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Isolation of storage fungi from seeds of *Vigna radiata, Cajanus cajan* and *Lens culinaris,* evaluation of their biodegradation by *Aspergillus niger,* the dominant isolate and its serological detection

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Seeds of mungbean (*Vigna radiata*), cowpea (*Cajanus cajan*) and lentil (*Lens culinaris*) were stored for varying periods and under different conditions of temperature and humidity. The storage fungi from these seeds were isolated, frequency determined and identified. A large number of fungi were isolated and identified, but the most dominant one was found to be *Aspergillus niger*. Storage conditions also affected the frequency of fungi, with least frequency being obtained when stored at low temperature with low humidity. Protein and carbohydrate contents decreased when seeds were either stored or separately inoculated with *A.niger*. Proteinase and amylase activities also declined. Protein pattern analysis by SDS-PAGE rvealed the loss of several protein bands during storage. Polyclonal antibodies (PAbs) were raised against *A. niger* in white male rabbits and IgG was purified by ammonium sulphate precipitation and DEAE-cellulose chromatography. Initial optimization was done by agar double diffusion and PTA-ELISA formats. Antigens from seeds of healthy and stored pulses (cowpea, mungbean, lentil) collected from various sources were reacted with PAb of *A. niger* using PTA-ELISA format and also reacted on nitrocellulose paper following dot immuno-binding assays. In PTA-ELISA, the highest absorbance values at 405 nm were obtained in reaction of *A. niger* PAb with antigens from stored seeds of *V. radiata*, followed by *L. culinaris* and the least from *C. cajan*. Among seeds from different localities, there were variations in ELISA values. The PAb was further used to detect the fungus in stored seeds following indirect immuno-fluoresence and immune-cytochemical staining. Localization of the fungus was confirmed either on the seed coat surface or the parenchymatous cells just below the seed coat as evidenced by bright apple green fluorescence.

Keywords: *Aspergillus niger*,Biodeterioration, cowpea, immunodetection,lentil,mungbean

INTRODUCTION

A mature, fertilized ovule with an embryo, stored material in the form of cotyledons or endosperm, and a seed coat is referred to as a seed. Because seeds typically go through a number of stages after being harvested, whether for current or future usage, they are the easy targets of many external agencies. These include gathering, sizing or grading, drying, cleaning, transporting, and storage.

Internally and externally, seeds have pathogenic and saprophytic microorganims that degrade their quality, interfere with germination, and produce aberrant seedlings (Amza, 2018). Of all these procedures, storage has the greatest potential for damaging seeds. An essential component of the human diet is pulses. Pulse crops including lentil, mungbean, and cowpea are widely grown in India and utilized in soups and vegetables. These three economically significant pulses suffer significant degradation while in storage. Microorganisms cause seeds to deteriorate. Fungi are the most significant of the microbes responsible for the microbial degradation of seeds because they have the highest capacity to infect seeds. The mycoflora associated with black gram (*Vigna mungo*) seeds have been studied (Agarwal *et.al*. 2011, Biswal *et.al*. 2019 and Kandhare, 2020). Bakr and Rahaman (2001) reported a number of mycoflora such as *Alternaria* sp., *Aspergillus flavus*, *A. niger*, *Fusarium oxysporum* associated with one of the

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most important leguminous crop seeds of mung bean or green gram (*Vigna radiata*). Seed fungi reduced germinability of mung bean seeds and also affected seedling health with mortality percentage 8.6 to 37.6% (Kushwaha, 2021). Fungal pathogens and seed storage in the dry state were reviewed by Martin *et.al*. (2022) with special emphasis on types of seed-borne fungi, paths of infection and transmission, seed health methods, fungi longevity, risk of pathogen dissemination, the effect of fungi on seed longevity and procedures to reduce the harmful effects of fungi in gene banks. Predominant fungi isolated from different seeds of black gram, mung bean and chilli were analysed by Roy and Ali (2023). Fungi associated with seeds may be pathogenic, which on germination of seeds cause diseases systematically to the new plants.

Detection of pathogen in seeds is very important for quality control. Common practices are time consuming and may not be specific. Immunodetection of fungal pathogens (Chakraborty and Charaborty, 2002) is considered as an early step in developing management strategies which lads towards sustaining optimum yield (Chakraborty and Chakraborty, 2021). Immunodetection of specific fungi in seeds using the antibodies raised against them would be an important step for seed storage. In an earlier work, Chakraborty *et al*. (2008) have developed polyclonal antibody based immunodetection of *Aspergillus flavus,* isolated from stored seeds of pulse crops. In the present investigation, we have evaluated the biodegradation of seeds of mungbean (*Vigna radiata*), cowpea (*Cajanas cajan*) and lentil (*Lens culinaris*) by *Aspergiluus niger,* the dominant storage fungus isolated from these seeds and developed its immunodetection in stored seeds which would be of help in seed storage management.

