Studies on bio-degradation of pulses by storage fungi

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This is to certify that Ms Paramita De has carried her research work under my supervision. Her thesis entitled " **Studies on bio-degradation of pulses by storage fungi**", is based on her original work and is being submitted for the award of Doctor of Philosophy (Science) degree in Botany in accordance with the rules and regulations of the University of North Bengal.

Usha Chakraborty

(Usha Chakraborty)

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Paramita De.
(Paramita De)

Contents

INTRODUCTION

Cultivation of food grains for consumption by man started in the Neolithic period of the Stone Age (about 8000 B.C.). Storage has been a necessity ever since man started cultivation, as the produce is seasonal, but its consumption is spread throughout the year (Mehrotra 1992). The importance of seed storage however, goes much beyond consumption, because good seeds are the basis of any agricultural programme. There is thus, no doubt that seed storage is an important aspect of any sound seed programme. Stored seeds will not be of much help if on planting, they do not yield healthy and vigorous plants (Saxena *et al.*, 1992). About 90% of world food crops are grown and propagated by seeds. The seed quality is greatly affected by the prevailing environmental conditions, as well as the time the seeds reach physiological maturity from harvest.

Seed storage is greatly influenced by the temperature and relative humidity. In a tropical country like India, the problem of storage is aggravated by weather. Conditions of both high temperature and humidity, with tremendous increase in production of grains over the years, and the increasing need to feed the ever increasing population of man and cattle, storage of grains has to be on a larger scale than ever before. Often a substantial amount of produce is lost between harvest and consumption, essentially in countries where proper storage facilities do not exist.

Deterioration of seeds is brought about by microorganisms. Among the microorganisms responsible for microbial deterioration of seeds, fungi are, by far, the most important, as they possess the greatest seed infecting ability (Mehrotra, 1992). Fungi associated with seeds may be the pathogenic ones which on germination transmit the diseases systemically to the new plants. A majority of fungi associated with seeds, on the other hand, are those from the atmosphere which colonize the seeds during storage. These do not cause plant diseases, but deterioration of seeds. Some of these also secrete toxins, which are harmful, if consumed.

Pulses are consumed in India, next only to cereals. Being protein rich, these form the diet of a majority of Indians, who are pre-dominantly vegetarians. Some of the major pulses grown in India are mung bean, pigeon pea, pea, lentil, black gram etc. *Cajanus cajan* (L.) Millsp., commonly called as pigeon pea, is one of the most common pulses cultivated in India. It probably originated in India, but may have come from Africa. It is clear that the species has been under cultivation for a long time and was spread by traders, thousands of years ago. Today, pigeon pea is cultivated throughout the tropics and has naturalized in many other regions. Pigeon pea (Arahar) is an important food in developing tropical countries, including India. Being an excellent source of protein, the seeds are consumed in several forms. Among world cultivation about 88% is cultivated in India. *Lens culinaris* Medik, commonly known as lentil is one of the oldest known pulses. Intensively cultivated from the earliest days of civilization, lentils have been found in Egyptian tombs dating back to 2200 B.C. though their cultivation goes back atleast 8000 years. Lentils are also cultivated widely in India, and the seeds are consumed in several preparations. Among the legumes, lentils are the richest in protein, iron and vitamin B_2, and lowest in fat. Mung bean, *Vigna radiata* (L.) Wilczek is also one of the most important pulse crops. It is grown in almost all parts of the country. It is an excellent source of high quality protein. It is consumed either sprouted, or cooked in various ways. Ascorbic acid (Vit. C) is synthesized in sprouted seeds of mung bean with increment in riboflavin and thiamin. The centre of origin of mung bean is believed to be India. It is grown throughout the southern Asia including India, Pakisthan, Bangladesh, Sri Lanka, Thailand, China etc. and has extended to parts of Africa, USA and Australia.

It is quite natural that all the above pulses are stored extensively, either to be used for cultivation in the next season, or to be consumed as food (Plate I). Storage of seeds are known to cause bio-deterioration due to the association of fungi, and hence, the present study was undertaken to determine the extent of association of storage fungi with the three pulses and

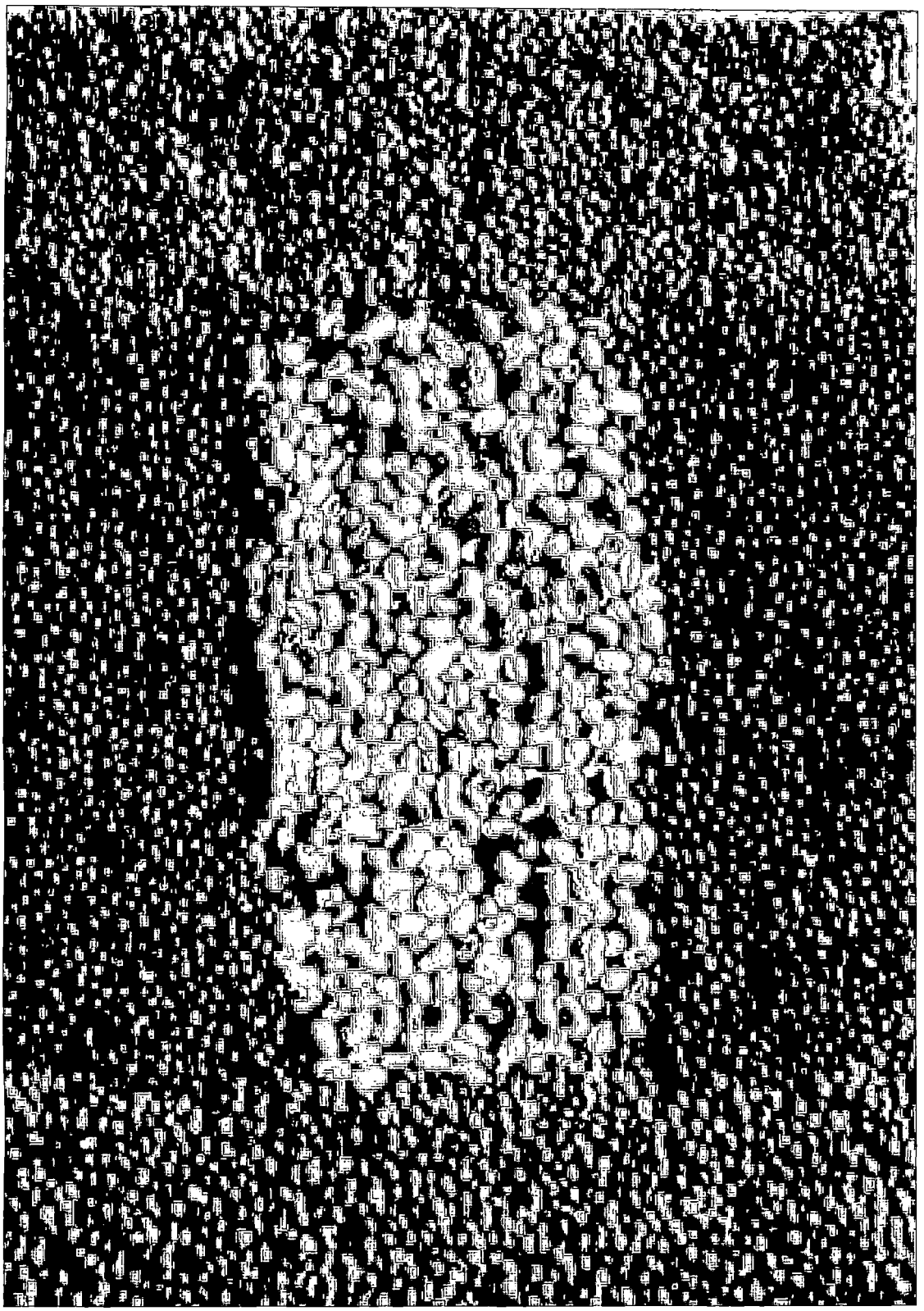

Plate-I: Seeds of pulses

the bio-deterioration caused by these. The main objectives of the present investigation has been:

(i) To isolate the fungi associated with stored seeds of different pulses- Mung bean (*Vigna radiata*), Arhar (*Cajanas cajan*) and Lentil (*Lens culinaris*), (ii) To identify the fungi and select a few most commonly occurring ones from each type, (iii) To determine the effect of specific fungi an seed germination and seedling growth, (iv) To determine changes in protein contents and carbohydrates in the seeds following seed treatment with the fungi, (v) Determination of changes in enzyme activities of seeds and seedlings due to storage fungi, (vi) SDS-PAGE analysis of proteins to determine changes in protein pattern, (vii) Preparation of antigens of fungi and raising of antisera against one selected fungus each from each seed, (viii) Detection of the fungus in the seeds by DAC ELISA using the antisera.

REVIEW OF LITERATURE

Seeds are the basic inputs for production. Most of the world food crops are sown and propagated by seeds. Besides, seeds also form important constituents of human diet. These necessitate that seeds harvested from the field should be stored for varying periods until use. During storage, seeds are subjected to deterioration, most of it microbial, which is enhanced by conditions of storage. Since majority of microbes causing deterioration are fungi, it is enhanced by environmental conditions, such as light, high temperature and moisture. Fungal association with the seed may be either during post harvest or diseases may be transmitted through the seeds. Whatever be the nature of fungi associated with seeds, a general deterioration in quality is observed. Storage fungi are known to affect the germination potential of the seeds; cause discoloration of its parts, produce heat, mustiness and a general decline in metabolic activity and even total decay of the grains.

