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IDENTIFICATION AND ISOLATION OF SOME PROTEOLYTIC FUNGI FROM STORED PULSES SEEDS

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Abstract

The present investigation was undertaken to isolate, identify, and assess the frequency of proteolytic fungi associated with stored seeds of *Vigna radiata* (Green gram), *Cajanus cajan* (Pigeon pea), *Vigna mungo* (Black gram), and *Cicer arietinum* (Chickpea). Stored pulses are highly susceptible to fungal contamination, leading to qualitative and quantitative losses. Many storage fungi secrete proteolytic enzymes that degrade seed proteins, thereby reducing nutritional value and germination capacity.

Seed samples were collected from local storage facilities and markets. Fungi were isolated using the standard blotter and agar plate techniques on Potato Dextrose Agar (PDA). Identification was carried out based on macroscopic and microscopic characteristics using standard mycological keys. Proteolytic activity was screened using skim milk agar medium. A total of sixteen fungal species were recorded. Among these *Aspergillus flavus*, *A. niger*, and *Penicillium citrinum* were the most dominant species across all pulse seeds. The results indicate a high prevalence of proteolytic fungi in stored pulses, emphasizing the need for improved storage practices to minimize post-harvest losses.

Keywords: *Stored pulses, Proteolytic fungi, Seed mycoflora, Aspergillus, Protein degradation*

INTRODUCTION

The pulses are next to cereals as the most important source of food for humans and animals. (Rathod et. al. 2012). Pulses are the important sources of dietary carbohydrates, proteins and some micronutrients. Therefore pulses like Green gram, (*Vigna radiate*), Black gram (*Vigna mungo*),Chick pea(*Cicer ariatinum*) Pigeonpea (*Cajanas cajan*) are cultivated in most of the part of Maharashtra during kharif and rabbi seasons by different methods. Pulses constitute an essential component of the human diet, particularly in developing countries, due to their high protein content, minerals, and vitamins. In India, pulses such as *Vigna radiata*, *Vigna mungo*, *Cajanus cajan*, *Vigna unguiculata* and *Cicer arietinum* play a significant role in nutritional security. However, during storage, pulse seeds are prone to fungal infestation, resulting in deterioration, loss of viability, reduced nutritive value, and contamination with mycotoxins. Many of these fungi produce extracellular enzymes such as

proteases, amylases, and lipases, which degrade seed reserves. Proteolytic fungi are particularly harmful as they hydrolyze seed proteins into amino acids, adversely affecting seed quality and germination potential.Among different types of plant pathogens, seed borne fungi are one of the most crucial pathogen determining the quality of seed. Today when the world facing problem of malnutrition, protein rich sources are of having great importance.

The aim of the present study was to identify and isolate the proteolytic fungi of pulses seeds which can deteriorate the seed during storage.Various seed borne fungi produce proteolytic enzymes in variable quantity.The proteolytic enzymes cause degradation of protein content of the seed and reduce its protein content affecting the seed quality.Similarly Chary and Reddy (1982) found that, fungi isolated from infected Green gram seeds showed presence of proteases. Ivanov et al. (1989) studied loss in protein content due to seed bone fungi in Groundnut and Sunflower. Reduction in protein content due to *Fusarium oxysporum* and *Aspergillus flavus* was significant. Functional role of proteolytic enzyme includes hydrolysis of macromolecular substrates, initiation and maintenance of pathogens **(**Kutryavtseva et.al. 2008) Attack of *A flavus* and *Penicillium* citrinum caused cell wall degradation due to production of enzymes. (Oluyemisi et al. 2006).Earlier studies have reported the occurrence of proteolytic fungi in stored cereals and oilseeds, but comprehensive information on pulse seeds is limited. Therefore, the present study aims to isolate and identify proteolytic fungi associated with stored pulse seeds and to analyze their frequency of occurrence in different pulse varieties.

MALERIAL AND METHODS

Collection of seed samples –

Stored seed samples of *Vigna radiata, , Vigna mungo , Cajanus cajan*, and *Cicer arietinum* were collected from local markets, farmers' storage structures, and warehouses in the study area. Samples were collected in sterile polyethylene bags and brought to the laboratory for further analysis.The collection and testing of samples were done as per the guidelines of the international seed testing association (ISTA, 2019).

