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Study of seed-borne microflora of previously stored *Sorghum vulgare* seeds in Rajasthan, India

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ABSTRACT

An experiment was conducted at Rajasthan to determine the type of seed borne fungal pathogens associated with farmer-saved *sorghum* seeds. The *sorghum* seeds were obtained from *sorghum* farmers in five districts (Ajmer, Alwar, Jaipur, Jodhpur and Nagaur) of Rajasthan State. Isolation and identification of stored seed microflora by using blotter and agar plate method. Seven fungal species associated with five genera and two bacterial genera were isolated from the seed samples. Higher percent incidence of fungi was of *Aspergillus niger* followed by *A. flavus, Curvularia lunata, A. fumigatus, Penicillium chrysogenum, Fusarium oxysporum, Helminthosporium pennisetii* and bacteria *E. coli, Pseudomonas aeruginosa*. The most frequently isolated fungal species from seed samples each from blotter as well as agar plate method were *Aspergillus niger* followed by *A. flavus, A. fumigatus,* C*urvularia lunata, Penicillium chrysogenum, Helminthosporium pennisetii* and *Fusarium oxysporum*. It is clear from the survey that the yields of *Sorghum vulgare* are declining in the arid and semi-arid regions of the state. This is because, the naturally occurring seeds are not healthy (with reduced viability) and generally either infected with disease causing pathogen or such microflora which damage the seeds resulting into biodeterioration under storage conditions.

1) INTRODUCTION

Sorghum vulgare is the first most important crop grown in the world based on the area planted [1] The cultivation of grain had been used for consumption of both humans and livestock, and different genes of the plant serve also for many other important uses. It is cultivated for emergency purposes and widely consumed due to its ability to compensate for the nutrient deficiencies of rice such as the lack of vitamins and minerals [2,3] In many Countries, the functional products of millets and Sorghums have a great potential same 2\as therapeutic agents [4,5,6,7] shows the antimicrobial and Awika, and Kwak,[8] reported the anti-carcinogenic effects of sorghum, whereas millets have an anti-diabetic action by improving the cholesterol metabolism of the body [9,10]. Other economic importance of Sorghum grains is production of ethanol, starch, adhesives and manufacturing of paper other than being used as food and feed [11].

Near about 90% of the entire world's food crops are seeds oriented [12]. Seeds are regarded as highly effective means for transporting plant pathogens over long distances [13]. The growth and productivity of crop plants affected by seed borne diseases. A seed borne pathogen present externally or internally may cause seed abortion, seed rot, seed necrosis, reduction in germination capability as well as seedling damage, loss in weight, mustiness and biochemical alterations resulting in development of disease at later stages [14, 15].

Frowd reported that in sorghum (*Sorghum bicolor*), covered smut (*Sphacelotheca sorghi*), head smut (*Sphacelotheca reiliana*) and long smut (*Tolyposporium ehrenbergii*) are the most destructive pathogens and causing heavy losses in third world countries [16]. *Peronosclerospora sorghi*, the downey mildew pathogen of sorghum causing 30 to 70% losses in seed production in the semiarid tropics [17]58 to 70% yield loss of hybrid sorghum and millet with 60 to 76% ergot severity has been reported in the growing countries [18]. Besides, these losses in potential yield, mycotoxins produced by toxigenic fungi which grow on the seed substratum are hazardous to man and animals [19,20,21,22,23]. Commercially, discolored sorghum seeds caused by fungi are of poor quality [24,25,26,27, reducing their acceptability and thus, the market value of the produce. Grain mold causes crop loss by reducing seed size and weight, the food value and keeping quality of grains [28,29]. Several diseases that cause reduction in yields of sorghum have seed-borne phases. Seed borne inoculum therefore, has severe implications for yield, seed production and distribution systems, trade, human nutrition and germplasm. The management of these pathogens during the seed-borne phase is considered to be the cheapest disease control strategy [30]. However, details on the role of seed born fungi and their metabolites in the deterioration of seed quality and viability are meager. The effective management can only be implemented effectively if the pathogens are correctly identified. The importance of the current study aimed to detect seed borne pathogens on sorghum seeds. In present work isolation of microflora with recent techniques, from the seeds

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collected from different districts of Rajasthan state. Fungi were isolated from the naturally discolored, rotten, immature and shriveled seeds, collected from the previously stored seeds. Isolation of seed microflora using standard blotter method as well as agar plate method is studied.