Considering the importance of proper seed storage, several studies have been conducted world wide to determine the types of mycoflora associated with stored seeds and deterioration caused by these. Studies on seed deterioration and loss of viability have been reviewed by many previous workers (Saxena & Pakeeraiah 1986; Saxena *et al.* 1992; Malvick 2002).

The present review has been carried out on mycoflora of various types of seeds. In keeping with the line of work review has been subdivided into two categories i.e. mycoflora of pulses and mycoflora of other seeds.

2.1. Pulses and legumes

Seeds of *Vigna mungo* collected from three localities around Agra and maintained at 30, 60 and 90 per cent pH and room conditions were tested by blotter paper and agar plate methods for the occurrence of seed borne fungi by Sharma and Roy (1991). Only 4 fungi were found to be pathogenic in soil test.

Differential). liberation of reducing sugars, proteins and polyphenols were evident from the seed coat leachates of five varieties of pigeon pea (*Cajanus cajan* (L.) Millsp. The variety GC-11-6 (white seeded) liberated considerably less amount of reducing sugars, proteins and polyphenols into the leachates when compared to GC-11-39, GC-11-54-2, GPC-7643 and ICP_2-87 (reddish brown seeded varieties). The antifungal properties of these seed coat leachates were tested again. Eight fungi including seed borne pathogens were tested and the results correlated with their resistances / susceptibility. Germination of seed borne pathogenic fungi when placed in seed leachates of resistant cultivars was drastically suppressed. (Reddy *et. al.,* 1994).

Simay-El (1994) reported that when 442 seed samples of Faba bean were tested by incubation on a moist blotter with or without surface sterilization, 69 taxa of fungi were identified. Rates of occurrence were generally higher on seeds tested without sterilization. Predominant potential seed pathogens included *Alternaria* spp., *Fusarium* spp. and *Trichothecium roseum. Ascochyta fabae, A.* [*Mycosphaerella*] *pinoides. Botrytis cinerea* and *B. fabae* were also observed on several seed samples. Rates of occurrence were correlated with total infection, except for *Ascochyta.* Some storage fungi were also observed.

Pereira *et. al* (1994) investigated the occurrence of storage fungi in soybean in the state of Minas Gerais, Brazil, and the implications for reduced seed quality were studied. Seeds were incubated on salt agar and absorbent paper, and *Aspergillus candidus, A. flavus, A. glaucus, A. niger, A. ochraceus, A. restrictus* and *Penicillium* spp. were identified. Samples from seeds produced in the Traingulo Mineiro region, where the majority had a standard germination rate of >75% were the most infected. As the correlation coefficient between percentage germination and fungal occurrence was always <0.5, it was concluded that the fungi were not the main cause of the low seed quality.

Fifteen field fungi belonging to 10 genera and 5 storage fungi of the genera *Aspergillus, Penicillium* and *Rhizopus*, were isolated from 25 seed samples of 9 cultivars of soybean by Anwar *et. al.* (1995). The field fungi causing reduction of seed germination or seedling emergence were *Alternaria alternata, Cercospora kikuchii, Colletotrichum truncatum* and *C. dematium. Fusarium equiseti, F. moniliforme* [*Gibberella fujikuroi*], *F. pallidoroseum, F. oxysporum, F. solani, C. dematium, C. truncatum, C. kikuchii, Curvularia lunata* (*Cochliobolus lunatus*). *Phomopsis sojae, Myrothecium verrucaria* and *Stemphylium botryosum* [*Pleospora tarda*] caused foliage and pod diseases. All seed samples of cultivars NARC-III, Crawford, William-82 and Century were infected, whereas some cultivars had no infection. The percentage fungal infection in individual samples varied from 0 to 14. High incidence of storage fungi (*Aspergillus, Penicillium* and *Rhizopus* spp.) reduced seed germination potential *in vitro*.

The incidence of fungi was determined on samples of 21 soybean cultivars harvested in 7 regions of Mato Groso do Sul in 1992/93 and incubated at 22^0C for 7 d before examination by stereoscopic microscope : 23 fungal genera were identified in 142 seed samples by Goulart *et. al.* (1995). The most widespread fungus, detected in 96.5% of seed samples, was *Fusarium semitectum* [*F.pallidoroseum*] which also showed high incidence. Fungal contamination was affected by region of cultivation and climatic conditions during growth and harvesting. The storage fungi, *Aspergillus* sp. and *Penicillium* sp. were detected at relatively high levels in seed samples from all regions.

Soybean seeds (cv. Doko) and *Phaseolus vulgaris* (cv. Monte Rico) stored for 1 year with no RH or temperature control were evaluated for storage fungi incidence using a selective medium. Of *Aspergillus* spp. identified (*A. flavus, A. niger* and *A. ochraceus*), *A. flavus* was predominant after 1 year of storage (6 and 9% for soybean and *P. vulgaris*, respectively). *Penicillium* spp. showed a high rate of occurrence (69 and 24% for soybean

and *P. vulgaris,* respectively). The soybean (12%) and *P. vulgaris* (15%) seed moisture content, checked at the end of the storage period, had an influence on *Aspergillus* occurrence, but not on that of *Penicillium* spp. One year storage was unsuitable for soybean seed conservation, but suitable for *P. vulgaris.* Field fungi, such as *Fusarium* spp., maintained their pathogenicity over a long storage period (Braccini and Dhingra, 1996).

Murthy and Raveesha (1996) investigated the mycoflora of soybeans in Karnataka, India. *Penicillium* and *Rhizopus nigricans* [*R. stolonifer*] were the most commonly occurring storage fungi *and Alternaria, Chaetomium, Colletotrichum, Cladosporium, Diaporthe, Fusarium, Macrophomina, Myrothecium, Phoma* and *Trichothecium* were the most commonly occurring field fungi. Most of the fungi also reduced seed germination and seedling vigour and caused a variety of symptoms on seedlings.

In a field trial in monsoon 1993, *Vigna unguiculata* cv. CO4 was given 0.25% endosulfan, 0.1% malathion, 0.1% dithane M-45 (Mancozeb), 0.1% carbendazim, endosulfan + dithane M-45, endosulfan + carbendazim. Malathion + dithane M-45 or malathion + carbendazim at 30 + 45 days after sowing. Incidence of pod borer (*Lampides boeticus*) was highest in the untreated control (35%) followed by dithane M-45 treatment (31.5%) and it was lowest with endosulfan + carbendazim (1.7%). Seed yield and seed vigour index were highest with endosulfan + carbendazim. Initial seed germination was highest with malathion + dithane M-45 (97%) followed by endosulfan + carbendazim (96%) and seed germination after 5 months storage was highest with malathion or endosulfan + carbendazim (90%). The incidence of live pulse weevils in seed stored for 5 months was not affected by treatments, however incidence of dead weevils was lowest with malathion or endosulfan + carbendazim. Dithane M-45 gave the lowest incidence of storage fungi, i.e. *Aspergillus* and *Rhizopus* spp. (Sasikala and Krishnasamy 1996).

The mycoflora of soybean in Karnataka India, was investigated by Murthy *et. al.* (1996). Of the 38 species of fungi identified, *Aspergillus, Penicillium* and *Rhizopus nigricans* [*R. stolonifer*] were the most commonly occurring storage fungi and *Alternaria, Chaetomium, Colletotrichum, Cladosporium, Diaporthe, Fusarium, Macrophomina, Myrothecium, Phoma* and *Trichothecium* were the most commonly occurring field fungi. Most of the fungi also reduced seed germination and seedling vigour and caused a variety of symptoms on seedlings.

Seeds of winged bean (*Phosphocarpas tetragoulobus* (L.) D.C.) varieties IIHR-80, NERI GRWB-14 and GRWB-10 were evaluated following the standard blotter and agar plate methods to determine the fungal flora associated with these seeds. (Kumar and Singh, 1996). Eighteen fungal species from eight genera were found associated with the seeds. Nine of these were recorded for the first time; variety NERI carried maximum number of colonies of almost all the fungi. *Alternaria alternata, Fusarium oxysporum* and *Rhizoctonia bataticola* were found associated with the internal tissues of seeds. Standard blotter method was found more suitable than agar plate method for the detection of most of the fungi. Chlorine pre-treatment of seeds reduced the appearance of fast growing fungi and facilitated development of *F. moniliforme, Aspergillus flavus* and *A. niger.* Eight fungi *A. alternata, A. flavus, A. candidus, Cladosporium cladosporoides, Curvularia geniculata, F. oxysporum, Drechslera tetramera* and *R. bataticola* were found pathogenic causing seed rot, root rot, seedling blight, leaf spot and blight under artificial inoculation. None of the nine fungicides used for seed treatment could eliminate the entire mycoflora associated with seeds. However, Emison (2g/1kg seed), Carbendazim-Bavistin, Jkstein and Bavistin 25 SD were effective in controlling most of the fungi and increasing seed germination.

Dayal *et. al.* (1997) reported that a significant difference in chlorophyll (a, b) was obtained where seedling raised from seed stored at 71.4% and 80.0% RH. Biomass of the seedling raised form the control seeds increased

due to increase in RH level from 52.0 to 71.4% and decreased due to storage at 80.0% RH.

