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PREVALENCE OF AIRBORNE POLLEN GRAINS AND FUNGAL SPORES IN A CITY OF THAR DESERT, INDIA

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ABSTRACT

Monitoring of bioaerosols *i.e.,* pollen grains and fungal spores, was undertaken for two consecutive years in a city of Thar Desert- Bikaner, using Gravity Slide Method. A total of 32 different pollen taxa were identified with Annual Pollen Integral (API) of 890 pollen grains per sq. cm in the first year and 906 pollen grains per sq. cm in the second year. The pollen count of non-arboreal plants was much more than that of the arboreal plants. The most prominent annual average pollen percentage was credited to Poaceae (25.91%), followed by Chenopodiaceae/Amaranthaceae (17.57%), Papilionaceae (7.86%) and Cyperaceae (5.95%) among non-arboreal plants: and to *Prosopis juliflora* (14.84%) amongst arboreal taxa. Two peak seasons were observed in both the years *i.e.,* February to April and August to October for both pollen grains as well as fungal spores. 32 types of airborne fungal spores were reported from the atmosphere of Bikaner during the study. A total number of 6717 fungal spores per sq. cm were counted in the first year and 7117 spores per sq. cm in the second year. The commonest fungal spores encountered were of the species of *Cladosporium,* followed by *Alternaria* and *Curvularia* with annual mean percentage of 24.95%, 23.02% and 11.53 respectively.

Keywords: Aerobiology, Airborne pollen, Airborne fungal spore.

1. INTRODUCTION

F.F. Meier, while portraying about the life of microbes in the atmosphere introduced the term aerobiology in the year 1930. Aerobiology is an interdisciplinary field which includes various aspect of airborne biological particles from their induction as aerosol to their transmission and deposition, as well as their influence on the wellbeing of man and the environment. The biological aerospora comprises of pollen grains; fungal and plant spores; bacteria, protists (protozoa), viruses and remanent of biologic material. It is worth noting here that during the process of respiration these bioaerosols get inhaled and are deposited in bronchial and nasal air passage causing serious and everlasting health hazards. In India, almost 20-30 percent population is affected by allergic rhinitis and hay fever; and approximately 15 percent of this group develops asthma [1, 2]. Therefore, the study of dispersal pattern of these bioaerosols has assumed greater significance. Airborne fungal spores and pollen grains have been found to be the major cause of allergy in humans. Concentration of pollen grains in the atmosphere greatly depends upon vegetation of the region, phenology of the local flora, seasonal variation, and weather conditions. Similarly, airborne fungal spores' distribution is greatly dependent upon the local meteorological variables, fungal growth substratum, and anthropological activities. These allergens have been found to be seriously impairing the human health, resulting thus, in substantial socio-economic loss. Pollen grains and fungal spores are the main airborne allergen which can be held responsible for asthma, rhinitis, and atopic dermatitis in human beings [3-5]. It is because of this reason that the monitoring of these airborne allergens becomes mandatory to regulate allergy.

2. MATERIAL AND METHODS

2.1. Study Area

The Thar Desert is present in the North-Western Part of the Indian subcontinent making a natural border between India and Pakistan. It covers an area of 200,000 sq. km and is also known as the Great Indian Desert. According to Bhandari [6], the Thar Desert has a very rich floral diversity including about 628 species, 352 genera and 87 families and mainly consists of grassland intermingled with trees and thorny bushes.

The present study has been carried out in Bikaner City situated in the middle of the Thar desert. It is spread across an area of 30,247.90 sq. km and is located between 27°11' N and 29°03' N and 71°54' E and 74°12' E. Major part of the area is occupied by sandy plains and dunes with a height of 61 to 305 metres above sea-level. It has a hot desert climate with very little rainfall and extreme temperatures. The summers (April to June) are very hot with maximum temperature exceeding 48°C whereas in winters (November to February) the temperature may drop below freezing point at night. Also, there is a steep contrast between the maximum and minimum temperature of different months of the year. The annual precipitation in the area is not more than 352 mm with maximum rainfall occurring in the months of July to September (Rainy season) when the maximum temperatures are also high. Sand and dust storms during hot and dry summers are very common. The wind velocity is often as high as 110 km/hrs weeping away old sand dunes and creating new ones. Low relative humidity and high evaporation are other common aspects of the climate with frequently occurring famine.