MATERIALS AND METHODS

Seeds and storage

Freshly collected seeds of three pulses-mungbean (*Vigna radiata* (L), cowpea (*Cajanas cajan* (L.) Mill) and lentil (*Lens culinaris* Medik) were obtained from Pulses and Oils Seed Research Station, Berhampore, West Bengal. The following 2-3 varieties of each of these legumes were obtained: Mungbean – B1 and Pusa Baishakhi, Cowpea – ICPL-87, Rubi-20/105, and Lentil – Asha, Ranjan, and Subrata. These were then kept in the lab for different lengths of time and used for experiments. The seeds were kept in 3 different storage environments: ambient, where they were kept at room temperature and humidity, 30°C regulated humidity at room temperature and controlled humidity at low temperature.

Isolation and identification of fungi

Every three months, seeds were sampled, and on PDA medium, fungi were isolated from various stored seeds. On PDA, the isolated fungi were kept alive with frequent subculturings. On the basis of morphological and microscopic analyses, along with literature consultation, fungi were identified.

Testing of viability

The seeds of the various pulses were surface sterilized with 0.1% $Hgcl_2$, cleaned with sterile distilled water, and then soaked in sterile distilled water for an entire night. After that, seeds were removed and put on previously sterilized, moist blotting paper kept on 9 cm petri plates. After 4-5 days, the seeds were allowed to germinate, and the percentage of germination was recorded.

Determination of Percentage frequency

The number of times a specific fungal species appeared out of all observations was counted in order to determine the frequency of appearance of that species. The percentage of observations in which a species appeared relative to all observations was then used to calculate percentage frequency.

Inoculation with selected fungus

In investigations where seeds that had already germinated were used, the fungal species was inoculated by preparing a spore suspension and dipping seeds for 48 hours. Following this, seeds were stored in petri dishes and permitted to

germinate as previously indicated. The seeds used for the uninoculated control were steeped in sterile distilled water.

Extraction and estimation of biochemical component

Soluble protein

Following the technique described by Chakraborty *et al*. (1995), soluble proteins were isolated from healthy and treated seeds. Seed tissues (1g) were crushed in a mortar and pestle at 40°C with 0.05 M sodium phosphate buffer (pH 7.2), 10 mM $Na_2S_2O_5$, and 0.5 mM $MgCl_2$. The homogenate was centrifuged at 10,000 rpm for 20 min at 40^0 C . The supernatant after centrifugation was then used as crude protein extract, while the pellet was discarded. Lowry *et al.* (1951) method was used to estimate the amount of soluble protein, with bovine serum albumin (BSA) serving as the standard.

Carbohydrates

The method of Harborne (1973), which involved extraction in alcohol, was used to extract carbohydrates. Following Plummer's (1978) description of the Anthrone method, estimates of total sugar were made.

SDS-PAGE analysis of total soluble protein

For the thorough examination of the protein profile, sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out following the method described by Sambrook *et al.* (1989).

Extraction and assay of enzymes

Amylase

Weighing out 1 g of fresh tissue, 10 ml of ice-cold citrate buffer (25 mM, pH 5.0) was added to a mortar and pestle. The brei was cold centrifuged at 10,000 rpm for 10 minutes, and the supernatant was collected for examination of the activity that degrades starch. The assay was conducted using a residual starch estimate, and the activity was represented as mg starch hydrolyzed.

Protease

The procedure outlined by Jayaraman (1996) was used to extract protease from seed samples. With a mortar and pestle and sodium phosphate buffer (0.05M, pH 7.2), 1 g of seed was weighed and crushed. The homogenate was spun at 10,000 rpm for 10 minutes in a cooled centrifuge. The supernatant was gathered and used as a source of enzymes. The method recommended by Jayaraman (1996) was used to conduct the assay, which was based on the amount of protein that the enzyme was able to break down.

Preparation of antigens

Following the procedure outlined by Chakraborty *et al*. (1995), antigens were prepared from seeds and fungal mycelia. In order to do this, soluble proteins were extracted using a centrifuge and a sodium phosphate buffer (0.05 M, pH 7.2). The supernatant was then employed as an antigen source.

Raising of Polyclonal antibodies

Polyclonal antibody (PAb) against mycelial antigen of *A. niger* was raised using the techniques described by Alba and DeVay (1985) and Chakraborty *et al*. (1995). Normal sera from rabbits were taken before to vaccination. Each rabbit received intramuscular injections of 1 ml antigens (1 mg/ml protein) emulsified in equal volume of Freund's complete adjuvant (Difco) 7 days after pre-immunization bleeding. The doses were repeated at intervals of 7 days for 14 days, and then Freund's incomplete adjuvant (Difco) was administered at intervals of 7 days for as long as necessary, up to 70-98 days. By puncturing a vein in the outer ear, blood samples were obtained. Three days after the first six injections, the first bleeding was performed, and then seven more times every 14 days after that. The blood samples were stored at 37^0 C for 1 h to aid in coagulation and then stored overnight at 4^0C. Then the clot was slightly loosened with sterile needle and antiserum was taken in another sterile centrifuge tube and clarified by centrifugation at 2000 g for 10 min at room temp and finally antisera were stored at – 20^0C until required.

Purification of IgG

IgG was purified using Clausen's (1988) method of ion exchange chromatography on a DEAE cellulose column. The concentrtaion of IgG was determined using a standard formula after absorbance measurements were made for a few selected fractions at 280 nm and 260 nm (Jayaraman 1996).