According to Medina *et. al.* (1997) soybean seeds of early maturing cultivars, produced at the traditional time (sown in November) in Soa Paulo, Brazil, often present problems associated with seedborne pathogens. The study was carried out with seeds of 5 early maturing cultivars (IAC-100, IAC-15, IAC-Foscarin-31, BP-4 and IAS-5) planted at 2 different dates (November and March/April) in Campinas and Votuporanga. The seed health tests were conducted immediately after harvest and at the time of next planting. The results showed that the healthiest soybean seeds were obtained when the sowing date was delayed until the end of summer / beginning of autumn, for both seedborne pathogens and storage fungi. The seeds obtained were less infected by storage fungi at the time of next sowing due to the shorter storage period.

Different levels of asymptomatic, seedborne infection by storage fungi (*Aspergillus* and *Penicillium* spp.) or *Phomopsis* seed decay (PSD) (caused by *Phompsis longicolla, Diaporthe phaseolorum* var. *sojae* and *D. phaseolorum* var. *caulivora*) were induced in sub-lots of separate soybean seed lots. Seeds or pods were incubated for different times at 25^0C and at a RH of >95%. Seeds were then air-dried to a constant moisture content in the laboratory, and each sub-lot was tested for incidence of infection, germination, and moisture content. Individual seeds in each sub-lot were dropped 10 cm onto a transducer in an ultrasound analyzer. The average peak value of the ultrasound signals for each sub-lot, which indicates the weight of seeds, decreased linearly as the incidence of seed infection by storage fungi or PSD increased. The slope and width of the signal, which indicates seed softness, increased as seed infection increased for both groups of fungi, although coefficients of determination were lower. Germination values, which decreased as seed infection for both pathogens increased, showed similar but inverse relationships to ultrasound parameters.

Peak values of ultrasound signals decreased, and slope and width increased, as seed moisture content increased for sub-lots of soybeans at 3 levels of PSD infection. It is concluded that ultrasound technology has the potential to identify soybean seeds with a symptomatic infections of seed borne pathogens (Walcott, *et. al.* 1998).

Thakur (1999) reported that fifteen fungi belonging to 12 genera were found associated with soybean seeds obtained from different fertilizer doses. The frequency of mycolora was highest in treatments with cent per cent NPK+YM. However, it was minimum (63) in cent percent NPK+S. The predominant mycoflora associated were the species of *Aspergillus, Penicillium Fusarium, Phoma* and *Colletotrichum.* Of 10 seed samples tested, 8 had the association of *C. dematium* f. sp. *truncatae* and other seedborne fungi. The frequency of total mycoflora associated with different grades of seeds were 10, of which *C. dematium* f. sp. *truncatae*, *Fusariam oxysporum* and *Phoma* sp. were pathogenic and others were non pathogenic. The association of *C. dematium* f. sp. *truncatae* with different grades of seeds was either alone or in combination with other seed borne fungi.

The soybean seed is considered to be the most important vehicle of spread, survival and introduction of important pathogens into non infested areas. This work was carried out by Goulart (2000) in the Seed and Plant Pathology Laborartory at Embrapa Agropecuaria Oeste, in order to determine the incidence of fungi on soybean seeds produced in the state of Mato Grosso do Sul, Brazil, during the 1992/93, 1993/94, 1994/95, 1995/96 and 1996/97 crop seasons. One thousand fifteen hundred and thirty seven samples of soybean seeds were analyzed. The fungal incidence was determined using the blotter test with 200 seeds per sample. The most prevalent fungus associated with soybean seeds was *Fusarium semitectum*, detected in 93.2% of the analyzed samples. *Phomopsis* sp. was observed in 68.1%, *Cercospora kikuchii* in 64.2% and *Colletotrichum truncatum* in 52.3%.

The average incidence of those pathogens on soybean seeds was registered at low levels : 8.2% for *F. semitectum,* 4.4% for *Phomopsis* sp., 2.0% for *C. kikuchii* and 1.2% for *C. truncatum.* The storage fungi, *Aspergilus* sp. and *Penicillium* sp., were detected at relatively high levels. It was observed that soybean mycoflora was probably influenced by factors such geographic location, environment during maturation and harvest period, varietal resistance and chemical control. During the period covered by this study, the soybean seeds produced in Mato Grosso do Sul showed high sanitary quality, with low levels of the major pathogens of the soybean crop.

Several tests were carried out with the objective to evaluate the effects in fungal occurrence on dry *Phaseolus vulgaris* seed and on the air in the storage environment. The percentage of seeds with fungi from the field decreased significantly (P>0.01) during storage, in both treatments. On the other hand, the percentage of seeds with storage fungi increased significantly (P>0.01) in both treatment, with no significant differences between their average levels at the end of the experiment. The percentage of fungi in the storage air increased in both storage treatments. (Borem, *et. al.* 2000).

The antifungal activity of plant essential oils on storage fungi commonly associated with cowpea (*Vigna unguiculata* (L.) Walp) seed was investigated by Aveling and Marasas (2002). The antifungal activity of the essential oils of thyme, clove, peppermint, soybean and peanut were tested against *Aspergillus* flavus, *A. niger, Fusarium oxysporum, F. equiseti* and *Penicillium chrysogenum in vitro.* Only thyme and clove significantly inhibited growth of all five fungi at 500 and 1000 ppm whereas peppermint oil successfully inhibited growth at 2000 ppm. Thyme, clove and peppermint were tested *in vivo* against storage fungi on naturally infected (cultivars PAN 325 and PAN 311) and artificially infected (cultivar CH 14) cowpea seed. Only thyme oil (1000 ppm), showed antifungal activity against storage fungi associated with PAN 325 cowpea seeds. In the PAN 311 cultivar, clove and

thyme at 1000 ppm and peppermint at 2000 ppm exhibited antifungal activity against the storage fungi. In cultivar CH 14, thyme, clove and peppermint significantly reduced growth of *P. chrysogenum* whilst thyme and peppermint inhibited growth of *F. oxysporum.* Only thyme had an antifungal effect on *F. equiseti.* No treatement showed antifungal activity against *A. flavus* and *A. niger.* None of the oils showed harmful effects on the germination and emergence of cowpea seeds. The storage fungi significantly reduced percentage germination and emergence of the white (IT 93K452-1) seed but had little or no effect on the brown (CH 14) seed. Furthermore, all three oils significantly inhibited the storage fungi on the white seed thereby increasing the percentage germination and emergence.

Seeds of 28 soybean cultivars were screened by Muthu *et. al.* (2002) for seed mycoflora in 1997 and 1998. Three fungi namely *Aspergillus flavus, Aspergillus niger* and *Alternaria alternata* were found dominant. Thiram seed treatment @ 2g /kg seed significantly improved germination and field emergence and reduced the seed mycoflora. Thus it is recommended to be used in routine to improve the field emergence in soybean

The seed germination rate, seed mortality, seedling length, and infection rate of alfalfa (*Medicago sativa* L. cv. Longdong) were measured by Li. *et al.* (2002) at a constant temperature of 20^0C every 60 days during one year storage period after inoculated or not inoculated by *Fusarium avenaceum* under room temperature (RT), 35^0C, and 35^0C + 10% seed moisture content (SMC) conditions. Field emergence rates of seeds under above treatments were also observed, and seed-borne fungi were detected under the conditions mentioned above and controlled deterioration (CD) as well. The results sowed that the percentage of isolated alfalfa seedborne fungi increased from 10% under room temperature and 35^0C to 29% under 35^0C+10% SMC. Disease resistance declined, and seed mortality and seedling infection rate under 35^0C + 10% SMC were significantly higher than those under room temperature and 35^0C respectively ($P<0.05$). The

percentages of both seedborne fungi isolated and field emergence decreased, and that of seedling infection increased with storage period extending from 60 to 360 days. Compared to not inoculated control, the percentage of seed germination, seedling shoot and root length were decreased and seed mortality and seedling infection rate were increased after inoculation by *F. avenaceum*.

The fungi namely *Aspergillus flavus, A. fumigatus, A. niger, Curvularia lunata, Fusarium equiseti, F. longipes, F. pallidoroseum, Fusarium* sp. *Paecilomyces variotii, Penicillium pinophilum, Rhizoctonia solani* and *Rhizopus stolonifer* were recorded from the seed coat, endosperm and embryo of normal and discoloured seeds of Horse bean (*Parkinsonia aculeata*), whereas *Apispora montagnei, Aspergillus aculeatus, A. flavus, A. fumigatus, A. niger, Curvularia clavata, C. lunata, Fusarium equiseti, F. pallidoroseum, F. moniliforme, Fusarium* spp. *Rhizoctonia solani* and *Rhizopus stolonifer* were found associated with the seed coat, endosperm and embryo of the normal and discoloured seeds of lead tree (*Leucaena leucocephala*). On the other hand, *Aspergillus flavus, A. fumigatus, A. niger, A. ustus, Aspergillus* sp., *Curvularia lunata, Fusarium compactum, F. longipes, F. pallidoroseum, Fusarium* sp. and *Penicillium aurantiogriseum* were detected in the seed coat, endosperm and embryo of the normal and discoloured seeds of rusty shield bearer (*Peltophorum ferrugineum*). In general, the per cent incidence of fungi recovered from the discoloured seeds was greater in comparison to the normal seeds (Sahu and Agarwal, 2002).