Isolation of seed borne fungi –

Blotter Method

Isolation of seed-borne fungi was carried out using the standard blotter method as recommended by the International Seed Testing Association (ISTA,1973, Mathur, S.B. and Kongsdal, O., 2003)). Sterile Petri plates were lined with three layers of sterilized blotting paper (Whatman No. 1) moistened with sterile distilled water. Excess moisture was removed to avoid water logging.

Seed samples were used in both surface sterilized and non-sterilized conditions. Surface sterilization was done using 1% sodium hypochlorite for 1–2 minutes, followed by three washings with sterile distilled water and air drying under aseptic conditions. Ten seeds were aseptically placed equidistantly on the moistened blotters in each Petri plate. Each treatment was maintained in three replications.

The plates were incubated at 25 ± 2°C for 7 days under alternating 12 h light and 12 h dark cycles to encourage fungal growth and sporulation. After incubation, fungal colonies emerging from seeds were examined under a stereomicroscope. Microscopic identification was carried out by preparing mounts in lactophenol cotton blue and observing morphological characters of mycelium and spores under a compound microscope. The fungi were identified using standard mycological keys.

Agar plate method

10 seeds were placed on petridish containing potato dextrose agar medium (PDA) by using sterilized forceps in laminar air flow. The inoculated plates were then incubated at 25 ± 2°C for 8 days under 12 hrs of alternating cycles of light and darkness. Thereafter on the subsequent day seeds were examined under microscope for the presence of fungi. The numbers of fungi isolated from different seeds were recorded in the table.

Identification of fungi –

Fungal colonies were identified based on colony morphology, pigmentation, sporulation, and microscopic characteristics using lactophenol cotton blue staining. Identification was carried out with the help of standard manuals, Monograph and available literature (Raper and Fennel 1965, and 1971) , Gilman (1957), Barnett and Hunter (1998), and Ellis (1971).

Percentage Frequency (PF) of fungal species

Fungal species found on the surface of seeds and frequency appearance of identified fungal species were recorded. Percentage Frequency (PF) of occurrence of fungal species was calculated by using following formula

$$PE = \frac{\text{Number of seeds on which fungi appeared}}{\text{Total number of seeds}} X\ 100$$

Screening for Proteolytic Activity

Proteolytic activity was tested using skim milk agar medium. Fungal isolates were inoculated on skim milk agar plates and incubated at 28 ± 2°C for 3–5 days. The appearance of a clear zone around the colony indicated proteolytic activity.

OBSERVATIONS AND RESULTS

Table 1 : Isolation of proteolytic fungi from pulses seeds by blotter and agar plate method

Sr. No.	Fungal species	Isolation of Fungi from pulses seeds by	
		Blotter Method	Agar Plate Method
1	*Aspergillus flavus*	+	+++
2	*A. fumigatus*	+	++
3	*A. niger*	++	+++
4	*A. terreus*	+	++
5	*Penicillium citrinum*	+	+++
6	*Rhizopus stolonifer*	+	+++
7	*Mucor mucedo*	+	++
8	*Fusarium oxysporum*	++	+
9	*F. moniliforme*	+	++
10	*Macrophomina phaseolina*	+	++
11	*Alternaria alternata*	++	++
12	*A. solani*	+	+
13	*Cladosporium* sp.	+	++
14	*Curvularia lunata*	+	++
15	*Drechslera tetramera*	+	+
16	*Chaetomium sp*	+	++

Table No.2: Frequency of fungal species isolated from pulses seeds.

It is evident from the results presented in table 1 that different types of fungi are found in stored pulses seeds. Before harvest fungi appearence is very least, but in majority of the storage food it has disastrous effects on not only seed but also on food and health of living beings which consume it. We know very well how contamination occurs through small quantities of spores contaminating the grain as it is going into storage from the harvest in handling and storage equipment or from spores already present in storage products (IRRI 2006).