2) MATERIALS AND METHODS

Experimental Information:

Table-1: A List of Previously Stored Seed Samples of *Sorghum vulgare* Collected During Survey from April-June 2003

S. No.	Sample No.	Place and Date of Collection		SP	Variety	SM
		Locality	District			
1.	Sv-1	Kishangarh	Ajmer	5	CSH-1	Jute
2.	Sv-2	Ajmer	Ajmer	4	CSH-4	Jute
3.	Sv-3	Thabda	Ajmer	4	CSH-5	Tin
4.	Sv-4	Mandore	Jodhpur	4	CSH-1	Jute
5.	Sv-5	Phalodi	Jodhpur	6	CSH-4	Jute
6.	Sv-6	Jodhpur	Jodhpur	8	CSH-5	Jute
7.	Sv-7	Somna	Nagaur	4	CSH-1	Cloth
8.	Sv-8	Tamau	Nagaur	4	CSH-4	Jute
9.	Sv-9	Nagaur	Nagaur	4	CSH-5	Jute
10.	Sv-10	Rainbara	Alwar	6	CSH-1	Jute
11.	Sv-11	Alwar	Alwar	7	CSH-4	Tin
12.	Sv-12	Chandoli	Alwar	7	CSH-5	Jute
13.	Sv-13	Dholai	Jaipur	4	CSH-1	Tin
14.	Sv-14	Sanganer	Jaipur	8	CSH-4	Jute
15.	Sv-15	Shahpura	Jaipur	6	CSH-5	Tin
16.	Sv-16	Jhotwara	Jaipur	8	CSH-6	Jute
17.	Sv-17	Amer	Jaipur	5	CSH-6	Polythene

(SP-storage period in months; SM- Storage medium)

*samples were preserved in polythene bags at laboratory temperature, for the isolation and detection of seed-borne fungi

Screening of Seed Microflora: Screening of seed-borne microflora from the given seed samples done by the procedure provided by Anonymous [31] and categorization is specified below

Table-2: Categorization of Seeds of Sorghum *vulgare* based on Dry Seed Examination

Categories	External Appearance of Seeds
A.	Seeds grey colored, healthy looking with shiny surface
B.	Seeds grey colored with holes and insect infection
C.	Seeds dull looking, seed coat with spotted black or covered with hyaline mycelium of fungus
D.	Seeds grey colored, small, dull looking, shriveled

Incubation Tests: Standard Blotter Method (SBM): 100 pretreated seeds (with 2% sodium hypochlorite for 5 minutes) were used in this test. Ten seeds were placed in a petri-dish of 9 cm. diameter, containing three discs of sterilized blotter paper which were well moistened with sterilized distilled water. These plates were then incubated at 28 ± 1°C temperature under 12 hours of alternating cycle of light and darkness for eight days. Seeds were examined for fungal and bacterial growth after eight days of incubation and identified with the help of standard monographs [32]

Agar Plate Method : Potato Dextrose Agar medium (200g peeled potato, dextrose 15g, agar 15g, distilled water 1000 ml, pH=7.0) was used in sterilized petri-dishes to study the colony characters of seed-borne microflora. Seeds were sterilized with 2 percent sodium hypochlorite solution for five minutes and ten seeds were aseptically placed in each petri-dish. Seeds were incubated under the same conditions as described under the blotter method. Data on percent occurrence of microflora and colony characters were recorded from 3rd day to the 8th day.

Percent incidence =Number of Seeds on which a fungal and Bacterial species appeared / Total number of Seeds examined X 100

Moisture Content of Seeds: Moisture content of seed samples was taken by oven dry method [33]. Firstly, weight of the empty petri-dishes with cover was taken in electric balance (W1). 5 grams of the previously weighed seed sample was taken in petri-dish and weighed (W2). The petri-dishes were placed in an oven, previously set at 130°C for 17 hours to dry the samples. After 17 hours, plates were cooled in desiccators for 30-45 minutes and weight was taken again (W3). The loss in weight represents the weight of water lost due to drying. The percent moisture content was calculated by the following formula.

