereomicroscope and microscopic slides. They were then sub-cultured and three-point inoculated on Czapek's Dox Agar medium and Malt extract Agar medium and identified to species level with the help of published literature (Raper & Thom 1949, Pitt 1979, Ramirez 1982, Singh et al. 1991). Some of the cultures were identified at Indian Type Culture Collection, Indian Agricultural Research Institute, Pusa, New Delhi.

Samples of maize, wheat, groundnut and soybean were surface sterilised with 1% sodium hypochlorite for two minutes. Five to ten seeds of each type (five for groundnut, maize and soybean, and ten for wheat) were plated on the Petri plates containing CDA in triplicates (Singh 1991). The plated seeds were incubated at $25 \pm 2^\circ\text{C}$ in the dark for seven days. Colonies of different fungi were observed visually and at different magnifications of a stereomicroscope. For *Penicillium*, all colonies showing brush-like structures were considered for microscopic mount and then sub-cultured on CDA and malt extract agar. The isolation frequency (Fr) and relative density (RD) of genus and species were calculated according to Gonzalez et al. (1995).

Isolates of different *Penicillium* species obtained during the present investigation were analysed for their mycotoxin producing potential. Plates containing CYA (Czapek Yeast Autolysate Agar) were three-point inoculated using a conidial suspension made from cultures grown on Czapek's agar for seven days. These plates were incubated for seven to 14 days at 25°C without illumination in an upright position. Cultures were analysed by TLC Agar Plug Method (Filtenborg et al. 1983). Small agar plugs were cut from the fungal colonies grown on CYA using a cork borer with inner diameter 4 mm from the centre of the colony. The plugs were wetted by a drop of chloroform/methanol (2:1, v/v), and immediately applied on to TLC plates (Silicagel 60, 20×20 , Merck) for few seconds, 2.5 cm from the bottom line. The diameter of the application spot was always less than 0.6 cm.

As an external standard, griseofulvin solution was applied in the centre lane of the TLC plate. All the spots were allowed to dry and then the plates were developed in TEF solvent (Toluene/ethylacetate/90% formic acid, 5:4:1, v/v/v). After the run, developed plates were allowed to dry and then viewed in daylight, under long wave (366 nm) and short wave (254 nm) ultraviolet (UV) light. The distance travelled by different spots from the application was measured in mm. The retardation factors of the different spots were calculated relative to griseofulvin (R_{fg} values) and compared with the R_{fg} values for different mycotoxins published by Frisvad & Thrane (1987) and Singh et al. (1991).

RESULTS AND DISCUSSION

Airborne fungi and *Penicillium*

Among the 13 species of *Penicillium* which were isolated, *P. citrinum* was the most prevalent species (24.2%), followed by

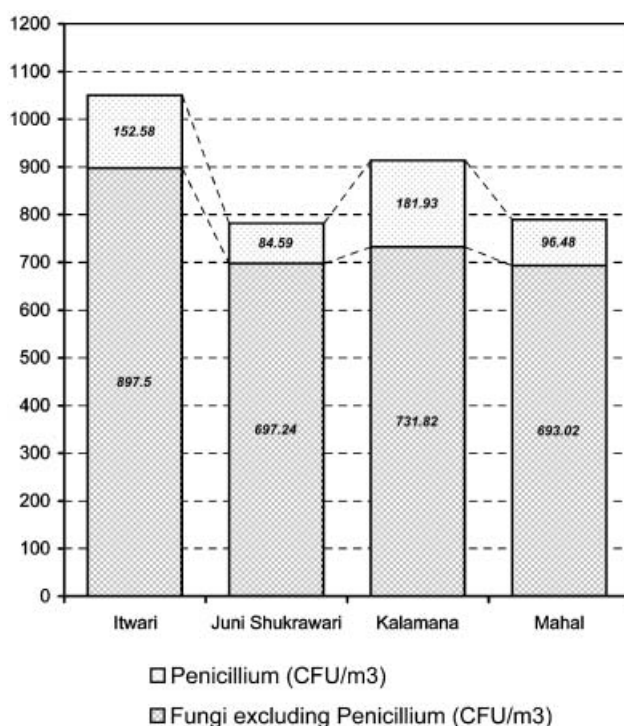


Fig. 1. Total fungi concentration (CFU/m^3) and *Penicillium* percentage in the air of grain shops.