The extreme climatic conditions do not support the growth and development of vegetation. While studying the ecology of Bikaner, Joshi [7] reported that *Laptadenia spartium, Crotalaria burhia, Aerua tomentosa* and several herbaceous annuals like *Trianthema pentandra, Euphorbia clarkeana,* and a number of grasses act as pioneer species in fixing the soil in sand-dunes during rainy season. Once the sand is consolidated, it gives way to the characteristic savannah formations of *Capparis decidua, Calligonum polygonoides* and *Ziziphus nummularia*. The climax community usually consists of an association of *Calligonum -Zizyphus* and *Capparis*. In 1980s, a part of the area of Bikaner started getting irrigation from the Indira Gandhi Canal, whereas in the remaining area, the agriculture is dependent on rainwater. Construction of Indira Gandhi Canal has greatly affected the area in many ways, such as the desolate barren sand dunes have been replaced by green fields; sand has been replaced by loamy soil; many new plants have been brought with the water along the banks of the canal as well as in the form of contaminants of the crop seeds; and moreover, many xerophytic species have vanished from the irrigated land [8].

2.2. Aerobiological Survey: Collection, Identification, and Counting

Aerospora was trapped using Gravity Slide Method. The sampler was an aeroscope designed and fabricated on the principles of "slide exposure-apparatus" used by Lakhanpal and Nair [9] with some modifications. Two aeroscopes were installed at a height of about 20 feet above ground level at two sites a few kilometres apart from each other for two consecutive years from January 2000 to December 2001. One of the sites was in the residential area of Bikaner City and the other one was in open fields. Both the sites were completely exposed on all sides and having a free display of air currents in all the directions. Microscopic slides smeared with glycerine jelly were exposed daily for 24 hours in the aeroscope and were changed daily at 10:00 am at the first site and at 11:00 am at the second site. Immediately after removing the slides from the aeroscope, the smeared slide is mounted using phenol & saffranine stained glycerine jelly under 22mm square microscopic cover slip. The total area covered under the cover glass was scanned under microscope at magnifications of x400 to study the aerospora deposited from the atmosphere.

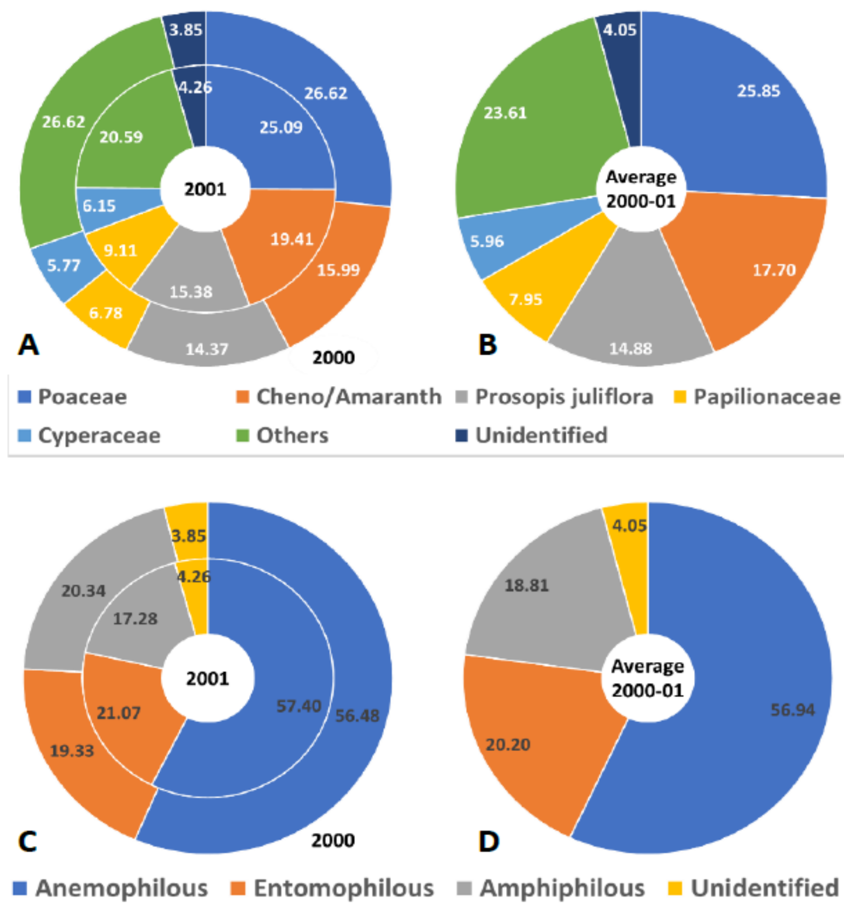
Airborne pollen grains were identified by comparing them with the corresponding pollen in the reference pollen herbarium as well as by using pollen atlases and the relevant literature available [10-12]. The taxonomic characters like number of apertures; form of aperture; distribution of apertures and patterns of stratifications of exine are the chief characters employed for the identification. Because of the limitations in identification, certain plants have only been described up to the generic level. The pollen of some stenopalynous families like Poaceae, Chenopodiaceae, Amaranthaceae and Cyperaceae are very identical, hence only differentiated up to the family level. Pollens of family Chenopodiaceae and Amaranthaceae are morphologically alike and are difficult to distinguish from one another, they are grouped together as Cheno/Amaranth. The fungal spores were identified mainly on the basis of spore morphology [13-15]. Daily count of aerospora deposited in the total area scanned (4.84 sq. cm) was converted into per sq cm counts and was used to calculate monthly and yearly concentrations for each species or taxon.