Paul (2002) reported that amongst various methods to detect seed borne fungi on French bean seeds collected from eleven locations in four districts of Himachal Pradesh, Standard blotter test was most effective in determining maximum number of fungi while washing test was superior in detecting most seed borne pathogens. Seeds collected from *Collectotrichum* infected pods resulted in maximum reduction in germination percentage. Germination studies of the seed infected artificially with different pathogens

revealed 100 per cent abnormal seedlings in case of *Rhizoctonia solani, Sclerotium rolfsii, Colletotrichum aruncatum* and *Fusarium roseum*. In general, most of the fungi caused decrease in total sugars but increase in protein and fat content of seeds with maximum effects due to *Scelerotium rolfsii* infection. It was found that 10 to 12 days old pods were most susceptible and resulted in significant effects on seed quality. Minimum bio-deterioration of seed was observed when it was stored at 7-12 per cent seed moisture level. Out of 10 fungicides tested as seed dressers, bavistin, thiram, dithane M-45 and hexacap were effective in eliminating maximum number of pathogens and improving seed germination and seedling vigour.

Rathour & Paul (2004) isolated thirty species of fungi belonging to 15 genera which were found associated with the pea seeds collected from different pea growing areas in Himachal Pradesh. *Alternaria tenuissima, A. tenuis, Ascochyta pinodes, A. pisi, Aspergillus flavus, A. niger, Aspergillus* sp. (White), *Cladosporium herbarum, Fusarium moniliforme, F. oxysporum, Penicillium* sp, *Phoma medicanginis* var. *pinodella, Rhizoctonia solani* and *Sclerotinia sclerotiorum* were the frequently encountered species. The fungal spectrum and seed lots from different locations. Among all the locatins surveyed the seeds from Solan and Bilaspur were comparatively free from the pathogenic fungi. Of the 17 seed borne fungi tested for their effect on seedling health of pea, *P. medicaginis* var. *Pinodella, S. scelerotiorum, A. pinodes, A. pisi, F. solani* and *F. oxysporum* were found highly pathogenic causing maximum root discolouration.*R. solani, A. tenuis* and *Stemphylium* sp. were moderately pathogenic. Seed dressing with *Carbendazim* @ 25% and flusilazole @ 0.1% was found to be highly effective in eliminating seed borne fungi. Three preharvest sprays each of mancozeb (0.2%) and carbendazin (0.05%) at 10 days interval, starting from pod initiation stage, proved to be the best in improving germination, seedling vigour, 1000 gram weight and in decrease in abnormal seedlings and incidence of seedborne fungi.

Singh *et. al.* (2005) reported that seeds of chick pea (*Cicer arietinum* L.) cv. Avarodhi were examined after harvesting, for external and internal seed mycoflora using agar plate and blotter metods. *Aspergillus niger, A. flavus, Penicillium citrinum, Trichoderma viride, T. harziamum, Rhizopus* sp. *Fusarium* sp. *Curvularia* and *Alternaria* sp. were isolated from Avarodhi varieties of chickpea by both techniques. The blotter techniques proved to be better in comparison to agar plate technique. The prominent field fungi recorded were *Fusarium* spp., *A. niger, A. flavus* and *Penicillium citrinum* during the summer season from fresh seeds. However in winter *A. niger, A. flavus, Fusarium* spp. *Penicillium* spp., *T. harzianum* were prominently present. The effect of different concentration of four common fungicides viz. Dithane M-45, Bavistin Thiram, Agrosan GN were studied on seed mycoflora and seed germination. All fungicides used were found to be effective in readucing seed mycoflora. However, the germinaiton percent of treated seeds was higher than the untreated seed. So these fungicides enhanced the germination rate of seed as well as controlling the seed mycoflora.

Urd bean (*Vigna mungo*) is one of the important short duration pulse crops which is extensively grown as catch as well as cover crops through out the year between the gaps of two main crops in Northern India. During the harvesting the seeds are contaminated by several seed borne mycoflora which deteriorate the germination of seed and reduce the yield of the crop. These problems are generally seen in the plain region because their method of harvesting is objectionable. *Alternaria alternata, Fusarium* spp., *Rhizopus nigricus, Aspergillus niger, Aspergillus flavus*, *Trichoderma harzianum* and *Penicillium* spp. were the seed mycoflora isolated fram the seed lot at 30, 60, 90 and 120 days of storage by using standard blotter and agar plate techniques. *Alternaria alternata, Fusarium* spp., *Rhizopus nigricaus, Aspergillus niger* and *A. flavus* predominantly occurred and these fungi were the major cause of rotting of seeds and the in the reduction in the germination of the seeds. The management of these microflora was done by using standard fungicides, likewise Zineb078, Mancozeb-45, Carbendazim . Among

three fungicides carbendazim, showed maximum efficacy and inhibited more than 95%. Population of seed mycoflora and thus enhance the percent germination of the seed (Singh and Sinha, 2005).

2.2. Other seeds

Dutta and Roy (1987) reported that altogether 19 and 20 fungi were associated with the seed samples of *Strychnos potatorum* and *S. nux-vomica* respectively. *Aspergillus flavus, Aspergillus niger* and *Penicillium citrinum* had maximum percentage of incidence on both the seeds. The extent of deterioration in total phenols, proteins and alkaloids was recorded at 96 percent RH and 30^0C temperature under various storage periods, that is 15, 30, 45, 60 days. Maximum loss in these principles in the seeds of both species was observed on 60th day of storage. *S. potatorum* seeds showed a decrease of 14.165 percent in phenol, 11.867 percent in protein and 10.185 percent in alkaloid whereas in case of *S. nuxvomica* it was 10.809, 10.046 and 6.333 percent respectively.

Isolation and identification of seed borne fungi were made from seeds of *Oryza sativa* through agar plate and blotter methods. (Jayaweera *et. al.* 1989). A total of 17 forms of fungi were isolated from the seeds. The frequency of occurrence was also determined. The fungi *Curvularia pallescens, Curvularia verruculosa, Curvularia eragrostidis, Curvularia offinis, Pyrenochaeta terrestris, Bipolaris oryzae, Alternaria padwickii, Sordaria fimilcola, Penicillium citrieoviride* and *Fusarium* sp. significantly reduced the germination of rice seeds.

Sao *et. al.* (1989) isolated 25 spp. of fungi from the seeds of vegetables belonging to Brassicaceae. The germinability of radish seed was suppressed considerably on storage for longer period at high RH. High percent of seedlings showed shriveling of the shoot, foot rot, stunting and chlorosis. The acetone extract of the seedlings was found to disturb the growth of healthy seedlings and reduce chlorophyll, starch, total sugar and

amino acid contents besides decreasing the activities of nitrate reductase and urease and enhancing that of AA oxidase, starch phosphorylase, pyruvic, α-ketoglutaric and succinic acid dehydrogenase, with concomitant uptake of more oxygens. The authors further reported that the O_2 uptake of radish Cv Pusa Reshmi seeds increased due to seed borne *Aspergillus flavus* with extension of the storage period upto twenty days and at specified as well as increased level of RH. The loss in dry weight and germination of seeds were also inflated with rise in RH level and duration of storage. The activity of starch phosphorylase, fructose diphosphatase aldolase, pyruvic. α-ketogluteric and succinic acid dehydrogenase, peroxidase, ascorbic acid oxidase, catalase and ATP ase was enhanced besides increase in pyruvic acid and total keto acid and decrease in α-ketoglutaric acid (Sao, *et. al.* 1990).

Eighty-two seed samples of mustard were collected from nine agro-climatic zones of Rajasthan and 15 species of fungi were isolated from these samples by Shivpuri *et. al.* (1990). Effect of these fungi were studied on the quantity and quality of mustard oil. Three fungi reduced, six increased and rest did not affect oil content. All the fungal species inflicted unpleasant odour in oil and changed the colour of oil significantly.

Maize seeds that had been hot-water-treated (30 min at 55^0C) to reduce inherent infection, were inoculated with the spores of four storage fungal species of varying xerotolerance.The less xerotolerant species (*Aspergillus oryzae* and *Aspergillus sydowi*) were characterized by vigorous growth on the six single carbon source media tested, and were also associated with rapid and extensive degradation of all the seed tissues. The more exotolerant species (*Aspergillus chevalieni* and *Penicillium pinophilum*), on the other hand, grew only slowly *in vitro* and were not located in the embryo despite six weeks storage of the artificially infected seeds, at 95% relative humidity. Germinability of infected seeds decreased with storage time, as did the dry mass of the resultant seedlings, the extent of the decline

increasing with decreasing xerotolerance of the fungal species. The rate of infection of and ultimate mycelial location in the seeds, are suggested to be related to the extracellular enzyme capabilities of the individual species (Mycock and Berjak, 1992).

Seed samples of hybrids / varieties of sunflower collected from different locations of Haryana yielded 17 fungi. There was qualitative and quantitative variation in the association of mycoflora on variety EC-68415C and hybrids, MSFH = 8, MSFH=17, MSFH = 31, HL-7, Jwala-mukhi and Sunbred over the locations (Raj and Saharan, 1994). Fungi like *Acremonium strictum, Aspergillus cerneus, Bipolaris tetramera* and *Fusarium chamydosporum* were new records on sunflower seeds. *Rhizopus oryzae, Sclerotinia sclerotiorum* and *Aspergillus parasiticus* caused 68-78 percent inhibition in seed germination. Head rot disease was caused by the former two fungi. Some fungi were potential pathogens of sunflower crop.

Changes in 2 cultivars (white and yellow) of maize were determined at harvest and on a monthly basis for 1 year. Kernel moisture decreased for the 1st 6 months of storage and increased thereafter. The predominant fungi at harvest were *Alternaria alternata, Botryodiplodia theobromae, Fusarium* spp. and *Macrophomina phaseolina*. Populations of these decreased with time until they finally disappeared after 5 and 4 months for white and yellow maize, respectively. Storage fungi, mainly *Aspergillus* and *Penicillium* spp. succeeded the field fungi on stored grain. The percentage seed germination decreased with storage time to the lowest after 12 months (Bankole, 1994).