P. oxalicum (16.5%), *P. digitatum* (8.9%), *P. janthinellum* (8.7%) and others (Figs 1 & 2).

The mean concentration of the total fungi was high in the air of grain shops (Table I) in contrast to the average indoor fungal concentration (2.9×10^2 to $4 \times 10^2 \text{ CFU}/\text{m}^3$) at different residential sites in Nagpur city (t test, $p < 0.01$), (Sawane & Saoji: Unpubl. data).

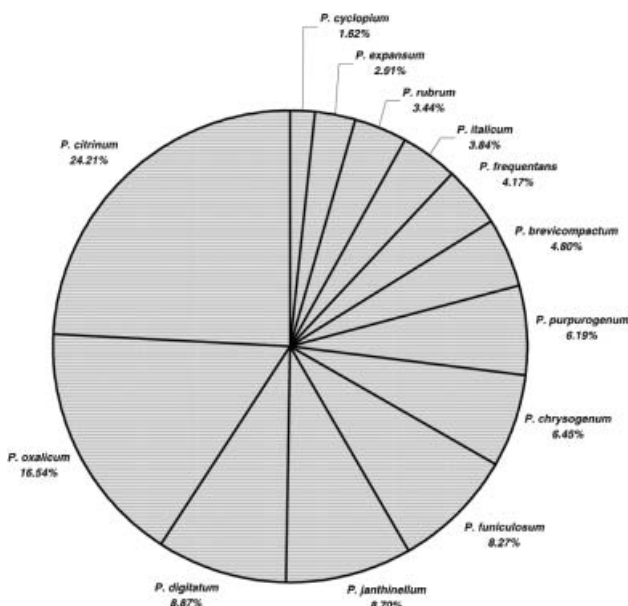


Fig. 2. Airborne *Penicillium* species isolated in the grain shops.

Table I. Airborne average total fungal spore and average *Penicillium* concentration (cfu/m^3) in grain markets (incl. statistical significance of the difference).

Site	Mean total fungi (CFU/m ³)	F value Anova	P value	Mean <i>Penicillium</i> (CFU/m ³)	F value Anova	P value
Itwari	1.1×10^3			1.5×10^2		
Juni Shukrawari	7.8×10^2			8.6×10^1		
Kalamana	9.1×10^2			1.7×10^2		
Mahal	7.9×10^2	19.95	2.5×10^{-8}	9.3×10^1	4.99	0.0045

The availability of high relative humidity, improper ventilation, manual handling and suitable organic substrates inside the grain shops are the factors responsible for the abundant occurrence of fungal airspora (Zahid et al. 1994, Vittal & Chinnaraj 1990). As a consequence of active liberation mechanism or more often due to agitation of substrates, spores of storage fungi inside the grain warehouse become airborne. Airborne spores then settle on stored bags, grains and walls and are redistributed during cleaning, handling, loading and unloading (Zahid et al. 1994). Fungi are a major cause of spoilage of the stored grains ranking next to insects, causing deterioration of grains leading to decrease in germinability, discoloration, heating, mustiness, production of toxins and loss in weight of the grains (Tuite & Christensen 1955).

The difference of the total fungal presence in the air of the different grain shops investigated may be accounted for by the differences in ventilation, surroundings and some other factors. Maximum fungal concentration observed in the grain shops of Itwari may be explained by the densely populated area, very poor ventilation, insufficient space, large number of grain shops in very narrow lanes, manually cleaning grains (removing unwanted material) all around the shops disseminating the spores, accumulation of spoiled grains surrounding the shops, and poor sanitation inside and in immediate vicinity of the grain shops.

The percentage of *Penicillium* isolated from air of grain shops in the present investigation (10.8 to 19.9%) is much higher than the percentage of *Penicillium* reported earlier (3.16%) by Zahid et al. (1994) in a grain warehouse in India. Different species of *Penicillium* were isolated throughout the year, with peak concentration being in June and November in contrast to the earlier report of Zahid et al. (1994) in which *Penicillium* was isolated only in August and September.

The genus *Penicillium* is reported to be highly allergenic

and its presence throughout the year in grain shops may pose a danger for the respiratory health of the workers. However, the correlation between *Penicillium* concentration and respiratory problems was not studied in the present investigation.

Seedborne fungi and *Penicillium*

In the present investigation, wheat, maize, soybean and groundnut were considered for evaluation of the presence of different species of *Penicillium*, by calculating percentage frequency and percentage relative density for genus *Penicillium* (Table II) as well as for individual *Penicillium* species isolated from these seeds (Table III).