Simultaneously, a reference pollen herbarium was prepared using Erdtman's [16] acetolysis technique. This technique is a combination of two steps, chlorination and acetolysation. Chlorination removes cellular content and intine of the pollen grains. Whereas, during acetolysation step pollen grains are treated with a mixture of acetic anhydride and concentrated sulphuric acid in a ratio of 9:1 to stain the pollen brown.

3. RESULTS AND DISCUSSION

3.1. Aeropalynological Studies

During the study period of 2-years, pollen grains were reported all the year round in the atmosphere of Bikaner. A total of 32 different pollen taxa were identified with 29 different pollen taxa in 2000 and 27 pollen taxa in 2001. The Annual Pollen Integral (API) was 988 pollen grains per sq. cm in 2000 and 845 pollen grains per sq. cm in 2001. Even after all attempts, an average count of 4.04% of pollen grains could not be identified due to their distorted nature and were placed in separate group 'unidentified'. A significant variation was reported in both quality as well as quantity of pollen grains throughout the study. These taxa were classified into two categories viz. arboreal and non-arboreal plants. Out of these 32 different pollen taxa, 13 taxa belong to arboreal plants whereas 19 taxa are from non-arboreal plants. The pollen count of non-arboreal plants was much more than that of the arboreal plants for both the years. The frequency of non-arboreal pollen was 63.77% and 66.04% in 2000 and 2001 respectively whereas, only 32.39% of arboreal pollen were recorded in 2000 and 29.70% in the year 2001 (Table 1). This is because, the prevailing habit wise vegetation in Thar dessert is herbaceous (60.10%) followed by shrubs (16.26%), trees (14.29%) and climber (9.36%) [17]. Sharma and Dharke [18] and Ahlawat et al. [19] have also reported flowering period of arboreal plants in March-April and October for non-arboreal plants from Agra city and Rohtak respectively.



(Data labels are in percentage of total pollen concentration)

Fig. 1: a) Variation of the five prominent pollen taxa; b) Annual mean relative contributions of the five major pollen taxa; c) Variations in the pollen taxa based on mode of pollination and d) Annual mean relative contributions based on mode of pollination present in Bikaner, India over a period of two-years