Seed samples of bhindi (*Abelmoschus esculenutus* (L.) Moench], brinjal (*Solanum melongena* L.) and chillies (*Capsicum annum* L.) were collected by Gupta and Basu Choudhury (1995) from different parts of Sikkim viz Tadong, Merchak, Majitar, Bermiok, Namthang, Namchi, Geyzing and Rumtek during 1984-86 and suitably stored in cool, dry place in cloth bags / polythene bags. These samples of fresh seeds were analyzed by standard

moist blotter technique (ISTA). Four hundred seeds were tested in each crop and incubated for 7 days at 20 ± 2^0C with 12 h photoperiod. The fungi developed on seeds were examined on 8^{th} day using the stereo binocular microscope and were identified by their growth characters. The fungi thus obtained were identified and further confirmed by C.M.I, Kew, Surrey England.

Milosevic, *et. al.* (1995) tested 1000 samples of wheat, 100 samples of barley and 600 samples of maize seeds obtained in Novy Sad, Yugoslavia, for the presence of fungi. A filter paper method was used for identification of genera and PDA medium for fungal growth and identification of species. Among the 21 species from wheat and barley and 34 from maize, the majority belonged to *Fusarium*, prevailing on wheat and maize, and *Helminthosporium* spp., dominating on barley. Storage fungi belonging to *Penicillium. Aspergillus, Rhizopus* and *Mucor* genera were present on wheat, maize and barley.

A survey of fungi associated with seed of birdsfoot trefoil [*Lotus corniculatus*] was undertaken in Colonia Uruguay, by Rubio and Altier (1995). from Oct. 1994 to May 1995. The mean incidence of fungi varied significantly among seed lots ranging from 1.7-38.2%. Storage fungi (*Aspergillus* and *Penicilium*) averaged 5.1% and seed contaminant fungi (*Alternaria tenuis,* [*A. alternata*]. *Cladosporium, Curvularia, Epicoccum,Helminthosporium, Pithomyces* and *Rhizopus*) averaged 5.3% and pathogenic fungi (*Colletotrichum, Fusarium, Leptosphaeria, Phoma, Rhizoctonia* and *Stemphylium*) averaged 0.3%. *Alternaria* was the most prevalent fungus, representing 37% of total fungi. Percentages of seed germination varied significantly. Seeds with low germination had the highest counts of storage fungi and / or *Alternaria*.

Vijaya Kumari and Mukewar (1995) surveyed the seed micro flora of cotton in India. A total of 113 and 133 cotton seed samples from two crop

seasons from different states of India were evaluated for the presence of seed microflora by Standard Blotter Method. The study indicated that none of the seed samples tested was totally free of microflora. Commonly present fungi were — *Alternaria macrospora, A. tenuis, Cladosporium* spp. *Colletotrichum indicum, Curvularia lunata, Dreschlera* spp., *Epicoccum purpurescens, Fusarium* spp. *Nigrospora amyzae, Trichoderma viride, Trichothecium roseum* and *Verticillium* spp. *Aspergillus* and *Penicillium* were present in most of the seed samples. Some of the potential and economically important cotton pathogens such as *A. macrospora* in herbaceum V797 (Viramgarm) hirsutum varieties- McII5VT Bikaneri Nernaa H 777, LH 886, (Coimbatore, Jaipur, and PAU, Ludhiana), *C. indicum* in herbaceum variety G. cot 13 (Indore) and KH 1002237 a hirsutum variety (Chharodi) *Pestalotia gossypi,* in herbaceum G.Cot 13 and hirsutum variety G. Cot 14 (Surat) were observed.

Fifty seed samples of different rice varieties stored in warehouses for varying periods (1-28 months) were collected and screened for their fungal flora, using standard blotter and agar plate methods, resulting in the isolation of 36 fungal forms. The samples were also studied for moisture content, germinability and seedling abnormalities. Significant correlations were found among the numbers of fungi, storage period and germinability. Fourteen samples, seven each of IR64 and IR66 were studied with regard to moisture content, germination test, abnormal seedlings, speed of germination, conductance of leachates, total dehydrogenase activity, total free amino acids, total soluble sugar, fat acidity, gelatinization temperature, gel consistency, amylose content, translucency and percentage whiteness. Significant relationship were found between the fungi and the parameters studied. (Misra, *et. al.*, 1995).

Freshly harvested paddy rice, with initial water activities (aw) of 0.84 and 0.90 (16.0% and 19.1% moisture content on a wet basis, resp.), was exposed to 0.01-1.5 mg phosphine (PH_3)/litre at 28^0C for 7 and 14d.

Fumigant concentration were determined after each exposure period. Fungal flora was evaluated before and after all treatments by direct plating onto two media. The results showed a decrease of total infected grains as the fumigant concentration increased. After all treatments, a better control was observed for field fungi at 92% relative humidity and for storage fungi at 85% relative humidity. The 0.5mg PH_3/litre concentration was probably sufficient for the control of *Eurotium* spp. development at both relative humidities. The lowest percentage of fungal infection of grains was obtained with 1.g mg phosphine/litre at the highest relative humidity. The protection period offered by the fumigant depended on the concentration applied. (Castro *et. al.* 1995).

Isolates (23) of field fungi encountered during the isolation of storage fungi from grains of different wheat varieties, were tested for xerophilic tendencies. Almost all the test strains grew on the malt salt (7.5% slat) agar medium However, some of the fungi showed >50% retardation in their growth on malt salt (7.5% salt) agar compared with their growth on malt extract agar medium, (Sijariya *et. al.* 1995).

Seeds of sorghum cv. SPV 351 with 8.00 per cent initial moisture content were treated individually and in combination with fungicides and insecticides and stored in cloth bags as well as polythene bags of 600 guage under ambient conditions (22.4-31.2^{0}C temperature and 50.5-75.0 per cent relative humidity) for a period of eighteen months. High germination and seedling vigour were recorded in seeds treated with Thiram (@3.0g/kg of seed), which apart from controlling seed born fungi effectively, protected seeds of sorghum from the attack of *Rhizoportha dominica* for a considerable period thereby indicating the possibility of controlling both seed borne fungi and the above stored grain pest by a single chemical (Savitri *et. al.* 1996).

Rice cv. Rasi seeds were inoculated with spores and treated with culture filtrates of *Aspergillus flavus, A. glaucus, A. niger, A. versicolor* and *Penicillium* sp. by Purushotham *et. al.* (1996). Seed deterioration was studied

based on germination, root and shoot length, total carbohydrate content and α-amylase activity. The surface sterilized seeds were inoculated with the spore suspension and incubated at 28± 2^0C for up to 30d. Carbohydrate content and amylase activity were measured. *A. flavus* inhibited seed germination and seedling growth the most followed by *A. glaucus* and *A. versicolor.* The fungi caused a significant decrease in the total carbohydrate content of seeds. Maximum reduction in carbohydrate content was observed in *A. flavus inoculated* seeds followed by *A. versicolor* and *A. glaucus.*

Fungi associated with rice grains in the field in Titaban, Assam, India and during storage were studied by Ali and Deka (1996) using different rice cultivars. Ten fungal species from 7 genera (*Curvularia, Drechslera, Nigrospora, Trichothecium, Fusarium, Aspergillus* and *Penicillium*) were associated with grain discoloration of 6 rice cultivars. The frequency of occurrence of these fungi varied considerably on different cultivars. The frequency of *F. moniliforme* (*Gibberella fujikuroi*) was highest among the field fungi, while *Aspergillus* and *Penicillium* spp. were most frequent among the storage fungi after 8-10 months of storage. Treatment with carbondazin (as Bavistin) at 1g/kg seed was effective in maintaining seed germination at >70% even after 8 months of storage.

Pathological symptoms, such as stunting and necrosis of radicle and fibrous roots, development of scanty fibrous roots, browning and blackening of the radicle and young leaf, and dwarfing and drying of the seedlings were distinct symptoms of diseases due to seed borne storage fungi, *Memnoviella Ichnata* appeared to be more pathogenic for seedling diseases. The number of seedlings with disease symptoms increased proportionately with increase in relative humidity level of storage seeds (Diwakar and Prasad, 1997).

The relationship between moisture content and storage period to fungal population, seed germination, grain whiteness and translucency was

determined by Paderes *et. al.* (1997) . Various fungal species predominated at different moisture conditions and storage periods. The fungi observed belong to the groups *Aspergillus flavus-oryzae, A. glacus, A. nidulans, A. candidus, A. versicolor, A. terreus* and *A. niger* and unidentified species of *Penicillium. Trichoconielle, Curvularia, Fusarium, Syncephalastrum* and *Verticillium.* The predominant storage fungi were *A. flavus-oryzae* and *A. candidus* whereas the predominant field fungi were *Trichoconiella* sp., *Curvularia* sp. and *Syncephelastrum* sp. A decrease in the number of field fungi and an increase in the number of storage fungi with storage time were observed. Storage fungi were noted as early as 5 weeks after storage at moisture contents from 9.3 to 18.33%. The % germination of paddy remained high when stored at moisture contents of 9.3 to 14% but decreased with reached a peak at 10-15 weeks of storage. A significant negative-correlation between % germination and moisture content was observed. At 14.5-10.33% moisture content, the germination of stored paddy decreased with a marked increase of storage moulds. Changes in grain whiteness was not affected by moisture content. However, a decrease in % whiteness and translucency was noted after 25 weeks of storage.