Percent moisture content = (W_2-W_3) X 100 / $(W2 – W_1)$

W1 = Weight in grams of dish and its cover

W2 = Weight in grams of dish + cover + sample

W3 = Weight in grams of dish + cover + sample after drying

Viability of Seeds

Viability of seeds was measured by incubating two hundred seeds of each sample on moist blotter paper and agar plate at 28 ± 1°C for eight days. Germination percentage of seeds was calculated by the following formula.

Percentage of seed germination = Total No. of Seeds Germinated X100 / No. of Seeds examined

Purification of Fungal Cultures: Each fungus was purified using single spore culture technique. To raise pure culture of fungal pathogen, a suspension of spores was prepared by adding sterilized distilled water to the test tube containing seven days old sporulating culture. These tubes were then vigorously shaken by hand to dislodge spores from the mycelium. The suspension was then diluted hundred times with sterilized distilled water successively and 0.5 ml of it was poured over plain agar medium (agar 15g, distilled water 1 litre, autoclave it and add 0.2 g of streptomycin to check bacterial contamination) plated in petri-dishes. The petri-dishes were set aside for 12 hours to allow the spores to germinate, after which they were examined under stereo binocular microscope and single germinating spore was picked by needle and transferred to the potato dextrose agar slants. For all kinds of experimental studies, only single spore pure cultures were used.

Raising Pure Cultures of Bacteria: To raise pure cultures of bacterial isolates, a bacterial suspension was prepared in a tube containing sterilized water. The tube was vigorously shaken to prepare bacterial suspension. The suspension was diluted to 10-6 times with sterilized water and 0.5 ml of it was poured over Nutrient Agar plate (peptone 5g, sodium chloride 5g, beef extract 3g, agar 15g and distilled water 1 lit, pH = 7.0). The petri-dishes were incubated for twenty four hours at

37°C. After incubation, the bacterial forms grow and form characteristic colony morphology. The isolated colony was again subcultured to obtain a pure culture. The purified bacterial isolates were then subjected to various morphological, cultural and biochemical tests according to the methods cited in the "Hand Book of Microbiology"[34] and were identified up to generic and specific levels with the help of "Bergey's Manual of Determinative Bacteriology" [32]

3) RESULT AND DISCUSSION

On the basis of external symptoms and morphology, the seeds were divided into different categories *viz.*, healthy, insect damaged, fungus infected and shriveled, dull looking seeds (Table 2). Such external symptoms were considered important to categories the seeds on the basis of physical appearance as observed by [35] in Mung (*Phaseolus aureus*); [36] in rice, wheat, black gram, green gram and soyabean; [37] in chick pea (*Cicer arietinum*); [38] and [39] in *Albizzia lebbeck*.

Ajmer district while minimum seed germination (37.0%) was recorded from sample no. Sv-14 (8 months old seeds stored in jute bag) collected from Jaipur district. Samples collected from Jaipur and Jodhpur district showed more infection with fungal species (7) and bacterial species (2) followed by Nagaur (7, 2), Alwar (6, 3) and Ajmer (5, 2) (Table 3). The reduction in germination percentage of seeds stored in cloth bags and jute bags could be due to increased seed moisture content during storage when compared to impervious containers [40]. [41, 42] also stated that the super quality of moisture impervious containers over the ordinary one for successful carryover of seeds. The above observations have also been supported by the observations of [43] in Cereals [44] in seeds of *Dalbergia sissoo* in seeds of *Butea monosperma*; [45] in seeds of *Albizzia lebbeck, Dalbergia sissoo, Prosopis odoratissima* and *P. julifora*, [46] in seeds of *D. sissoo*. [47] reported reduction in germination of seeds of *Pisum sativum* by *Aspergillus* spp. (*A. flavus, A. candidus, A. ruber, A.*

Table 3: Percent Incidence of Seed- borne Microflora and Seed Germination of Previously Stored Seeds of *Sorghum vulgare*