Table 1: Total pollen catch per square centimeter

Pollen Class		2000		2001.00		2000-2001	
		Annual Conc. (pollen/sq. cm)	%	Annual Conc. (pollen/sq. cm)	%	Annual Mean. (pollen/sq. cm)	%
A. Identified							
I Arboreal							
1	*Prosopis juliflora*(AM)	142	14.37	130.00	15.38	136.00	14.84
2	*Ricinus communis*(AN)	34	3.44	25.00	2.96	29.50	3.22
3	Moraceae(AN)	26	2.63	19.00	2.25	22.50	2.45
4	Meliaceae(EN)	23	2.33	17.00	2.01	20.00	2.18
5	Myrtaceae(EN)	31	3.14	8.00	0.95	19.50	2.13
6	*Ailanthus excelsa*(AM)	16	1.62	8.00	0.95	12.00	1.31
7	*Prosopis cineraria*(EN)	11	1.11	12.00	1.42	11.50	1.25
8	*Acacia nilotica*(EN)	11	1.11	11.00	1.30	11.00	1.20
9	*Albizia lebbeck*(EN)	9	0.91	8.00	0.95	8.50	0.93
10	*Ziziphus spp.*(EN)	2	0.20	7.00	0.83	4.50	0.49
11	*Lawsonia inermis*(AM)	8	0.81	-	-	4.00	0.44
12	*Salvadora spp.*(EN)	4	0.40	4.00	0.47	4.00	0.44
13	*Pinus spp.*(AN)	3	0.30	2.00	0.24	2.50	0.27
Arboreal Total Sum		**320**	**32.39**	**251**	**29.70**	**285.50**	**31.15**
II. Non-arboreal							
1	Poaceae(AN)	263	26.62	212.00	25.09	237.50	25.91
2	Cheno/Amaranth(AN)	158	15.99	164.00	19.41	161.00	17.57
3	Papilionaceae(EN)	67	6.78	77.00	9.11	72.00	7.86
4	Cyperaceae(AN)	57	5.77	52.00	6.15	54.50	5.95
5	Brassicaceae(EN)	21	2.13	23.00	2.72	22.00	2.40
6	*Parthenium hysterophorus*(AM)	25	2.53	4.00	0.47	14.50	1.58
7	Asteraceae(AN)	11	1.11	4.00	0.47	7.50	0.82
8	*Xanthium strumarium*(AN)	6	0.61	6.00	0.71	6.00	0.65
9	Apiaceae(EN)	3	0.30	4.00	0.47	3.50	0.38
10	Solanaceae(AM)	3	0.30	4.00	0.47	3.50	0.38
11	*Argemone mexicana*(EN)	4	0.40	1.00	0.12	2.50	0.27
12	*Tribulus terrestris*(EN)	-	-	3.00	0.36	1.50	0.16
13	*Boerhavia difusa*(AM)	3	0.30	-	-	1.50	0.16
14	*Glinus lotoides*(AM)	3	0.30	-	-	1.50	0.16
15	*Ipomoea spp.*(EN)	3	0.30	-	-	1.50	0.16
16	*Malvaceae*(EN)	2	0.20	1.00	0.12	1.50	0.16
17	*Heliotropium spp.*(EN)	-	-	2.00	0.24	1.00	0.11
18	*Corchorus tridens*(AN)	-	-	1.00	0.12	0.50	0.05
19	Capparaceae(AM)	1	0.10	-	-	0.50	0.05
Non-arboreal Total Sum		**630**	**63.77**	**558**	**66.04**	**594.00**	**64.81**
B Unidentified		**38**	**3.85**	**36.00**	**4.26**	**37.00**	**4.04**
Grand Total		**988**	**100.00**	**845.00**	**100.00**	**916.50**	**100.00**

AN: Anemophilous; EN: Entomophilous; AM: Amphiphilous

The most prominent annual average pollen percentage for 2000-01 was credited to Poaceae (25.91%), followed by Cheno/Amaranthaceae (17.57%), Papilionaceae (7.86%) and Cyperaceae (5.95%) among non-arboreal plants and to *Prosopis juliflora* (14.84%) amongst arboreal taxa (Table 1). A good quantity of pollen of Poaceae, Chenopodiaceae and Amaranthaceae were also reported from Lucknow [9], Jaipur [20], Delhi [21], Meerut [22], and Tamil Nadu [23]. Maximum quantity of pollen of Asteraceae were reported in Jabalpur [24]. These five major taxa with high frequency of airborne pollen grains together account for 69.53% of the total in 2000 and 75.15% in 2001 (Fig. 1 a, b). Other frequently occurring pollen grains with annual average pollen percentage were Moraceae (2.45%), Brassicaceae (2.40%), Meliaceae (2.18%), Myrtaceae (2.13%), *Parthenium hysterophorus* (1.58%), *Ailanthus excelsa* (1.31%), *Prosopis cineraria* (1.25%), and *Acacia nilotica* (1.20%), The pollen grains of *Albizia lebbeck*, Asteraceae, *Xanthium strumarium*, *Ziziphus spp*., *Lawsonia inermis*, *Salvadora spp*., *Apiaceae, Argemone mexicana, Pinus spp*., Solanaceae, *Boerhavia difusa, Glinus lotoides, Ipomoea spp*., Malvaceae, *Tribulus terrestris, Heliotropium spp.,* Capparaceae, and *Corchorus tridens* were very infrequently reported i.e., less than 1% (Table 1). The reported aero-pollens were also categorised according to their mode of pollination viz. Anemophilous, Entomophilous and Amphiphilous. Out of total 32 pollen taxa, 9 taxa were anemophilous with maximum average pollen count of 56.94% followed by entomophily comprising of 15 pollen taxa with average pollen count of 20.20%. Average pollen count of amphiphilous plants was lowest (18.81%) and was represented by 8 different pollen taxa (Table 1, Fig. 1c, d).