Immunodiffusion

Using antigen and antiserum, agar gel double diffusion tests were performed in accordance with Ouchterlony's (1967) methodology. The slides were cleaned, stained with Coomassie blue (R-250, Sigma), and then destaining was performed after immunodiffusion.

Enzyme linked immunosorbant assay (ELISA)

The ELISA procedure was carried out in accordance with Chakraborty *et al*. (1996)'s method description. A total of thirteen different West Bengal and Sikkim locales, including Raiganj, Malda, Rohini, Siliguri, Matigara, Bagdogra, Jalpaiguri (North Bengal), Berhampore, Burdwan, Bidhannagar, Khidirpur, Behala (Kolkata), and Gangtok (Sikkim), provided the stored seeds for the three pulses. With the aid of coating buffer, seed and fungal antigens were diluted before being loaded (at a rate of 200 l/well) into a 96-well ELISA plate (costar EIA/RIA, strip plate USA) that was mounted in 12 rows on a cassette ELISA plate. Antigens were treated with rabbit IgG goat antiserum labeled with alkaline phosphatase after being reacted with by an A. flavus PAb, and then with substrate. At 405 nm, the absorbance was measured using an ELISA Reader (Labsystem, Multiskan). Values for absorbance in wells not coated with antigens were considered as blanks.

Dot immuno-binding assay

The dot blot technique described by Lange *et al*. (1989) was used. Antigens were placed in droplets onto nitrocellulose membrane (NCM; Millipore, H5SMO 5255, 7 x 10 cm, pore size -0.45 m Millipore corporation, Bedford), which was then treated with previously raised PAb (IgG) and enzyme conjugate. The intensity of the drips was used to categorize the development of colour.

Indirect immunofluorescence

Mycelia, spores, and seed sections were stained with fluorescent antibodies using the technique described by Chakraborty and Saha (1994). The slides were examined and photographed using a Leica Leitz Biomed microscope with fluorescence optics fitted with an I-3 ultraviolet filter after treatment with goat antiserum conjugated with fluorescein isothiocyanate (FITC) and specific for rabbit globulins.

Immunocytochemical Staining

For immunocytochemical staining, the procedure described by Young and Andrew (1990) was used. Thin sections of seeds were first treated with a fungus-specific PAb, washed, and then incubated for 40 min at 37^0C in the dark with a solution containing naphtol-As-phosphate and the fast blue BB substrate. The sections were cleaned in PBS, mounted in glycerol jelly, viewed using a bright field microscope (Leica Leitz Biomed), and captured on camera.

RESULTS

Isolation of seed storage fungi and their characterization

Twenty six unique fungi in all were isolated from three different seeds that were stored in varying conditions. Up to 9 months of storage, when a drop was noticed, the number of fungal isolates grew in all cases. The most fungal isolates were found in low temperature storage with low humidity, while the most were found in laboratory conditions with ambient temperature and humidity. The frequency of appearance was measured by counting the number of times a given fungal species appeared, and the findings are shown in Table 1 to help identify the fungi that appear during storage the most frequently. According to the findings, *Aspergillus niger* and *Aspergillus flavus* had maximum frequency in all cases followed by *Aspergillus fumigatus.* This was followed by others

species of *Aspergillus, Rhizopus, Penicillium* etc. The other storage fungi had frequencies varying from 5-40%

Seed Viability

Every seed was removed and its viability was examined. On the basis of 50 seeds in each sample, the percentage of seeds that germinated was computed. It was observed that viability was consistently preserved to a high degree, even after 18 months of storage at low temperatures and low moisture. When kept in standard storage conditions, *Cajanas cajan* lost its whole viability after 12 months. However, after 18 months of storage at low temperatures and humidity, 80–90% germination was still visible (Fig.1).

Table 1: Frequency of appearance of fungal species isolated from seeds

Fungal Species	% Frequency		
	C. cajan	*V. radiata*	*L. culinaris*
Aspergillus flavus	81	83	80
Aspergillus fumigatus	60	57	61
Aspergillus niger	90	87	89
Aspergillus terreus	40	32	41
Aspergillus variecolor	30	37	41
Aspergilus tamarii	25	20	24
Chaetomium sp.	17	00	12
Cladosporium sp.	15	18	20
Epicoccum sp.	12	17	00
Mammaria sp.	05	00	00
Trichothecim sp.	15	10	12
Bipolariscarbonum	09	07	10
Cochliobolus sativus	05	08	5
Colletotrichum gloeosprioides	05	02	2
Curvularialunata	10	07	05
Curvulariageniculatus	12	18	10
Phomaexigua	05	02	07
Alternaria solani	07	11	08
Penicillium sp.	58	55	63
Stachybotrys sp.	05	07	00
Rhizopus stoloniforme	46	48	39
Mucor mucata	37	41	45
Fusarium oxysporum	10	05	07
Fusarium solani	10	17	15
Macrophominaphaseolina	21	20	15
Alternaria alternata	10	17	13

% Frequency calculated on the basis of 25 observations.