Immediately after harvest as it was transferred into grain bins, maize grain (19.8% moisture, wet basis) was treated with P-7 grain preservative (15% propionic acid, 85% inert ingredients) at rates of 0.0, 0.1, 0.2 and 0.3% on a wet basis. The grain was low temperature dried using ambient air. Samples were taken every 2 weeks for 42 weeks and fungal incidence was determined by plating on malt salt agar. Samples for the determination of percentage damaged kernels (DKT) were taken at weeks 2, 4 and 6, and then every 6 weeks until week 42. Treatment with P-7 was associated with a reduction in *Penicillium* spp. and *Aspergilus* spp. incidence for the first half of the experiment, but not for the second half. In the second half, treatment with P-7 was associated with an increase in incidence of *Penicillium* spp. Based on percentage damaged kernels, there was no economic benefit of P-7 application in this experiment (White and Coates, 1997).

Sunflower seeds naturally infected with *A. helianthi* and artificially inoculated by Prasad and Kulshreshta (1999), showed 32.8 and 19.0 percent reduction is germination respectively. Shoot and root length of seedlings was also reduced in both cases. The vigour index was very low (400) in naturally infected seeds, artificially inoculated seeds recorded a vigour index of 533.5 whereas in control it was 1799.8. With increase in spore load of *A. helianthi* on seeds there was an increase in number of seedlings showing blight incidence.

Laboratory studies were carried out by Bankole *et. al.* (1999) in the Department of Biological Sciences, Ogun State University, Ago-Iwoye, southwestern Nigeria to determine the extent of fungal deterioration, of melon seeds stored in two types of storage bags : jute and polyethylene bags under ambient conditions using the 2x2 factorial design (variety vs. type of bag) for 12 months. The moisture content (mc), incidence of visible mouldiness (ivm) and germinabillty of the stored seeds were determined monthly. The mc of Tc139 ranged from 6.1 to 6.7% in jute and 6.2 to 6.5% in polyethylene bags. The percentages of which was initially 2.1% increased to 10.7% and 5.5% in jute and polyethylene bags respectively, after 12 months in storage. The germination percentage decreased from 96.3% to 28.7% and 45.3% in jute and polyethylene bags, respectively. The mc of V2 stored in jute and polyethylene bags, respectively, after 12 months. The percentage seed germination declined from 98.0% to 37.3% in jute and 48.7% in polyethylene bags after 12 months. Decreased incidence of field fungi namely *Alternaria, Botryodiplodia theobromae, Cladosporium, Fusarium* and *Macrophomina phaselina.*

Hasan (1999) reported that fungal association of safflower seeds, and factors affecting their deterioration during storage were evaluated periodically for fungal invasion, viability, biochemical changes and mycotoxin accumulation. Safflower seeds were infected with different fungal sepecies, mainly *Aspengillus flavus, A. fumigatus, A. niger, A. sydowii, Emericella*

nidulans, Mucor circinelloides and *Penicillium chrysogenum.* Naturally infected seeds at 15.2 and 25% moisture contents were stored for 5 months at 10 and 25^{0}C. At 15% moisture content, germinability of seed changed slightly at 10^{0}C, however it significantly decreased at 25^{0}C. The depression in seed germination increased with increasing moisture content and length of storage. No germination in seed at 25% moisture content was observed after 5 month of storage at 25^{0}C. There was no noticeable change in protein content, but increases of free fatty acid contents were noted, especially at high moisture and temperatures. This promotion in free fatty acids was negatively correlated with percentage germination and positively correlated with invasion of seed by the storage fungi, *P. chrysogenum* at 10^{0}C and / or *A. flavus* and *A. niger* at 25^{0}C. In another experiment, the sterilized seeds at 25% moisture content were artificially infected by toxigenic *A. flavus* and stored for 5 months at 25^{0}C and 100% relative humidity. The ability of the fungus to produce aflatoxin reached the maximum level after 1 month of seed infection and then decreased. Degradation of oil and insoluble protein was accompanied by a corresponding rise in free fatty acids, free amino acids and soluble protein. Wheat grains infected by *A. flavus* were accompanied by lowered levels of insoluble carbohydrates and insoluble protein, and raised levels of soluble sugars, soluble protein and free amino acids. In faba bean, the total carbohydrates and proteins reduced with the rise in free amino acids. The high levels of autolytic enzymes (amylase, cellulose, lipase and protease) detected in seed fungi clearly indicate the important role of these enzymes in deterioration of seeds. Percentage of free fatty acids in safflower, free amino acid in faba bean as well as soluble sugars and soluble protein in wheat are valid indicators for seeds quality.

Microorganisms associated with robusta coffee (*Coffee canephora* Pierre ex Froehner) seeds during the storage period were identified. The coffee seeds cv. Xonillon put into different packing bags (transparent plastic, kraft paper and cloth bags) and having different initial moisture contents (25 and 35% wet basis) were submitted to five storage periods (0,3,6,9, and

12 months), in controlled conditions. After each storage period seeds were evaluated in laboratory by the following tests blotter test, standard germination test and moisture content determination. Five different fungi genera were isolated and identified as infestation of robusta coffee seeds : *Fusarium semitectum* (63-73%) and *Altemaria* spp.(7-11%) were predominant at the beginning of the seed storage. During the storage period great increase of storage fungi (*Aspergillus* spp. and *Penicillium* spp.) incidence in seeds packed in different bags was observed. The seed infestation level showed great variation in relation to the type of packing during the storage. The increase of fungal incidence was associated to the reduction of coffee seed germination. *Fusarium semitectum* was observed after 12 months in seeds stored in Kraft paper and cloth bags. Plastic bags associated to higher initial moisture content were more efficient in preserving robusta coffee seeds. (Braccini *et. al.* 1999).

Seed samples of different cultivars of tomato viz. Arka Kusmakar, Madanapalli, Punjab Chores and five local varieties were subjected to standard blotter method for the analysis of seed mycoflora. *Aspergillus flavus* was recorded only in local varieties compared to the improved varieties. All the chemical fungicides inhibited the expression of *Fusarium oxysporum.* Biological treatments stood superior over any other treatment in controlling *Fusarium solani* reported by Kumar and Lokesh (1999).

A wide range of field and storage fungi were isolated by Freire *et. al.* (2000) from black pepper, white pepper and Brazil nut kemels from Amazonia . A total of 42 species were isolated from both peppers. *Aspergillus flavus* and *A. niger* were isolated more frequently from black than from white pepper. Other potential mycotoxigenic species isolated included : *A. ochraceus, A. tamarii, A versicolor, Emericella nidulans Chaetomium globosum, Penicillum brevicompactum, P. citrinum, P. istandicum* and *P. glabrum.* Species isolated from pepper for the first time were *Acrogenospera sphaerocephala, Cylindrocarpon licheicola, Lacellinopsis sacchari, Microascus cinereus, Petriella setifera* and *Sporormiella minima*..Seventeen

species were isolated from Brazil nut kermels. *A. flavus* was the dominant species followed by *A. niger. P. citrinum* and *P. glabrum* were the only penicilliam isotated. Species isolated for the first time included *Acremonium curvulum, Cunninghamella elegans, Exophiala* sp., *Fusariium oxysporum, Pseudiallescheria boydii, Rhizopus oryzae, Scopulariopsis* sp., *Thielavia terricola* and *Trichoderma citrinoviride*. Considerably more metabolites were detected from black than white pepper in qualitative analyses. *Chaetocin, penitrem A*, and *xanthocilln* were identified only from black pepper, and tenuazonic acid was identified from both black and white pepper. Aflatoxin G2, chaetoglobsin C, and spinulosin were identified from poor quality brazil nuts. Aflatoxin, B1 and B2 were also only detected in poor quality brazil nuts at concentrations of 27.1 μg kg^{-1} and 2.1 μg kg^{-1} respectively (total 29.2μg kg^{-1}).

Arafa *et. al.* (2000) reported that sixteen different fungal species representing nine genera were isolated from testa and endospermic tissues of sunflower seeds. Frequency of fungi isolated from the testa or endosperm varied according to the color of each. Among these fungi, *Nectria haematococca, Alternaria alternata, Gibberella baccata* and *Aspergillus parasiticus* were the most frequently isolated. The quality tests in discolored seeds showed a decrease in protein, oil content, and 1000 seeds weight, while an increase in free fatty acids were detected. Deep black endospermic and testa of seed colour contained the highest levels of mycotoxin production compared with slightly red and normal seeds. Alternariol (ADH), aflatoxin, Zearalenone (ZDN) and Zearalenols (ZOL) were detected in the blackish endospermic and/ or testa of seed colour, except the later mycotoxin was only found in endospermic tissues. Slight red seeds (endospermic or testa) were found to have most of the ZON or deoxynivalenol (DON) (vomitoxin) mycotoxins, but ZOL and aflatoxin mycotoxins was detected only in endospermic tissues. Normal seeds contained the least levels of ADH or ZON only. Inoculation trials proved that the storage fungi *Nectria haematococca* and *Aspergillus parasiticus* caused more serious losses than *Alternaria*

alternata, which decreased seed germination, increased seed invasion and produced toxins. Soil infestation with nine fungal species isolated from discoloured and normal seeds was carried out. These fungi could be arranged in descending order according to their virulence as follows: *Gibberella baccata, Nectria haematococca* and *Rhizoctonia solani.* However, *Macrophomina phaseolina* was less virulent in the pre-emergence phase, but more serious in the post-emergence phase and later stages of growth. The remaining fungi (*Aspergillus flavus, A. fumigatus, A. parasiticus* and *Alternaria alternata*) only caused pre-emergence rotting.