Two peak seasons were observed in both the years- February to April and August to October. Total mean annual pollen from February to April was 37.7% whereas, from August to October the total average annual pollen was 45%. However, different pollen taxa showed different peak seasons throughout the year according to their phenology and the environmental conditions. The maximum concentration of pollen grains in air was found in the month of September (24.83% in 2000 and 25.83% in 2001), whereas minimum concentration of pollen grains was reported in the month of December (1.57% in 2000 and 1.88% in 2001) (Fig. 2a).

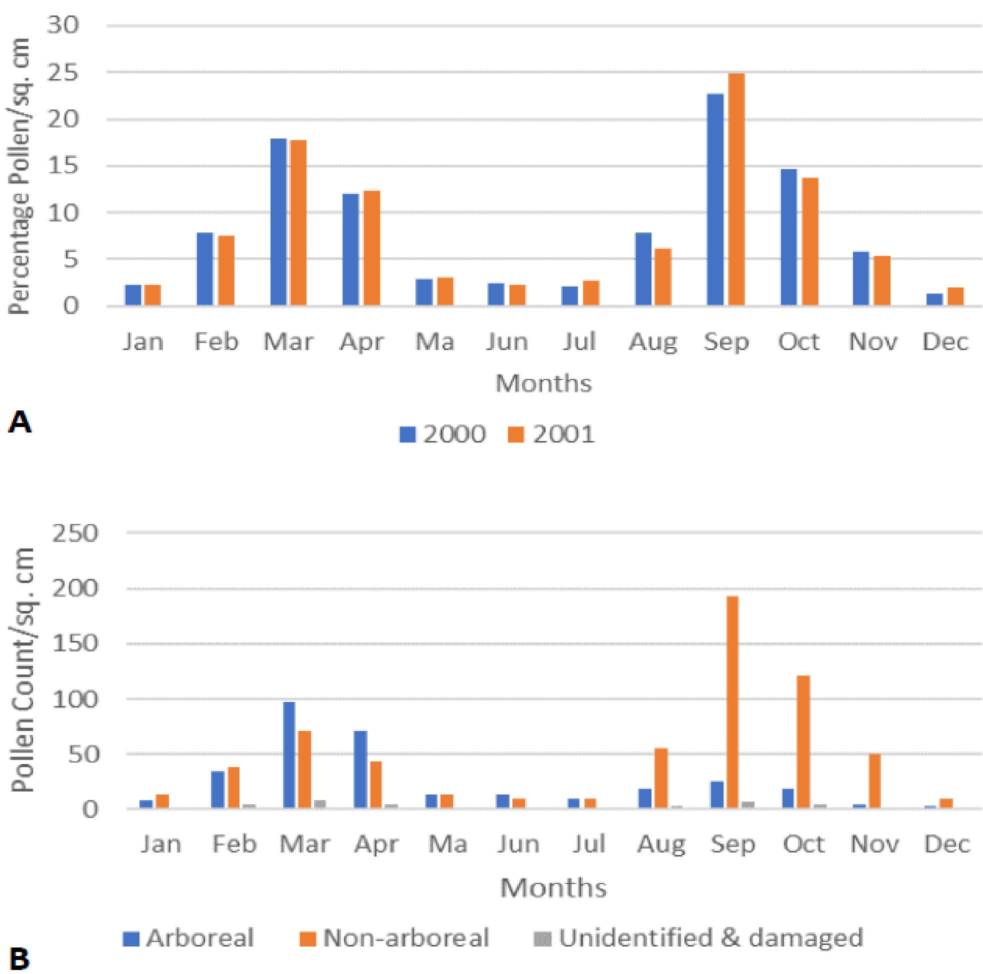


Fig. 2: a) Monthly variations in total pollen concentrations; b) Annual mean monthly variation in pollen concentrations based on habit of the plant in the atmosphere of Bikaner over a period of two years