Changes in biochemical components induced by storage fungi

Protein contents of both fresh and storage seeds as well as those *A. niger* inoculated seeds were determined. In all cases there was significant reduction in protein content (Fig 2) with the decreases being more than 50%, which was statistically highly significant. An overall decrease in total sugars following storage was also observed but it was not significant in all cases (Table 2).

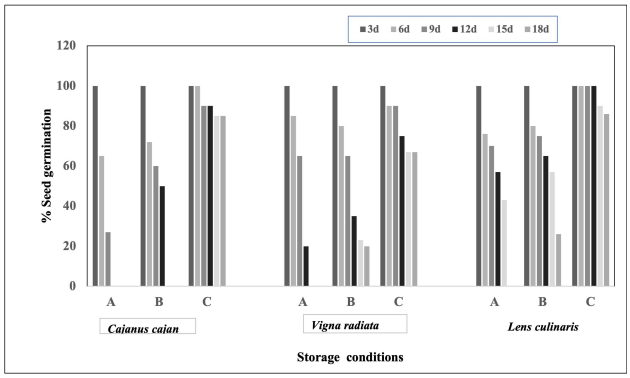

Fig.1: Germination of seeds of *Cajanus cajan, Vigna radiata and Lens culinaris* at different days and conditions of storage. A- Ambient: B- Low humidity; C- Low humidity + low temperature

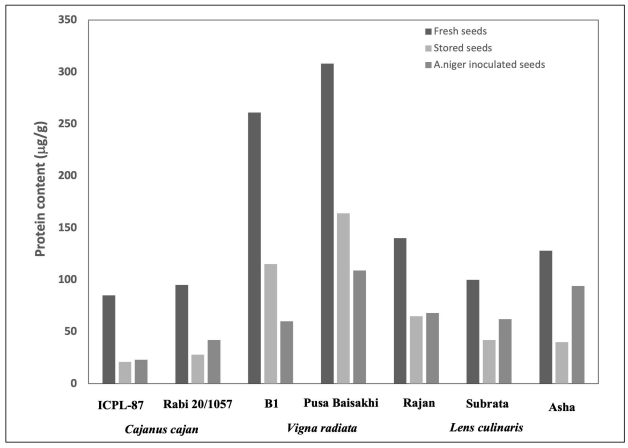

Fig.2: Protein content of fresh, stored and *A.niger* inoculated seeds of different varieties of *Cajanus cajan, Vigna radiata and Lens culinaris*

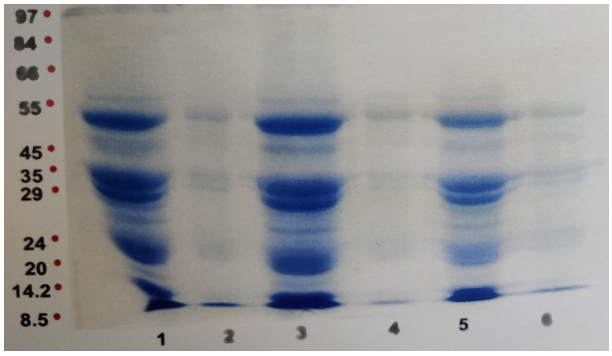

Fig. 3: SDS -PAGE analysis of seed proteins of 3 varieties of *Lens culinaris*. Lanes 1,3,5- fresh seeds; Lanes-2,4,6- stored seeds. Lanes 1&2- var. Asha; 3&4- var. Ranjan; Lanes 5 & 6 var. Subrata

Protein pattern

In order to determine the effect of storage fungi on protein patterns SDS- PAGE analysis was

Table 2: Total sugar content of fresh, stored and *A.niger* inoculated seeds of *C.cajan, V.radiata* and *L.culinaris*

Seeds	Variety	Protein content (mg / gm tissue)		
		Fresh seeds	Stored seeds**	*A.niger* inoculated seeds
Cajanas cajan	ICPL-87	42·5 ± 0·54	28.0 ± 0·23	40·8 ± 0·54
	Rabi 20/105	37·5 ± 0·54	30.0 ± 0·23	43.0 ± 0·72
Vigna radiata	B_1	56·7 ± 0·47	34·2 ± 0·47	41.0 ± 0·23
	Pusa Baisakhi	70·2 ± 0·72	30·1 ± 0·23	40.0 ± 0·54
Lens culinaris	Rajan	94.0 ± 1·18	64.0 ± 1·18	60.0 ± 0·72
	Subrata	72.0 ± 0·72	30.0 ± 0·54	38.0 ± 0·23
	Asha	94.0 ± 0·72	60.0 ± 0·23	51.0 ± 0·54

* Storage period –12 months, Results are Average of 3 replicates.
Difference in values between fresh and stored seeds significant in all cases in students 't' test at P = 0.01; ± = Standard error.