Sample No.	Sv-1		Sv-2		Sv-3		Sv-4		Sv-5		Sv-6		Sv-7		Sv-8		Sv-9	
Method Employed	**B***	**A***	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**
A. Fungal flora																		
Aspergillus flavus	8.3	12.5	6.8	11.2	2.8	4.5	7.2	8.4	8.2	14.0	10.2	15.2	7.8	12	10.0	12.2	-	-
A. fumigates	2.4	6.4	-	-	-	-	4.2	9.4	4.2	4.5	2.3	3.5	1.5	5.2	1.0	2.5	1.0	2.2
A. niger	10.0	12.5	7.5	6.3	1.2	6.5	10.2	15.4	10.0	11.5	10.5	15.4	5.8	14.8	10.0	8.2	14	5.2
Curvularia lunata	2.1	6.3	2.4	5.5	-	-	-	-	-	-	8.2	12.0	-	-	4.2	7.6	2.7	2.4
Helminthosporium pennisetii	1.5	3.5	1.0	2.4	-	-	-	-	2.4	4.0	-	-	-	-	1.0	-	6.4	7.3
Fusarium oxysporum	-	-	-	-	-	-	-	1.5	-	-	-	-	-	2.0	-	-	-	5.4
Penicillium chrysogenum	-	-	-	-	-	-	-	3.0	-	-	3.6	8.0	-	2.2	1.0	2.2	-	-
B. Bacterial flora																		
Pseudomonas aeruginosa	5.0	6.5	2.0	3.4	1.0	1.0	2.4	4.6	3.0	6.2	1.5	5.0	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	3.6	5.5	7.5	9.0	2.8	3.5	1.6	2.8	2.0	4.8	3.2	7.6
% of Seed germination	66	67	75	78	89	88	69	68	65	68	48	44	68	76	67	74	67	68
Moisture content	11.94		12.62		11.54		11.73		11.52		12.98		11.25		11.29		11.56	

Sample No.	Sv-10		Sv-11		Sv-12		Sv-13		Sv-14		Sv-15		Sv-16		Sv-17	
Method Employed	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**
A. Fungal flora																
Aspergilkus flavus	5.4	7.6	-	2.4	14.0	14.5	8.4	10.4	12.5	8.6	9.2	7.2	7.5	15.0	6.2	7.5
A. fumigatus	-	-	-	-	4.0	6.5	3.0	5.5	2.0	4.2	-	-	1.0	2.5	2.4	6.2
A. niger	8.4	16.5	5.8	10.8	10.0	13.2	11.5	13.8	7.4	14.5	9.5	12.6	8.2	14.5	15.2	16.2
Curularia lunata	1.8	1.1	1.0	3.6	2.4	6.5	2.0	3.6	-	6.0	-	-	5.0	7.5	-	-
Helminthosporium pennisetii	2.0	4.5	1.0	3.4	4.2	4.0	-	-	1.0	4.0	-	-	-	-	-	-
Fusarium oxysporum	-	-	-	-	2.2	2.8	-	2.0	-	2.5	-	-	5.0	7.5	-	-
Penicillium chrysogenum	-	-	-	-	-	-	1.0	3.2	-	4.0	-	4.2	-	6.5	-	1.2
B. Bacterial flora																
Pseudomonas aeruginosa	3.1	7.6	4.2	8.2	3.2	8.0	1.0	3.2	2.5	4.2	1.5	2.0	2.0	3.5	1.8	2.4
Escherichia coli	3.0	8.0	2.3	4.8	2.5	5.6	3.2	5.8	7.8	9.3	1.2	4.4	1.0	5.4	2.0	4.4
% of Seed germination	63	64	78	75	58	60	77	75	37	40	77	78	52	58	68	65
Moisture content	11.84		7.00		12.17		11.03		12.82		7.04		12.63		7.12	

B* = Blotter Method, A* = Agar Plate Method

Maximum seed germination (89.0%) was recorded in sample no. Sv-3 (4 months old seeds stored in tin box) collected from

amstelodami and *A. ochraceous*) [48,49,50]. According to them, the reduction in germination was governed by four

factors: moisture content of stored seeds, period of storage, storage temperature and species and density of fungi involved.
Present investigation also revealed that as the storage period advanced, fungal incidence increased leading to reduced germination of seeds. Reduction in germination percentage may partly be due to extensive fungal growth and partly due to the toxic metabolites produced as observed by Harman [51]. The viability of the stored seeds tended to decrease progressively with longer storage periods due to predomination of storage fungi as reported by Tuite and Christensen [52.53] in seeds of barley.
A total number of seventeen seed samples collected, were previously stored (Table 1). Seven fungal species associated with five genera were isolated from the seed samples (Table 3).