Vegetation in Thar desert mostly depends upon rains. The monsoon hits this area in the mid of July and last up to mid-September. Ephemeral vegetation starts germinating with the onset of rainy season. The non-arboreal plants have abundance of flowering, increasing the pollen count in the atmosphere in the month of August to September. Therefore, the peak season of August-October was dominated by non-arboreal taxa. In the month of October, the days are warmer and relative humidity is very low, the pollen count starts decreasing. Winter season starts in the month of November and lasts till January. By November the non-arboreal plants pollen season is already over and the pollen season for arboreal plants has yet to start. Most of the trees start flowering by the end of January and are at their peak during spring season i.e., March and April. Weeds and grasses of spring season also bloom during these months. Therefore, both arboreal and non-arboreal plants equally contribute to the pollen load in the atmosphere during February to April peak season. The spring season is followed by warm and dry summer with windy storms in the months of May-July leading to decrease in the pollen count (Fig. 2b).

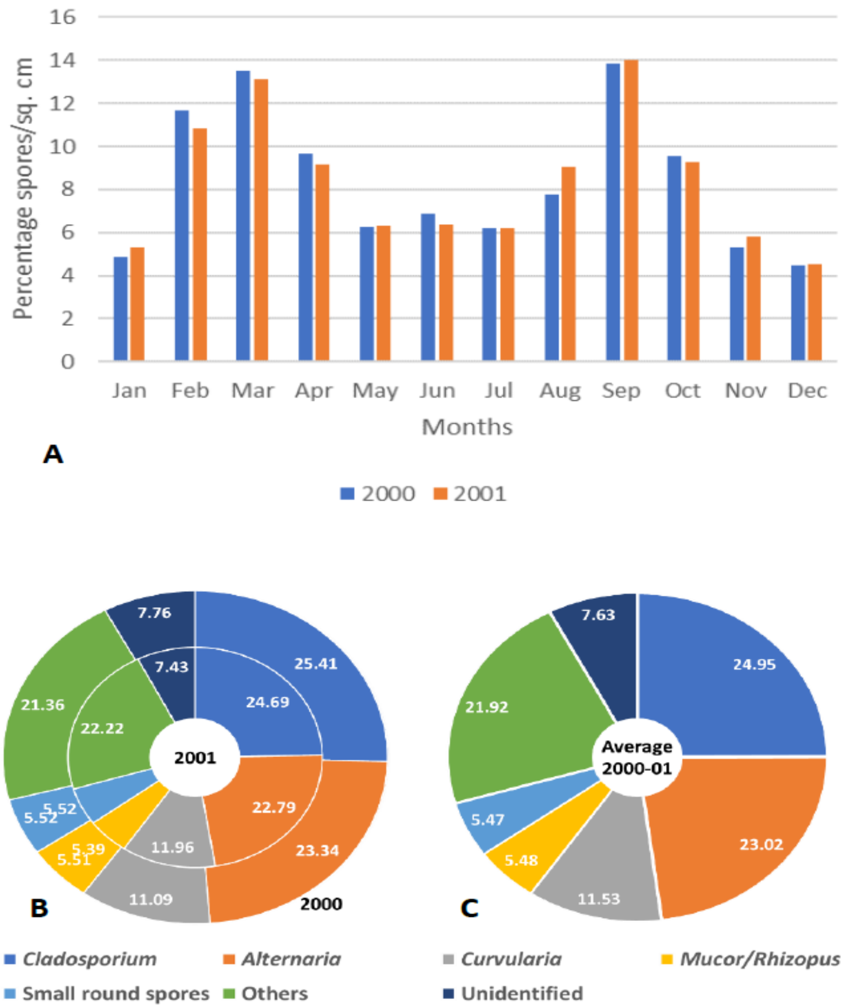
3.2. Aeromycological Studies

The 33 types of airborne fungal spores, including one unidentified, had been counted from the atmosphere of Bikaner- a city in Thar desert of Rajasthan, during the study spread over two years (January 2000-December 2001). A total number of 6717 spores per sq. cm were counted in 2000 and 7117 spores per sq. cm in 2001. Out of these, 29 types were common for both the years, 2 types were present only in 2000 and 2 types only in 2001. The commonest fungal spores encountered were of the species of *Cladosporium* amounting to 25.41% in 2000 and 24.69% in 2001 followed by *Alternaira* with 23.34% in 2000 and 22.79% in 2001 and *Curvularia* with 11.09% in 2000 and 11.96% in 2001 of the total fungal count. Verma and Chile [25] also observed higher percentage of *Cladosporium* and *Alternaria* in the atmosphere of Jabalpur and Gupta et al. [26] from Jaipur. When percentage contribution of different classes of fungi was studied, the Deuteromycotina group, in which 14 spore types were identified, dominated amounting 67.55% in 2000 and 66.80% in 2001; followed by Ascomycotina with 14 types contributing 11.63% in 2000 and 12.76% in 2001; Zygomycotina represented by *Mucor/Rhizopus* amounting 5.51% in 2000 and 5.39% in 2001; and Basidiomycotina represented by smut spores and basidiospores accounted for 7.56% in 2000 and 7.62% in 2001 of the total spores catch. The contribution of unidentified types was 7.67% in 2000 and 7.43% in 2001. The annual variations studied for two years is not much but variations in individual components of fungal air spora are significant. In qualitative variations it was noted that spores of *Melanospora* and *Tetracoccosporium* were present in 2000 only whereas, spores of *Botrytis* and *Fusarium,* were present in 2001 only (Table 2).