Table 3 : Protease activity of fresh , stored and *A. niger* inoculated seeds of *C. cajan, V. radiata* and *L. culinaris*

Seeds	Variety	Protease activity (? g protein hydrolyzed / gm tissues/ min.*		
		Fresh seeds	Stored seeds**	*A.niger* inoculated seeds
Cajanascajan	ICPL-87	125.0 ± 0·72	90.0 ± 0·5	120.0 ± 0·47
	Rabi 20/105	205.0 ± 0·72	120.0 ± 0·5	137.0 ± 0·47
Vigna radiata	B_1	180.0 ± 0·54	80.0 ± 0.23	137.0 ± 0·72
	Pusa Baisakhi	120.0 ± 0·54	100.0 ± 0·23	100.0 ± 0·72
Lens culinaris	Rajan	292.0 ± 0·47	180.0 ± 0·5	275.0 ± 0·5
	Subrata	278.0 ± 0·47	130.0 ± 0·5	230.0 ± 0·5
	Asha	250.0 ± 0·47	140.0 ± 0·5	230.0 ± 0·5

* Storage period –12 months; Results are Average of 3 replicates; ± = Standard error.

carried out with freshly harvested seeds, stored seeds as well as artificially inoculated seeds of three pulses. Results (Fig.3) revealed that protein bands decreased in number and intensity with storage in the seeds.

Enzyme changes associated with seed storage fungi

As seed storage fungi were found to cause bio deterioration affecting the biochemical components as well as germination of seeds it was decided to further investigate the effect of these fungi on two common hydrolytic enzymes i.e. protease and amylase. In both cases artificial inoculation of seeds with *A. niger* and *A. flavus* was done and allowed to germinate. Uninoculated seeds were also allowed to germinate. Protease and amylase activities were estimated from the germinated seedlings. It was observed that artificial inoculation did not lead to significant decreases in activity of both the enzymes (Table 3 and Fig.4).

Optimization of Pab

Soluble antigens were prepared from mycelia of *A. niger* and analysed on SDS-PAGE.(Fig. 5A) Polyclonal antibodies (PAbs) were raised against mycelia antigens of *A. niger* for detecting the presence of the fungus in seeds even in minute quantities. Initially effectiveness of antigen preparations from *A. niger* for raising PAbs were checked by homologous cross reactions following agar gel double diffusion test. Good precipitin arcs were observed (Fig. 5B). In case of ELISA, maximum absorbance values were obtained at 5th

Table 4 : Dot-blot analysis of antigens from *L. culinaris, V.radiata and C.cajan* from different sources reacted with PAb of *A. niger.*

	**Colour intensity of dots with antigens of		
Antigen source*	*V. radiata*	*C. cajan*	*L .culinaris*
1	+++	+++	+++
2	+++	+++	+++
3	+++	++	++
4	+++	+++	+++
5	+++	+++	+++
6	++	++	++
7	+++	+++	+++
8	+++	+++	+++
9	++	++	+++
10	++	++	++
11	+++	++	++
12	+++	++	++
13	+++	+++	++

*Antigen Source = Different locations of West Bengal & Sikkim 1 = Raiganj; 2= Malda; 3= Rohini; 4= Siliguri; 5= Matigara; 6= Bagdogra;7= Jalpaiguri; 8= Berhampore; 9= Burdwan; 10= Bidhannagar (Kolkata);11= Khidirpur (Kolkata); 12= Behala (Kolkata); 13= Gangtok
** + = Light pink; ++ = Dark pink; +++=Deep pink

Table 5 : ELISA reaction of antigens from seeds of pulses with PAb of *A. niger*

Antigen source*	A 405 nm with antigens of		
	V.radiata	*C.cajan*	*L. culinaris*
1	2.76	0.82	1.20
2	2.40	0.71	1.49
3	2.31	0.44	1.28
4	2.46	0.57	1.03
5	2.29	0.51	1.11
6	2.00	0.37	1.29
7	2.10	0.78	1.20
8	2.00	0.56	1.19
9	2.20	0.89	1.53
10	2.40	0.42	0.94
11	2.10	0.46	1.13
12	2.20	0.51	1.00
13	2.40	0.67	1.20

*Antigen Source = Different locations of West Bengal & Sikkim 1 = Raiganj; 2= Malda; 3= Rohini; 4= Siliguri; 5= Matigara; 6= Bagdogra;7= Jalpaiguri; 8= Berhampore; 9= Burdwan; 10= Bidhannagar (Kolkata);11= Khidirpur (Kolkata); 12= Behala (Kolkata); 13= Gangtok.A405 nm for homologous reaction was 3.30

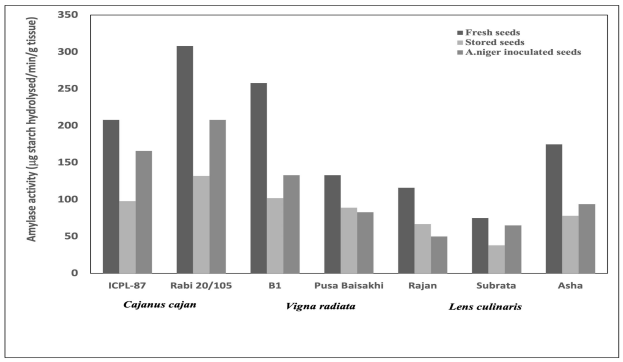

Fig. 4: Amylase activities of fresh, stored and *A.niger* inoculated seeds of different varieties of *Cajanus cajan, Vigna radiata and Lens culinaris*