Mehrotra (1983) has reviewed the seed borne fungi and has reported that *P. urticae*, *P. cyclopium*, *P. citrinum* and *P. islandicum* are the common *Penicillium* species associated with wheat in India. Abdel-Hafez (1984) isolated nine species of *Penicillium* from cereal grains and found that *P. notatum* (*P. chrysogenum*) was common in barley, sorghum and wheat grains. Mislivec & Tuite (1970) isolated 14 different species of *Penicillium* from stored corn with maximum incidence of *P. cyclopium* followed by *P. brevicompactum*, *P. oxalicum* and *P. funiculosum* with average *Penicillium* infection to be 21%. Gonzalez et al. (1995) reported that amongst the external mycoflora, percentage frequency of *Penicillium* on corns ranged from 62.5% to 80%, while the percentage relative density ranged from 27.9 to 43.2. These authors have reported isolation of 19 *Penicillium* species with *P. decumbens* and *P. funiculosum* as the dominant species. Jimenez et al. (1984) reported that *Penicillium* is the most abundant genus after *Fusarium*, with an incidence of 71.5% in infected samples and that most frequently isolated and dominant species were *P. chrysogenum*,

Table II. Number of samples infected, frequency, relative density of *Penicillium* obtained from seeds.

Fr (%) – Percentage frequency of *Penicillium* isolation – Percentage of samples infected with *Penicillium*.

RD (%) – Relative density of *Penicillium* – Percentage of *Penicillium* isolates out of the total fungi isolates obtained from seeds.

	Wheat	Maize	Soybean	Groundnut
<i>Penicillium</i> Isolates	314	204	189	260
Total fungal isolates	3727	1736	1865	2674
Samples infected with <i>Penicillium</i>	39	37	48	38
Total samples	96	74	82	96
Fr (%)	40.6	50	53.6	39.6
RD (%)	8.4	11.7	10.1	9.7

Table III. Frequency and relative density for different *Penicillium* species in the grains.Fr (%) – Percentage frequency of *Penicillium* isolation – Percentage of samples infected with *Penicillium*.RD (%) – Percentage relative density of *Penicillium* – Percentage of *Penicillium* isolates out of total fungal isolates obtained from seeds.

Species	Wheat		Maize		Soybean		Groundnut	
	Fr (%)	R.D. (%)	Fr (%)	R.D. (%)	Fr (%)	R.D. (%)	Fr (%)	R.D. (%)
<i>P. chrysogenum</i>	1.2	1.9	8.1	8.8	4.2	10.0	—	—
<i>P. citrinum</i>	18.3	33.8	24.3	41.7	13.1	29.1	10.4	29.2
<i>P. cyclopium</i>	18.3	31.8	—	—	3.7	5.3	—	—
<i>P. funiculosum</i>	6.1	9.9	8.1	15.2	6.1	7.9	4.2	10.0
<i>P. oxalicum</i>	6.1	12.7	1.3	3.4	6.1	12.7	19.8	46.5
<i>P. purpurogenum</i>	2.4	4.5	10.8	12.7	3.7	5.8	7.3	5.8
<i>P. rubrum</i>	—	—	9.5	13.2	4.9	9.5	5.2	3.1
<i>P. janthinellum</i>	1.2	1.0	—	—	—	—	—	—
<i>P. frequentans</i>	3.7	4.5	2.7	4.9	9.7	12.2	2.1	5.4
<i>P. brevicompactum</i>	—	—	—	—	6.1	7.4	—	—

P. steckii and *P. purpurogenum*. Hesseltine et al. (1981) have reported *P. oxalicum* and *P. funiculosum* to be dominant species associated with maize. Halfon-Meiri & Barkai-Golan (1990) isolated *P. frequentans* as the most dominant *Penicillium* species associated with corn and also reported that corn shows brown discoloration and loss of germination ability as a result of invasion by *Aspergillus* and *Penicillium* species.

Abdel-Hafez (1984) reported that the percentage frequency of *Penicillium* on pea seeds was 60% while the percentage relative density was 10.71% with most prevalent species being *P. chrysogenum*, *P. citrinum* and *P. funiculosum*. Vishnuvat & Shukla (1983) reported that *Penicillium oxalicum* was the prevalent species on lentil seeds in India. El-Maghraby & El-Maraghy (1988) isolated 11 species of *Penicillium* from groundnut with *P. funiculosum*, *P. citrinum* and *P. chrysogenum* being the dominant species with very high density.

Mycotoxins of *Penicillium* spp. isolated

Many of the species of *Penicillium* associated with the grain seeds have been reported to produce mycotoxins that cause mycotoxicoses of domestic animals and man (Scott et al. 1972, Scott 1978). In the present investigation, secondary metabolites production on Czapek Yeast Autolysate Agar was studied for some of the *Penicillium* species isolated from seed samples (Table IV).