Monthly and seasonal variations in the quantity and quality of the fungal spores were also well marked. Aerospora of an area greatly depends upon its ecology. It is therefore likely that the seasonal variations recorded for the different elements of the fungal airspora depends on the seasonal growth of vegetation and concurrent weather [27-29]. In present study, there were two peak periods when incidence of total mean of fungal spores was higher and these two peaks correspond to sporulation of fungi, good vegetation, and suitable climatic conditions. It was found that too hot and too cold seasons were unfavourable for the concentration of fungal spores in the atmosphere of Bikaner. The first peak period was seen from February to April with the onset of harvesting period. During this phase, the temperature was moderate, which was necessary for the growth of the fungi. A marked decrease in the total number of spores was seen during summer when the temperature was maximum and humidity was minimum. Even though the relative humidity was maximum, similar decrease continued during the rainy season due to the cleansing effect of rainfall on the atmosphere [30]. The second peak period was immediately after the rainy season, *i.e.,* from August to October, before the onset of winter. Considerable decrease was seen in the air incidence of fungal spores with the advancing winter during November to December. These two peaks together contributed 66.01% in 2000 and 65.46% in 2001. A small peak was also observed in the month of June because of strong winds carrying spores along with the sand (Fig. 3a).

The most prominent annual average fungal spore percentage for 2000-01 was credited to *Cladosporium* (24.95%), followed by *Alternaria* (23.02%), *Curvularia* (11.53%), *Mucor/Rhizopus* (5.48%) and Small round spores (5.47%). These five major taxa with high frequency of airborne fungal spores together account for 70.88% of the total in 2000 and 70.35% in 2001 (Fig. 3 b, c). Other frequently occurring fungal spores with annual average fungal spores percentage are *Drechslera* (2.96%), *Rosellinia* (2.85%), *Epicoccum* (2.26%), *Chaetomium* (2.00%), *Helminthosporium* (1.73%), *Pleospora* (1.50%), Smut spores (1.37%) and *Didymosphaeria* (1.17%). The spores of *Periconia*, *Torula*, *Herpotrichia*, *Basidiospores*, *Nigrospora*, *Pseudotorula*, *Stemphylium*, *Leptosphaeria*, *Sporormia*, *Pithomyces*, *Bispora*, *Botrytis*, *Fusarium, Didymella, Melanospora, Tetraploa, Phoma, Melanoconium* and *Tetracoccosporium* are very infrequently reported i.e., less than 1% (Table 2).