bleed, with antigen and IgG concentrations of 40 mg/ml

Detection in seeds

In the present study detection of *A. niger* was carried out by two immuno-detection methods i.e. Dot immunobinding assay (DIBA) and plate trapped antigen coated (PTA) -ELISA. In both cases IgG obtained from PAbs raised against *A. niger* was used. Antigens were prepared from these seeds and tested against PAb of *A. niger* by Dot-Blot and ELISA. In dot blot, colour intensity of reaction was high when the seed antigens were reacted with PAb of *A. niger* (Table 4). Variations were also obtained in seeds from different localities. Differences in colour intensity of dots was an indication of lesser association of the particular fungus with the seeds from a locality. PTA-ELISA tests revealed the presence of *A. niger* in the stored seeds of all three pulses, could be detected and A_{405} values were in the range of 0.37-0.89 in seeds of *C. cajan*, 2.00- 2.76 in case of *V. radiata* and 0.94 - 1.54 in case of *L. culinaris* (Table 5). Among the three pulses, seeds of *V. radiata* showed high reactivity to the PAbs. Differences in ELISA values as well as intensity of colour of dots in dot-blots also varied with the locations. It is therefore possible to use these PAbs for the detection of Aspergilli in the seeds. Having detected the presence of *A. niger* in stored seeds by immuno-detection methods in the present study, it was decided to study the association of these fungi with the seeds internally.

Localization of fungal antigens in seed tissue

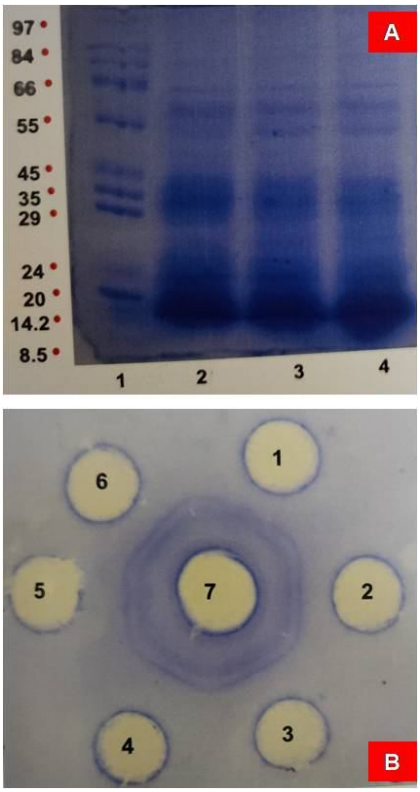

Fig.5 : A- SDS- PAGE analysis of *Aspergillus niger* proteins. Lane 1- Marker; Lanes-2,3 & 4- Antigens of *A.niger*. **B**- Agar-gel double diffusion test of antigens of *A.niger* with its homologous PAb. Peripheral wells (1-6) were loaded with antigens and central well with PAb.

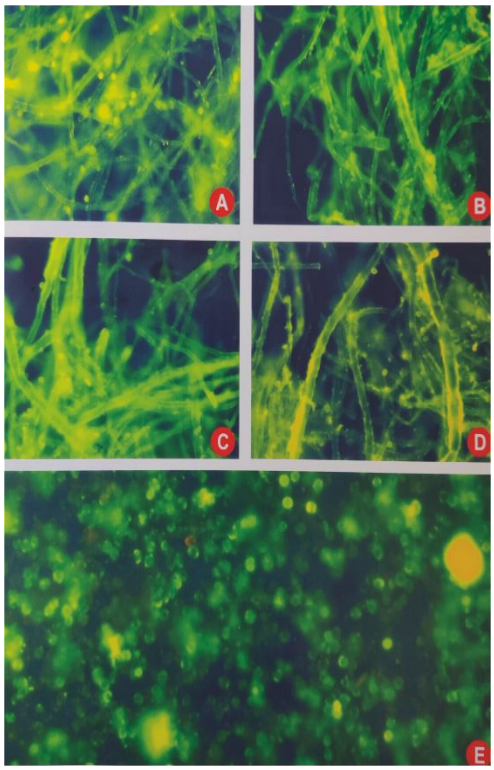

Fig. 6: Immunofluorescence **of** (A-D) hyphae and (E) conidia of *Aspergillus niger* treated with PAb of *A. niger* and reacted with FITC labelled antibodies of goat specific for rabbit globulin