have been isolated from the *Sorghum vulgare.* It is probable, that the cumulative effect of both bacteria and fungi are responsible for the degradation of seeds.
This may be due to that slow growing fungi and weak competitors could not grow. It has been observed that field fungi decreased along with increase in storage time. It is evident from observation that maximum fungi were recorded in rainy season and summer season and minimum fungi were recorded in winter season [54]. Similar observation was also recorded [55] while studied the succession of fungi on wheat and maize seeds during storage condition. It also has been recorded [56] in Wheat seed during storage [57]. Amongst the species *Aspergillus niger, Aspergillus flavus* and *Macrophomina phaseolina* were the most dominant. Decreasing in number of fungal species during storage has

Table 4: Fungal and Bacterial Flora Isolated from the Previously Stored and Deteriorating Seed Samples of *Sorghum vulgare*

S.No.	Microflora	Number of sample found positive	Maximum percent incidence	Sample number with highest percent incidence	District
A. Fungal flora					
1	**Aspergillus flavus**	16	15.2(A*)	Sv-6	Jodhpur
2	*A. fumigates*	12	9.4(A)	Sv-4	Jodhpur
3	*A. niger*	17	16.5(A)	Sv-10	Alwar
4	*Curularia lunata*	11	12.0 (A)	Sv-6	Jodhpur
5	*Helminthosporium pennisetii*	9	7.3(A)	Sv-9	Nagaur
6	*Fusarium oxysporum*	7	7.5(A)	Sv-16	Jaipur
7	*Penicillium chrysogenum*	9	8.0(A)	Sv-6	Jodhpur
B. Bacterial flora					
8	*Pseudomonas aeruginosa*	14	8.2(A)	Sv-11	Alwar
9	*Escherichia coli*	14	9.3(A)	Sv-14	Jaipur

Higher percent incidence was of *Aspergillus niger* (16.5%) followed by *A. flavus* (15.2%), *Curvularia lunata* (12%), *A. fumigatus* (9.4%), *Penicillium chrysogenum* (8%), *Fusarium oxysporum* (7.5%) and *Helminthosporium pennisetii* (7.3%) (Table4). The most frequently isolated fungal species from seed samples each from blotter as well as agar plate method were *Aspergillus niger* (17) followed by *A. flavus* (16), *A. fumigatus* (12), C*urvularia lunata* (11), *Penicillium chrysogenum* (9), *Helminthosporium pennisetii* (9) and *Fusarium oxysporum* (7). The number and percent incidence of fungi isolated are given in Table 3. Of these, highest number (7 fungal species) was isolated from sample no. Sv-14 (8 months old seeds stored in Jute bag) collected from Jaipur district and only two species were isolated from sample no. Sv-3 (4 months old seeds stored in tin box) collected from Ajmer district. Two bacterial genera viz., *Pseudomonas aeruginosa* and *Escherichia coli* were isolated from blotter as well as agar plate method. Highest percent incidence was of *E. coli* (9.3%) and *Pseudomonas aeruginosa* (8.2%) (**Table 4).**
Among these micro-organisms fungi play an important role in the degradation of seeds under storage. Bacteria are less instrumental in the degradation process. [53] stated that bacteria generally do not take part in the deterioration of stored seeds because they require free water to grow and seeds are seldom stored under such condition. In the present investigation *Pseudomonas aeruginosa* and *Escherichia coli* been reported [58,59]. Similar observations that aspergilli being principle storage fungi have also been made by Swaroop and Mathur, [60,61,62,63]The dominant fungi and fungal growth depend on period of storage and environmental conditions.

4) CONCLUSION

Seven fungal species and two bacterial genera were isolated from different varieties of *Sorghum vulgare* seeds. The study revealed the presence of diverse microflora of both pathogenic and non –pathogenic in *Sorghum vulgare* seeds in different district in Rajasthan state. To conclude, the findings suggest that there is a need for scientific storage of *Sorghum vulgare* seeds to minimize the fungal and bacterial infestation and their mycotoxin production in near future.

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