None of the six isolates of *P. chrysogenum* obtained from maize and soybean produced any of the secondary metabolites. Nielsen et al. (1999) also reported the absence of toxin production in any of the five isolates of *P. chrysogenum*. Swelim et al. (1994) however, reported aflatoxin B₁ and G₁ production by 33 isolates of *P. chrysogenum* out of 72 isolates obtained from the stored poultry fodder.

P. citrinum produced citrinin in five out of six isolates tested of which two isolates were obtained from wheat, two isolated from groundnut and one from maize. El-Maghraby & El-Maraghy (1988) reported citrinin production by *P. citrinum* isolated from groundnut seeds. Malmstroem et al.

(2000) reported citrinin production by 25 isolates of *P. citrinum* in addition to production of tanazawaic acid.

None of the isolates of *P. funiculosum* produced any of the known or unknown secondary metabolites. Three of the six isolates of *P. frequentans* produced unknown metabolite with retardation factor of 0.95 relative to Griseofulvin. Wunder et al. (1997) reported formation of metabolites identified as 1-hydroxypyrene, 1,6- and 1,8- dihydroxypyrene, 1,6 and 1,8-pyrenyl sulphate by a *Penicillium frequentans* strain.

P. oxalicum produced two metabolites, roquefortine C by three isolates and an unknown (with R_f=1.57) by two isolates. These isolates were obtained from wheat and groundnut. Singh et al. (1991) have reported that oxaline, roquefortine C, and secalonic acid are the mycotoxins produced by *P. oxalicum*. The incidence of these species on both wheat and groundnut is high, suggesting that these toxins may be present in the infected seeds, but the confirmation of presence of toxins in seeds is needed.

Four out of six isolates of *P. rubrum* produced a known metabolite rubratoxin B (R_f=0.32, blue fluorescence). Singh et al. (1991) and Zohri et al. (1995) have reported the production of rubratoxin B by *P. rubrum*. *P. purpurogenum* also produced rubratoxin B along with mitorubrinic acid and one unknown metabolite with R_f value of 1.772. Natori et al. (1970) have reported production of rubratoxin B by *P. purpurogenum*. Rubratoxin B is a hepatotoxin and is also responsible for multiple haemorrhages (Wilson & Wilson 1962).

In this investigation, cyclopiazonic acid and verrucosidin were produced by three and two isolates of *P. cyclopium* respectively. Again these isolates were obtained from the wheat and soybean. Scott (1978) has reported production of cyclopiazonic acid by *P. cyclopium*. It is a neurotoxin when intraperitoneally injected into rats. With oral administration to rats, lesions in liver, kidney, spleen and other organs are observed (Scott 1978).

As the mycotoxin potential of different species of *Penicillium* isolated from the grains in this region is lacking, even a preliminary study of mycotoxins would add to the existing knowledge on the subject and highlight the hazards of fungal contamination.

Table IV. Secondary metabolites produced by *Penicillium* isolates obtained from seed grains.

Rfg=Distance travelled by a spot in mm/Distance travelled by Griseofulvin in mm.

Species	Rfg values of spots on TLC plates	Secondary metabolites after comparison with published data	No. of isolates tested	No. of isolates producing sec. metabolites
<i>P. chrysogenum</i>	—	—	6	0
<i>P. citrinum</i>	1.23	Citrinin	6	5
<i>P. funiculosum</i>	—	—	6	0
<i>P. frequentans</i>	0.95	Unknown ₁	6	3
	0.19	Roquefortine C	6	3
<i>P. oxalicum</i>	1.57	Unknown ₂		2
<i>P. rubrum</i>	0.32	Rubratoxin B	6	4
	0.32	Rubratoxin B,	6	3
<i>P. purpurogenum</i>	1.03	Mitorubrinic acid,		2
	1.37	Cyclopiazonic acid,	6	3
<i>P. cyclopium</i>	1.45	Verrucosidin		2

CONCLUSIONS

In conclusion, the high percentage of *Penicillium* in the air of grain shops, by causing increased inoculation on seeds, may be responsible for the storage losses as well as production of toxins. It may become a potential high risk factor for allergenic disorders for shop personnel, though this aspect was not studied in the present investigation. Thus, the higher fungal concentration found inside the grain shops calls for some corrective measures, such as better sanitation of the shops and antifungal spraying to reduce the spore load in the air.

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