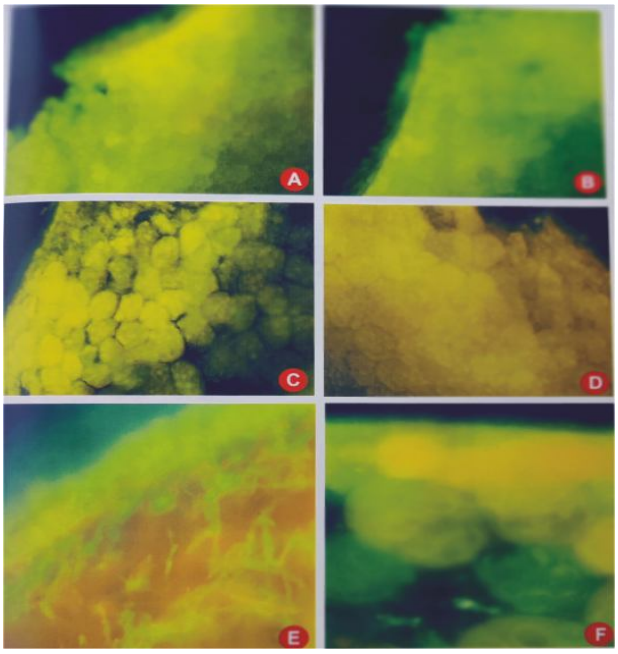

Fig. 7: (A-F): Fluorescent antibody staining of cross section of seeds (stored for 12 months) of various pulses. Seed tissues of (A&B) *Vigna radiate,* (C&D) *Lens culinaris* and (E&F) *Cajanus cajan* treated with PAb of *A.niger* and labelled with FITC conjugates

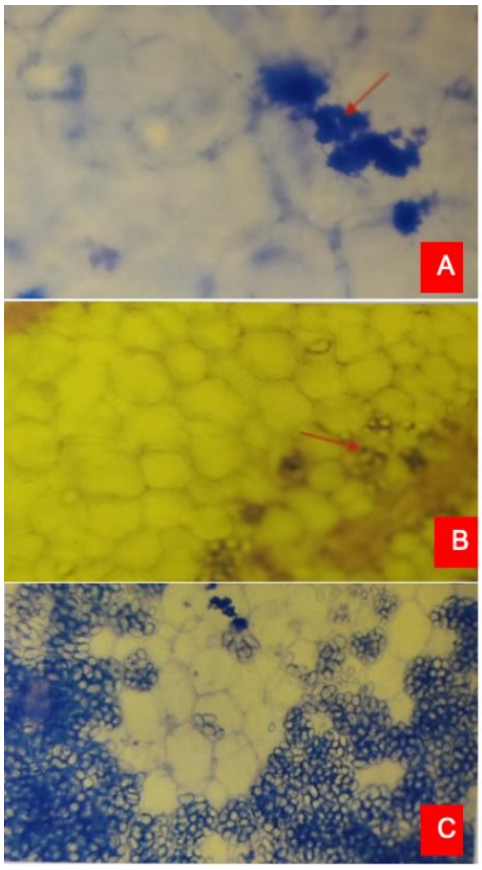

Fig. 8: Immunocytochemical staining of section of stored seeds treated with PAb of *A.niger*. A- *Cajanus cajan*; B- *Vign radiata*; C- *Lens culinaris*.

Indirect immuno-fluorescence staining and immunocytochemical staining techniques were used. The effectiveness of the PAb was tested by homologous reactions. Bright green fluorescence was observed when mycelia of *A. niger* were treated with homologous PAb followed by FITC labeling (Fig.6 A-D). Strong fluorescence was also detected when conidia of *A. niger* treated with homologus PAb and reacted with FITC labelled antibodies of goat specific for rabbit globulin (Fig. 6E). For detection of *A. niger* in the tissue, sections of the stored seeds were treated with the PAb of *A. niger* and reacted with FITC labeled conjugates of goat specific for rabbit globulin. Observations under the microscope revealed that most reactions occur either on the seed coat surface or in parenchymatous cells just below the seed coat. Clear attachment of *A. niger* on seed coat of *Vigna radiata* (Fig. 7A-B), *Lens culinaris* (Fig. 7 C-D) *Cajanus cajan* (Fig. 7 E-F) could also be visualized by immuno-fluorescence. Fungal mycelia could also be localized in tissues through another approach- immunocytochemical staining based on specific PAb produced against *A.niger* which provided a means of visualizing hyphae within seed tissues. Cross section of all the pulses were stained immunocytochemically as described under Materials and methods and observed under light microscope. Presence of *A. niger* within the seed tissues of *Cajanus cajan* (Fig.8A), *Vigna radiata* (Fig. 8B) and *Lens culinaris* (Fig. 8 C) was evident on the basis of deeply stained regions.

DISCUSSION

The management of seed quality in general and viability in particular is of extreme importance in order to obtain good quality seeds for plantation or for consumption During storage of seeds, conditions like temperature, moisture, durations of storage and the kind of seed being stored determine to what extent microbial deterioration occurs. In the present study, three common pulses grown in India i.e. pigeon pea (*Cajanas cajan*) lentil (*Lens culinaris*) and mungbean (*Vigna radiata*) were selected for seed storage studies. Seeds were stored for upto 18 months under three different conditions i.e. (i) ambient temperature and humidity (ii) ambient temperature and low humidity (iii) low temperature and low humidity. Isolation of fungi revealed that maximum number of fungal colonies appeared when seeds were stored under ambient conditions. Storage under low temperature and low humidity recorded minimum fungal colonies.Twenty six fungal species were isolated from the three seeds and these were characterized and identified. Species of *Aspergillus* predominated in all the seeds with other genera like *Penicillium, Alternaria, Fusarium* showing lesser frequency. Previous studies have also shown the predominance of different sp. of *Aspergillus* on several seeds (Kumar and Singh, 2004). Out of hundred and fifty-five tests, *A. niger, A. flavus, Fusarium oxysporum, F. solani, Penicillium chrysogenum* and several other species were also found associated with lentil seeds (Verma and Lahori, 2004). Though the authors reported the predominance of *Fusarium* speices in three cultivars (PD.L-2, L-4046 and L-4147), in the present study with three cultivers Asha, Ranjan & Subrata of lentil, *Aspergillus* sp. were found predominant. Among other pulses Bagri *et al.*(2004), isolated *Alternaria alternata,* different speices of *Aspergillus, Fusarium* species, *Macrophmina* sp. etc. from chick pea.