Many fungi were reported by Brown *et, al.* (2000) from flowers, capsules and seeds, of various species of *Corymbie* and *Eucalyptus.* Some fungi, such as *Ramuleria* spp., *Colletotrichum gloeosporioides* (*Glomerella cingulata*) and *Dothiorella eucalypti* have caused diseases of capsules, resulting in reduced seed production while many others have been reported from capsules without mention of their pathogenicity. The fungi found on eucalypt seeds were either s field or storage fungi. The field fungi associated with eucalypt seeds include common soilborne fungi such as *Fusarium* spp. *Macrophomina phaseolina, Pythium* spp. and *Verticillium albo-atrum.* Their association with seed was suggested to be probably due to contamination of the seed with soil during harvesting on processing. Other field fungi include known foliar pathogens of eucalypts such as *Botrytis cinerea, Coniell australiensis, Curvularia* spp., *Cylindrocladium scopanium, Dothiorella eucalypti, Fairmaniella leprosa, Harknessia* spp. and *Pestalotiopsis* spp. Storage fungi are those that grow on or infect the seed during storage. They are specialized fungi that are able to grow without free water and on substrates of high osmotic potential. Most storage fungi of eucalypt seeds were species of *Aspergillus* and *Penicillium.* According to the authors, appropriate hygiene procedures during seed harvesting and processing, and the storage of seed at low temperatures and humidity will usually provide satisfactory control of seed borne fungi. When necessary, treatment with hot

water or hydrogen peroxide can be used to reduce the effect of seed borne fungi on seedling establishment.

Seeds of *Dendrocalamus strictus, Phyllanthus emblica, Hardwickia binata.*, and *Dalbergia latifolia* were screened for mycoflora incidence and showed both field and storage fungi. Dominant mycoflora were isolated by Mamatha, *et. al.* (2000) and their effects were studied on quality aspects like germination and vigour. Seeds inoculated with dominant fungi showed significant decreases in germination and seedling vigour. Seed samples were subjected to four different pre-treatments. Treatment with *Trichoderma* spp. was most effective both in reducing the incidence of mycoflora and enhancing the germination and vigour.

Seeds of *A. catechu* were treated with four fungicides in order to study the effects of treatment on storage fungi and seed germinatin. Fungicides were applied either individually : Bavistin (carbendazim) (0.05%); captan (0.2%); Indofil M-45 [Mancozeb + thiophanate-methyl (0.2%); and streptocycline (100 ppm); or in combination; Bavistin (0.025%) – Indofil M-45 (0.1%); Bavistin (0.025%) + captan (0.1%); Bavistin (0.025%) + streptocycline (50 ppm); captan (0.1%) + Indofil M-45 (0.1%); captan (0.1%) + streptocycline (50 ppm); and Indofil M-45 (0.1%) + streptocycline (50 ppm). Seed germination was highest for those treated with Indofil M-45 (52%) followed by Bavistin + Indovil M-45 (48.50%) and Bavistin + streptocycline (47%). Data were aso tabulated for the incidence of *Penicillium* sp. and *Aspergillus* sp. fungi (Pathania, *et. al.* 2000).

Damage by fungi (especially *Aspergillus* spp. and *Penicillium* spp.) to stored sorghum seeds in Brazil was briefly described by Almeida and Almeida (2000). Different seed treatments or cv. BR-304 were compared, including acetic acid (15%), urea (3.5%), thiabendazole (53.2 g/100 kg seeds), iprodione (22.0 g/100 kg) and mineral oil (1%).

Untimely and frequent rains (29.0 mm in April, 100.2 mm in May and 105.2 mm in June 1997) at the time of seed maturity in pea cv. Bonnevile,

onion cv. Pb. Naroya, carrot, cv. Selection 21, radish cv. Pb. Safed, turnip cv. L-1 and cauliflower cv. Pb. Giant –26 caused heavy losses due to rotting and discoloration of the seeds. For each of the species, data were tabulated on frequency of 7 fungal species after 0, 2 and 4 months of storage under ambient conditios, beginning in June 1997. Pea seeds suffered the greatest damage during storage and their viability declined from 86.6 to 28.5%. The major damage in stored seed was caused by *Aspergillus flavus, A. niger, A. fumigatus, Mucor* spp. *Rhizopus* spp. *Penicillium* and *Fusarium* spp. After storage the seeds were treated with 0.2% Thiram 75WP, captan 75WP and Bavistin [carbendazim] 50WP before sowing. Treatment with Bavistin in pea and with Thiram and Captan in the other crops significantly improved seed germination. (Sharma, *et. al.* 2000).

Jojoba oil and its derivatives have diversified uses. One of the major threats to Jojoba cultivation is the onslaught of seed mycoflora. Since no information was available on seed mycoflora of Jojoba and attempts were made to find out seed borne fungi and their control (Sharma and Champawat, 2000). A total of five fungal species were isolated from Jojaba seeds. Among these *Aspergillus flavus, A. niger* and *Fusarium palidoroseum* were found pathogenic which caused significant reduction in seed germination, root and shoot length of seedlings. These fungi also caused pre and prost emergence mortality of seedlings. Among five seed dressing fungicides tested, bavistin, vitavax and thiram were found effective against these mycoflora resulting in increased germination and seedling vigour.

To know the incidence and the extent of various fungi associated with different varieties and lots of chilli seed about 50 samples were collected from Maharastra state seed corporation, Akola and subjected to standard blotter test as per ISTA method. Observations revealed that *Aspergillus flavus, Rhizopus stolonifer, Fusarium moniliforme, Colletotrichum* capsici and *Aspergillus niger* were the predominant seed borne fungi, the incidence of individual fungus being in the range of 11.56 to 31.42 per cent. When artificially inoculated on ton seed, the individual isolate of the seed borne

fungus was capable of causing pre-emergence as well as post-emergence mortality to a significant extent reported by Asalmol *et. al.* (2001).

The growth inhibitory effect of *Cymbopogon nardus* (L.) W. Watson var. *nardus* essential oil on *Aspergillus niger* (Van Tieghem) mycelium was determined on agar medium by Billerbeck *et. al.* (2001). The mycelial growth was completely inhibited at 800 mg/L. This concentration was found to be lethal under the test conditions. Essential oil at 400 mg/L caused growth inhibition of 80% after 4 days of incubation, and a delay in conidiation of 4 days compared with the control. Microscopic observations were carried out to determine the ultra structural modifications of *A. niger* hyphae after treatment with *C. nardus* essential oil. The main change observed by transmission electron microscopy concerned the hyphal diameter and the hyphal wall, which appeared markedly thinner. These modifications in cytological structure might be caused by the interference of the essential oil with the enzymes responsible for wall synthesis which disturb normal growth. Moreover, the essential oil caused plasma membrane disruption and mitochondrial structure disorganization. The findings thus indicate the possibility of exploiting *Cymbopogon nardus* essential oil as an effective inhibitor of biodegrading an storage contaminating fungi.

During storage, several kinds of fungi can remain associated to corn seeds, either causing their deterioration or simply remaining viable to infect germinating seedlings. Tanaka *et. al.* (2001) studied the survival of fungi in corn seeds stored for twelve months in a cold chamber (14^0C and 40% RH) or stored under uncontrolled conditions. A larger frequency of *Altermaria alternata, Bipolaris maydis, Cephalosporium acremonium, Cladosporium herbarum, Fusarium moniliforme, Rhizoctonia solani, Rhizopus* spp. and *Trichoderma* spp. occurred in the cold condition. The survival decreased with the storage period mainly under uncontrolled atmosphere as compared to the cold environment. The incidence of *Aspergillus* and *Penicillium* increased with the storage, mainly under the uncontrolled ambient. *Fusarium moniliforme,* on the other hand, decreased sharply under ambient conditions at the end of the

period. In the cold chamber, the *F. moniliforme* viability was less affected. *Bipolaris maydis* remained viable in most of the seed lots during the entire storage period in the cold chamber. Under uncontrolled conditions, this fungus survived for four to twelve months, depending on the lot studied. The seed storage under uncontrolled ambient may reduce the inoculum of *F. moniliforme* and other important fungi for the maize crop, but this condition may accelerate seed deterioration. In the cold condition, otherwise, the ambient is favorable to preserve the physiological quality of the seeds, but the maintenance of fungi viability may reduce their sanitary quality.

Purple blotch (*Alternaria porri*) used to be a major disease of onion seed crop in Punjab till late 1980s. However, during the past one decade, downy mildew (*Peronospora destructor*) has emerged as a major disease. Onion seed production, in Punjab, has suffered heavily due to severe outbreaks of this disease causing 60-70% losses due to low seed recovery, poor seed health and vigour. Investigations carried out by Sharma, *et. al.* (2002) on disease control during 1996-97 to 1998-99 indicated that four sprays of Dithane M-45 at 12 days interval, commencing from the time of disease appearance, resulted in significant disease control. The incidence of the disease was higher in unweeded field. Out of seven genotypes screened at 0-9 point scale against this disease, Punjab Naroya showed the minimum (2.0) disease reaction whereas ADR the maximum (7.0). The seed mycoflora studies indicated that species of *Fusarium, Alternaria, Stemphylium,* and *Aspergillus* were found to be associated with the seeds of all the varieties, which got eliminated during storage and were replaced with a storage fungus, *Rhizopus* sp. There was a significant decline in seed germination in all the varieties during storage. Varieties N-53 an ADR were the poor storers whereas PRR and Punjab selection as good storers Though the field and storage fungi accounted for 3-22% seed/seedling mortality, the major cause of loss of germination was physiological (ageing). While studying the effect of pre-storage seed treatments, it was observed that Thiram and Captan had positive effect on seed storability and viability. The germination of all the

treated seed lots, in general, was maintained significantly at a higher level than the control. The fungicidal treatment inhibited the occurrence of storage fungi resulting in upgradation of seed germination.