Sr. No.	Fungal Species	% Frequency of fungal species			
		V.radiata	*C. cajan*	*V mungo*	*C arietinum*
1	*Aspergillus flavus*	72	70	71	65
2	*A. fumigatus*	50	55	-	40
3	*A. niger*	68	80	60	60
4	*A terreus*	22	30	-	10
5	*Cladosporium sp.*	12	10	-	15
6	*Curvularia lunata*	07	09	-	10
7	*Penicillium citrinum*	45	50	-	36
8	*Rhizopus stolonifer*	42	40	-	20
9	*Mucor muceda*	35	30	-	10
10	*Chaetomium sp.*	00	15	-	06
11	*Fusarium oxysporum*	05	07	50	13
12	*Fusarium moniliforme*	10	11	13	11
13	*Macrophomina phaseolina*	20	20	30	10
14	*Alternaria solani*	10	8	-	6
15	*Alternania alternata*	15	11	27	8
16	*Drechslera tetramera*	16	12	25	10

Aspergillus niger is commonly associated with all the pulses with a frequency of 3-48% since its distribution of the world wide. It is commonly found all over the world with the pulses. Alternania toxin have been detected infrequently in grains (Androus 1986,Champ et. al. 1991) Alternaria can cause discoloration of the grains by their abundant presence on the grain called black (sooty) heads and other spp also play a key role in spoilage of pulses by producing proteolytic enzymes, that show a clear impact on their quality or nutrient content.

Among all detected fungi *Asperigillus niger* and *A.flavus* were having drastic effect on pulses followed by *Drechslera tetramera* and *Rhizopus stolonifer* proteolytic enzymes produced by these fungi causes seed deterioration and are responsible for reduction in protein content of pulses seeds.

On the basis of present study it is clearly evident that some seed borne fungi in pulses produces proteases in valuable quantity during storage, which degrade the seeds by affecting its quality.

DISCUSSION

The present investigation clearly demonstrates that stored pulse seeds act as an excellent substrate for the growth of proteolytic fungi, particularly under tropical storage conditions. The predominance of *Aspergillus* species, especially *A. flavus* and *A. niger,* across all pulse samples corelates earlier findings

that these fungi are the most common storage contaminants due to their xerophilic nature and ability to grow at low moisture levels (Christensen and Kaufmann, 1969; Pitt and Hocking, 2009).

Proteolytic fungi play a significant role in seed deterioration by secreting extracellular proteases that hydrolyze storage proteins into simpler peptides and amino acids. This enzymatic degradation leads to loss of nutritional value, reduced seed vigor, and poor germination. The high frequency of *A. flavus* observed in *Vigna radiata* and *Cajanus cajan* is particularly alarming, as this species is not only strongly proteolytic but also toxigenic, capable of producing aflatoxins under favorable storage conditions.

The occurrence of *Penicillium citrinum* and *Rhizopus stolonifer* further indicates suboptimal storage practices, such as high relative humidity and inadequate aeration. These fungi are known to rapidly colonize damaged or improperly dried seeds and accelerate biochemical deterioration. Similar observations were reported by Neergaard (1977), who emphasized the role of storage fungi in enzymatic degradation of seed reserves.

The detection of *Fusarium oxysporum*, *F. moniliforme*, and *Macrophomina phaseolina* suggests that some of the fungal contamination may originate from the field and persist during storage. These fungi are moderately proteolytic and can survive in dormant form within seed tissues. Their presence supports the concept that seed-borne fungi represent a continuum from field to storage environment (Agarwal and Sinclair, 1997).

Differences in fungal diversity among pulse species may be attributed to variations in seed coat thickness, chemical composition, and moisture absorption capacity.*Cajanus cajan* exhibited the highest fungal diversity and infestation, indicating its greater susceptibility during storage. In contrast, *Vigna mungo* showed comparatively lower fungal diversity, possibly due to inherent antifungal compounds or structural barriers in the seed coat.

The use of both blotter and agar plate methods proved effective for the isolation of seed mycoflora. The agar plate method was superior for fast-growing fungal species such as *Aspergillus niger, Penicillium citrinum*, and *Rhizopus stolonifer*, whereas the blotter method facilitated better detection of slow-growing and internally seed-borne fungi like *Fusarium moniliforme, Alternaria alternata* , and *Drechslera tetramera*. These findings are in agreement with ISTA recommendations and earlier reports by Ellis (1971).

Overall, the study highlights the significant role of proteolytic fungi in post-harvest losses of pulses and underscores the necessity of adopting improved storage technologies, including proper drying, moisture control, and periodic monitoring of seed health.

CONCLUSION

The study confirms that stored pulse seeds are highly susceptible to colonization by proteolytic fungi, predominantly species of *Aspergillus*, *Penicillium*, and *Fusarium*. *Cajanus cajan* showed maximum fungal diversity and infestation. The prevalence of proteolytic fungi highlights the need for improved storage conditions, proper drying, and regular monitoring to prevent seed deterioration and economic losses.

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