The most important environmental conditions affecting seed storage are temperature and moisture content. It has been reported that a moisture level below 13% is safe for most seeds. Seed moisture content fluctuates with the changes in relative humidity, which is again dependent on temperature. In the present study, it was observed that viability depended on a number of factors i.e. storage period, temperature and humidity as well as type of seeds. Among the three pulses tested *L. culinaris* was the most resistant to deterioration as viability was lost only after 18 months of storage under ambient conditions whereas in case of *C. cajan* it was lost after 12 months and in case of *V. radiata* after 15 months. Storage under low temperature and humidity increased germination percentage in all seeds. Patra *et al.* (2000) reported that in groundnut harvesting and drying as well as storage are critical operations and play a crucial role in determining the seed viability. They observed highest seed viability in polythene lined gunny bags containing calcium chloride after three

and six month of storage. After six month of storage the seed viability was as high as 80.3% in this storage method. However, viability decreased gradually with advancement of storage period and became nil after 9 months of storage. In soybean it was observed that at eleventh month of storage a significant decline in seed germination occurred (Gupta and Aneja, 2004). They observed that seed treatment with fungicide prolonged viability to about 15 months. Thus temperature and moisture of seeds during storage are determining factors of the quality of seeds at the end of storage period. Loss in protein content of soybean seeds following storage was also documented.

Since loss of viability of seed following storage is generally accompanied by changes in metabolism of seeds and hence of biochemical components, in the present study the effect of seed storage on two major seed components i.e. proteins and carbohydrates were determined initially. It was observed that after a period of storage of one year when analyses were done significant reduction in both protein and total sugar contents occurred in all cases. Results of present study are in confirmity with some of the earlier reports. In French bean (*Phaseolus vu*lgaris), loss in total sugar content and increase in protein and fat content due to seed borne fungi were reported by Paul (2002). However Gupta *et al.* (2004) reported that in *Albizzia lebbek* soluble proteins, phenols and soluble sugar increased gradually during storage while starch content decreased. Similar results were also reported by Kheroda-Devi *et al.* (2004) in rice grains during storage. Results of present study and that of previous workers taken together point to the fact that the nature of biochemical changes in seeds vary during storage. This may be due to differences in seeds themselves, condition of storage as well as the microorganisms causing deterioration. Decrease in the protein content of seeds during storage observed in the present study was also confirmed by SDS-PAGE analysis of protein pattern. Significant reduction in the number of protein bands was observed in all seeds both during storage as well as artificial inoculation. It has also been reported previously that nonviable seeds of vegetables, pulses, cereals and all seeds had lesser bands of functional proteins as well as iso-enzymes as compared to viable seeds.

Activities of amylase as well as protease in seedlings obtained from seeds artificially inoculated with *A.niger* or *A.flavus* also showed a decline which was, however, not consistent. Previous reports on the activities of enzymes in seeds during storage are contradictory. Enzymes such as amylase, phosphatase, peroxidase, catalase and total dehydrogenase activities on germination decreased in stored seeds of different crops. On the other hand Chakraborty and De (2007) reported increased amylase activity in pulse seeds affected by *A. flavus* and other storage fungi. Thus in some cases seed deterioration may be accompanied by stimulations of enzymes which enhance the ageing process, whereas in other cases overall deterioration of metabolic activity may include loss of enzyme activities. In an exhaustive review, Martin *et.al*. (2022), have considered some basic aspects of seed-borne fungi, such as the fungal types and pathways of infection and transmission, whch could help the understanding of the interactions and processes that occur between fungi and seeds. They have also provided an overview of seed health methods is also provided, from conventional techniques to the latest molecular analyses, as early detection is a key aspect for controlling problems caused by seed-borne pathogens.

Various formats of ELISA using polyclonal antisera have found a wide application in plant pathology and are routinely used for the detection of fungi in various plant tissues (Viswanathan *et al* 2000). Kumar *et al* (2000) used immunodot binding assay to detect karnal bunt of wheat. Kumar and Singh (2004) detected the location of *A. flavus* in ultra thin sections of pigeon pea seeds by using microtone. They also observed that thick hyphal mat was formed in the region of seed coat parenchyma. Detection of pathogen in host tissues using antibody based immunofluorescence technique has been reported by several previous workers (Chakraborty *et.al*., 2008; Chakraborty *et.al*., 2016; Bhagat and Chakraborty, 2020; Das Biswas and Chakraborty, 2020; Das *et.al.,* 2022). Results of the present study clearly demonstrate

that it is possible to determine the presence of stored fungi within seeds by specific immuno-assays using the antibodies of fungi to be tested. Such testing can be routinely adopted for detection of toxin producing or harmful fungi in the seeds.

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