Fungal spores Class		2000		2001		2000-2001	
		Annual Conc. (Spores/sq.cm)	%	Annual Conc. (Spores/ sq. cm)	%	Annual Mean (Spores/sq.cm)	%
A. Identified							
I	**Zygomycotina**						
1	*Mucor/Rhizopus*	370	5.51	388	5.39	758	5.48
Total Zygomycotina		**370**	**5.51**	**388**	**5.39**	**758**	**5.48**
II	**Ascomycotina**						
1	*Rosellinia*	193	2.87	201	2.79	394	2.85
2	*Epicoccum*	165	2.46	148	2.06	313	2.26
3	*Chaetomium*	140	2.08	137	1.90	277	2.00
4	*Pleospora*	95	1.41	112	1.55	207	1.50
5	*Didymosphaeria*	59	0.88	103	1.43	162	1.17
6	*Herpotrichia*	45	0.67	69	0.96	114	0.82
7	*Pseudotorula*	29	0.43	41	0.57	70	0.51
8	*Stemphylium*	12	0.18	33	0.46	45	0.33
9	*Leptosphaeria*	9	0.13	27	0.38	36	0.26
10	*Sporormia*	17	0.25	16	0.22	33	0.24
11	*Botrytis*	-	-	17	0.24	17	0.12
12	*Didymella*	9	0.13	4	0.06	13	0.09
13	*Melanospora*	5	0.07	7	0.10	12	0.09
14	*Phoma*	3	0.04	3	0.04	6	0.04
Total Ascomycotina		**781**	**11.63**	**918**	**12.76**	**1699**	**12.28**
III	**Basidiomycotina**						
1	Small round spores	371	5.52	386	5.52	757	5.47
2	Smut spores	85	1.27	105	1.46	190	1.37
3	Basidiospores	52	0.77	46	0.64	98	0.71
Total Basidiomycotina		**508**	**7.56**	**537**	**7.62**	**1045**	**7.55**
IV	**Deuteromycotina**						
1	*Cladosporium*	1707	25.41	1744	24.69	3451	24.95
2	*Alternaria*	1568	23.34	1617	22.79	3185	23.02
3	*Curvularia*	745	11.09	850	11.96	1595	11.53
4	*Drechslera*	190	2.83	220	3.06	410	2.96
5	*Helminthosporium*	116	1.73	124	1.72	240	1.73
6	*Periconia*	57	0.85	71	0.99	128	0.93
7	*Torula*	88	1.31	33	0.46	121	0.87
8	*Nigrospora*	34	0.51	45	0.63	79	0.57
9	*Pithomyces*	13	0.19	10	0.15	23	0.17
10	*Bispora*	13	0.19	8	0.11	21	0.15
11	*Fusarium*	-	-	13	0.18	13	0.09
12	*Tetraploa*	4	0.06	4	0.06	8	0.06
13	*Melanoconium*	1	0.01	-	-	1	0.01
14	*Tetracoccosporium*	1	0.01	-	-	1	0.01
Total Deuteromycotina		**4537**	**67.55**	**4739**	**66.80**	**9276**	**67.05**
B. Unidentified		**521**	**7.76**	**535**	**7.43**	**1056**	**7.63**
Grand Total		**6717**	**100.00**	**7117**	**100.00**	**13834**	**100.00**



(Data labels are in percentage of total fungal spores' concentration)

Fig. 3: a) Monthly variations in total fungal spore concentrations; b) Variations in five major fungal spores and c) Annual mean relative contributions of five major fungal spores present in Bikaner, India over a period of two years

4. CONCLUSION

The present aerobiological investigation has given us an idea about the airborne pollen grains and fungal spores prevailing in the atmosphere of Bikaner-a city in Thar desert, India. A strong correlation has been observed between the meteorological conditions like temperature, humidity, precipitation, wind speed and the bioaerosol composition of the atmosphere. The highest aerosol concentrations were recorded during spring and autumn. The spring season is dominated by pollens from non-arboreal taxa. Pollens from arboreal plants were reported predominantly during autumn. In case of fungal spores, these two peaks correspond to sporulation of fungi, good vegetation, and suitable climatic conditions. Both summer and winter seasons are unfavourable for the incidence of pollen grains as well as fungal spores in the atmosphere of Bikaner. A small surge was observed in the airborne fungal spore concentration in the month of June because of strong winds carrying spores along with the sand.

Conflict of interest

None declared

Source of funding

None declared

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