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. Seeds storage is not just placing seeds in containers, but what is more important is how the seed and its internal biological-physiological, biochemical processes function and interact with its surrounding environment. Actually, seed storage starts right in the field because once seeds reach physiological maturity they do not receive the full protection of the mother plant any more. Rather, starting in that physiological point the seed depends on the external environment in terms of moisture, temperature and even biotic pressure. Hence prestorage factors also influence seed quality.

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. Sampling was done at regular intervals of three months. 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 (Dwivedi and Dubey 1992, Kumar & Singh, 2004). Lokesh *et al.,* (1987) isolated nine fungi

from *pigeon pea* of which *A. niger* and *A. flavus* were predominant. Kumar & Singh (2004) detected *A. flavus* in sixty six samples. 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). They reported that a considerable number of seeds were found carrying the fungi externally with *Fusarium* sp. dominating. Externally seed borne nature of these fungi were also previously reported by various workers (Prasad and Choudhury, 1987; Khare 1996; Ram *et al.,* 1997. Though Verma and Lahori (2004) 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. Ahmed and Reddy (1995) also reported *F. solani* and *M. phaseolina* from the seed of chick pea. In another study Rathour and Paul (2004) recorded thrity species belonging to fifteen genera from pea seeds. Most encountered fungi were Alternaria *tenuissima, A. terreus, Ascochyta* sp. and *Asperigillus niger.*

It was observed that a number of fungal isolates in all three seeds increased upto nine month of storage after which there was a decline. Adebajo and Popoola (2003) reported that n Cola nuts at collection time only about 2% of the nuts had visible mould infection which after three, six, and nine months increased to approximately 15, 39, and 88% respectively. Among the storage fungi *Aspergillus* sp. dominated followed by *Penicillium* sp. In this case also *A. niger* and *A. flavus* were predominant.

The most important environmental condition 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. Thus temperature and moisture interact to determine storage risk. At low temperatures, seeds can be stored for longer period. However, *Aspergillus*

and *Penicillium* can grow even at low moisture content. Within the range 12-18% loss of viability of seeds is one of the major problems encounter during storage. However, this can be overcome to a great extent if stored under low temperature and humidity. 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 ground nut 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. They suggested that initially polythene lining in gunny bags checked the flow of moisture from outside atmosphere and moisture from inside the seed environment was absorbed by anhydrous calcium chloride. Padma and Reddy (2004) suggested that seeds of Okra could be stored for upto 26 months in cloth bags, 32 months in polypouch and 50 months in polythene bags and aluminium foil pouch without losing viability. In soybean it was observed that at eleventh month of storage a significant decline in seed germination occurred (Gupta & 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.

In the temperature range of 5-10^{0}C storage moulds grow very slowly while at 27-44^{0}C growth is very rapid. Seeds that are to be stored for only a few weeks before processing may contain higher moisture content, have

more extensive invasion by storage moulds and be kept at a higher temperature without serious problems, than can seeds stored for longer periods. However seeds stored for only a few weeks under any combination of moisture content and temperature that permits even moderate invasion by storage fungi will be at high risk if kept in continued storage (Malvick 2002). Seeds moderately invaded by storage fungi or moulds develops damage at lower combinations of moisture content and temperature and in a shorter time than seed free or almost free of storage fungi. Once storage moulds become established they continue to develop at moisture and temperature level below those required for the initial invasion of healthy seeds. The damage caused by fungi growing in stored seed is the end product of storage condition. Storage moulds work like a "bucket-brigade". Each fungus is active within rather narrow limits. When those limits are reached, another fungus or other fungi begin to colonize the seed quickly resulting in succession of organisms colonizing the seeds.

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. This was further confirmed by analyzing seeds artificially inoculated with *A. niger* and *A. flavus*. In this case also significant reduction was obtained. Results of present study are in confirmity with some of the earlier reports. In a study on rice, Puroshotham *et al.* (1996) observed that while total carbohydrate content of uninoculated seeds remain unchanged during storage, a decrease was observed upto 10 days in the seed treated with different fungi. In their study greatest net loss of carbohydrate was recorded in *A. flavus* treated seeds after 30 days of incubation and lowest loss with *A. glaucus* and *A. versicolor*. They suggested that rapid loss of carbohydrate may be due to their utilization in respiration for energy requirement. In the

present study, *A. flavus* caused greater loss of carbohydrate in *C. cajan* in comparison to *A. niger.* However, in case of protein, in *C. cajan, A. niger* caused greater reduction. Therefore same storage fungi may cause deterioration of seed components differently which again depends on the types of seeds. In French bean (*Phaseolus vul*garis) loss in total sugar content and increase in protein and fat content due to seed borne fungi were reported by Paul (2002). However, *Alternaria alternata* caused a decrease in protein content. All the fungi were shown to decrease sugar content by more than 40%. In studies conducted with 3 seeds-maize, groundnut and soybean, Bhattacharya and Raha (2002) reported a gradual loss of both soluble and insoluble carbohydrate as well as protein. Oil contents also decreased in prolonged storage with simultaneously increase in fatty acid.Storage fungi were also shown to decrease the carbohydrate content of bread fruit (*Artocarpus commis*) during storage. In this study fat content was also observed to decrease during storage while crude fiber, crude protein and ash content of the fruit (Amusa *et al.* 2002). They opined that the decrease in carbohydrate content of breadfruit stored at room temperature might be due to fermentation caused by microbes and the respiratory loss of sugars as carbon-di-oxide. Loss in protein content of soybean seeds following storage was also reported by Gupta and Aneja (2004). 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 micro organisms 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. (Malhotra, 1990, Saxena *et al.* 1992).

Activities of amylase as well as protease also showed a declining trend during storage. 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 seed of barley, pea and Sesame (Pakeeraiah 1985). Similar findings were reported in other crops (Yadav, 1990; Srivastava, 1990; Paul, 1990; Arya, 1990). On the other hand Puroshottam *et al.* (1996) reported increased amylase activity in rice seedling caused 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.

Detection of storage fungi in seeds by conventional techniques like blotter method, agar plate method etc. is time consuming and lengthy. Recent techniques which involve either immuno detection or DNA based detection are much more sensitive and specific for detection of specific fungi in stored seeds and other plant tissues. These techniques make the detection possible even when the fungal concentration in the tissue is very low. The techniques generally used are ELISA. Dot-blot or PCR. Keeping this in mind in the present work polyclonal antibodies (PAbs) were raised against the two selected fungi and these PAbs were used in detecting the fungi in stored seeds obtained from various localities. Detection was done by ELISA and dot immuno binding assay. Initially the PAbs which were raised in rabbits were checked by Agar gel double diffusion, ELISA and dot-blot in homologous reactions to determine the optimum parameters. In ELISA very low concentration of antigen could be detected. ELISA reaction showed both PAbs to have very high titer of which *A. flavus* gave higher readings. However when seeds of all three pulses obtained from 13 localities were tested against

PAbs of *A. niger* and *A. flavus*, much higher reading were obtained with PAb of *A. niger.* These results were also confirmed by Dot-blot. This indicated the possibility that *A. niger* was more predominant in the seeds than *A. flavus.* Among the three pulses *V. radiata* showed most 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 detection of fungi in the seeds. Various formats of ELISA using polyclonal antisera have found wide spread application in plant pathology and are routinely used for detection of fungi in various plant tissues. (Sundaram *et al.* 1991, Lyons and Wite 1992, Chakraborty *et al.* 1996, and Viswanathan *et al.* 2000). Miles *et al.* (1998) detected endophytic fungus in the intercellular spaces of the leaves and seeds of *Echinopogon ovatus* using immuno blotting and ELISA techniques. Detection of *Plasmopora halstedii* in seeds of sunflower was done by the use of monoclonal antibodies raised against a fungus (Bouterige *et al.* 2000). ELISA is also routinely used in seed testing laboratories. Having detected the presence of *A. niger* and *A. flavus* in stored seeds both by conventional and immunodetection methods it was decided to study the association of these fungi with the seeds internally. For this fluorescence staining and immunocytochemical staining techniques were used. For these immunoasays, sections of the stored seeds were treated with the antibodies and suitably stained. Observations under the microscope revealed that maximum reactions occur either on the seed coat surface or in the parenchymatous cells just below the seed coat. Detection of pathogens in host tissues using antibody based immunoflorescent technique has been reported by several previous workers (Dewey *et al* 1989; Watabe 1990) Kumar & Singh (2004) detected the location of *A. flavus* in pigeon pea seeds by microtone sectioning. They also observed that thick hyphal mat was formed in the region of seed coat parenchyma.

In conclusion it can be stated that though a number of fungal isolates were obtained from the different varieties of the three pulses tested *A. niger* and *A. flavus* were found to be predominant. Further studies have brought out

the role of temperature, moisture and storage period in determining the association of storage fungi with the seeds. Attempts have also been made to determine seed viability and bio-deterioration of seeds during storage. Finally, modern immuno diagnostic techniques have been used to detect the presence of fungi in the stored seeds. All the results taken together can throw more light on the bio-deterioration of seeds by storage fungi and the importance of the storage conditions in keeping seeds healthier for longer period.