Seed samples of maize and sorghum from different agro climatic zones of Karnataka were evaluated by Rai *et. al.* (2002) for the occurrence of seed mycoflora and germination. Maize samples showed high incidence of *Fusarium moniliforme.* Fungi like *Verticillium albo-atrum, Trichoderma harzianum, Sclerotium rolfsii, Botrydiplodia theobromae* reduced seed germination to a higher extent. Maize seeds treated with *Sclerotium rolfsii* resulted in higher percentage of abnormality in seedlings. Extracts of *Thuja, Vinca*, lower dosage of cinnamom oil and higher dosage of clove oil favoured the colonization of *F. moniliforme.* Arecanut leachate and neem leaves extract proved to be promising in reducing the incidence of *f. moniliforme* in sorghum.

Bhattacharya and Raha (2002) studied fungal infection, moisture content, germinability and deterioration of three seeds, viz., maize (starchy), groundnut (oily) and soybean (proteinaceous) in storage at the locality of Santiniketan, West Bengal, India, under natural condition for 1 year. The airspora of storage environment was trapped using culture plate method. Different species of *Aspergillus* (*A. candidus, A. flavus, A. niger, A. terreus,* and *A. ruber*) were dominant followed by *Rhizopus, Penicillium, Curvularia, Fusarium, Altermaria,* etc. Seed moisture was maximum in the rainy season followed by a gradual decrease during longer storage. A gradual decrease in field fungi with simultaneous increase in storage fungi accompanied by a reduction in germinability occurred in all seeds as storage proceeded. A gradual loss of carbohydrate (both soluble and insoluble) content in all the seeds were recorded. A loss of protein content was recorded followed by a small increase. Oil content decreased in prolonged storage with simultaneous increase in fatty acid.

Investigation were conducted by Tagne *et. al.* (2003) to determine the presence of mycotoxigenic fungi in maize samples from Cameroon. The deep freezing blotter method, and the medium DG-18, with and without 1% sodium hypochlorite surface sterilization pre-treatment, were used. The plated grains were incubated for 7 days under a cycle of 12 h (NUV) daylight and 12 h darkness. *Fusarium pallidoroseum* was found infecting 1-2% of the tested grains. Eleven samples out of 65 tested were found infected, 2 samples from the locality of Melong in the humid forest agro-ecoogical zone with monomodal rainfall, 1 from Foumbot and 2 from Bamenda in the highlands agro-ecological zone, and 6 from Yaounde, in the humid forest with bimodal rainfall agro-ecological zone. An infection rate of 2% of the grains was found on blotter paper while only 1% was recorded on the reduced water activity medium DG-18, with or without surface sterilization. *F. pallidoroseum* was reported here for the first time from maize samples of Cameroon. This underlines the need for detailed research on toxigenic fungi and the improvement of common storage practices to avoid mycotoxin contamination and the resulting human and animal health problems.

Although fungi cause a recognized problem during storage of recalcitrant seed of many tropical species, there are no data to date on defence strategies of these seeds against fungal attack. To ascertain whether recalcitrant seeds of *Avicennia marina* elaborate compounds that might suppress fungal proliferation during hydrated storage, the production and efficacy of β-1, 3-glucanase (EC 3.2.1.39) an chitinase (EC 3.2.1.14) were studied by Merhar *et, al.* (2003) in relation to histopathological changes. Freshly harvested seeds had low β-1, 3-glucanase and chitinase activities and fluorescence microscopy revealed progressive deterioration of the internal tissues of these seeds associated with fungal infection during hydrated storage. In seeds treated to minimize associated fungi (clean seeds) β-1, 3-glucanase and chitinase activities increased significantly during 10 d of hydrated storage. Similar high levels of activity were observed when these seeds were experimentally infected with *Fusarium moniliforme* and subjected

to further storage. The histo pathological observations indicated delayed disease development in the 10-d clean-storage period, although the hypersensitive response was not observed. The results suggest that, although the recalcitrant seeds of *A. marina elaborate* some antifungal enzymes, there is a lack of effective defence strategies that might lead to successful responses against fungal infections.

The mycoflora levels of afllatoxins and the presence of ochratoxin A and zearalenoue in nuts of *Cola acuminata* and *C. nitida* were determined immediately after curing and after 3, 6, and 9 months of storage in leaf lined baskets. Five field fingi and 11 storage fungi were isolated. *Aspergillus, Penicillium* and *Fusarium* were the predominant genera. None of the target toxius was detected immediately after curing. Increasing quantities (5 to 160 Ppb) of each of the aflatoxius B_1, B_2, G_1 and G_2 were recorded as from the 3rd month while zearalenone and ochratoxin A were detected only after the 6th and 9th month, respectively. (Adebajo and Popoola 2003).

Seven fungal species viz. *Alternaria raphani, A. tenuissima, A. oryzae, Chaetamella hormida, Humicola* grisea, *Penicillium Ochracium* and *Stachybotrys tabulates* are new records on seasonal (*sesamum indicum* L.) Seeds from almora in India. *Alternaria* alternata, *Aspergillus flavus, A. niger curvularia lunata, Fusarium oxysporum* and *Penicillium chrysogenum* caused 30-61% inhibition in seed germination while *Fusarium oxysoporum* caused maximum 34% seedling rot. The vigour of seedling was found poor in case of seeds inoculated with *Alternaria alternata, A. sesami, Aspergillus flavus, A. niger, Curvularia lunata, Fusarium oxysporum* and *Penicillium chrysogenum. Alternaria alternata, A. sesami, cercospora sesami* and *corynospora cassiicola* weri found pathogenic on plant inoculation and caused leaf spot or blight diseases. Afternaria alternala, *A. sesani, Aspergillis flavous, A. niger, Fusarium oxysporum* and *Penicillium chrysogenum* were the main seed rotting fungi. (Khati and Pandey 2004).

Thirty six fungal species were isolated (by Singh *et. al* 2004) from 134 seed samples of pearl millet (*Pennisetum typhoides*) (Burm. F) (Stapft and Hubb) collected from 21 districts of Rajasthan. Among them *Alternaria alternata, Aspergillus flavus, A. fumigatus, A. niger, Curvularia lunata, C. pallescens, Drechshlera halodes, D. hawaiiensis* and *Fusarium oxysporum* were dominant pathogenic fungi which caused seed discolourations, loss is seed germination and seedling diseases.

Effect of relative humidity (RH) and temperature on seed microflora and biodegradation of *Albizzia lebbeck* (Sirris) seeds stored for three months was investigated by Gupta *et al.* (2004). Storage fungi succeeded the incidence of field fungi during initial period of storage, with the increase in relative humidity and temperature during storage. Among bacteria *Enterobacter, Proteus* and *Pseudomouas aeruginosa* were encountered at high RH. The levels of total soluble protein, phenol and soluble sugar increased gradually with increase in the RH and temperature while that of starch decreased. Aflatoxin production was higher at 28^0C and at 95% RH as compared to other temperature and relative humidity regimes.

Aspergillus flavus Link Ex. Fries commonly occurs in food grains as storage mould (Kumar and Singh, 2004). In asymptomatic seeds the infection was extra-embryonal. Mouldy symptomatic, weakly to heavily infected seeds carried intercellular mycelium in seed parts. The fungal inoculum in seed caused histological disorganization of embryonal cells. The seed borne inoculum during germination caused browning of radicale and root-shoot transition zone followed by brown to black streaks on hypocotyls. The rate of disease transmission increased proportionally with increase in extra and intra embryonal infection. But seeds with a high degree of intra embryonal infection (45-8.5%) mostly rotted.

MATERIALS & METHODS

3.1. Source of seeds

Freshly harvested seeds of three different pulses viz. Mungbean (*Vigna radiata* (L). R.Wilzek, Cowpea (*Cajanas cajan* (L.) Mill) and Lentil (*Lens* culinaris Medik) were collected from Pulses and Oils Seed Research Station, Berhampore, West Bengal.

For each of these 2-3 varieties were obtained as follows:

Mungbean — B_1 and Pusa Baishakhi.
Arhar (cowpea) — ICPL-87, Rubi-20/105.
Lentil — Asha, Ranjan and Subrata.

These were then stored for various periods in the laboratory and used for experimental purposes (Plate II).

3.2. Storage conditions

All seeds were sorted and distributed in small paper packets and stored under 3 different conditions.

(i) **Ambient condition :** In this case seed packets were left on the shelf of the laboratory under normal conditions of temperature and humidity. ($30+2^0C$).

(ii) **Controlled humidity:** In order to maintain seeds under low humidity, seed packets were stored in a desicator at a moisture level <15% using glycerol as described by Braun & Braun (1958) and the mouth of the desicator was sealed. In this case, the desiccators were kept under normal temperatures.

(iii) **Low temperature and controlled humidity:** Seeds were stored in desiccators as described above which were then kept in a refrigerator at a temperature of 10 ± 1^0C.

3.3. Isolation of fungi

Sampling of seeds was done after every 3 months. Seeds were removed from the different storage conditions and used for isolation. Initially

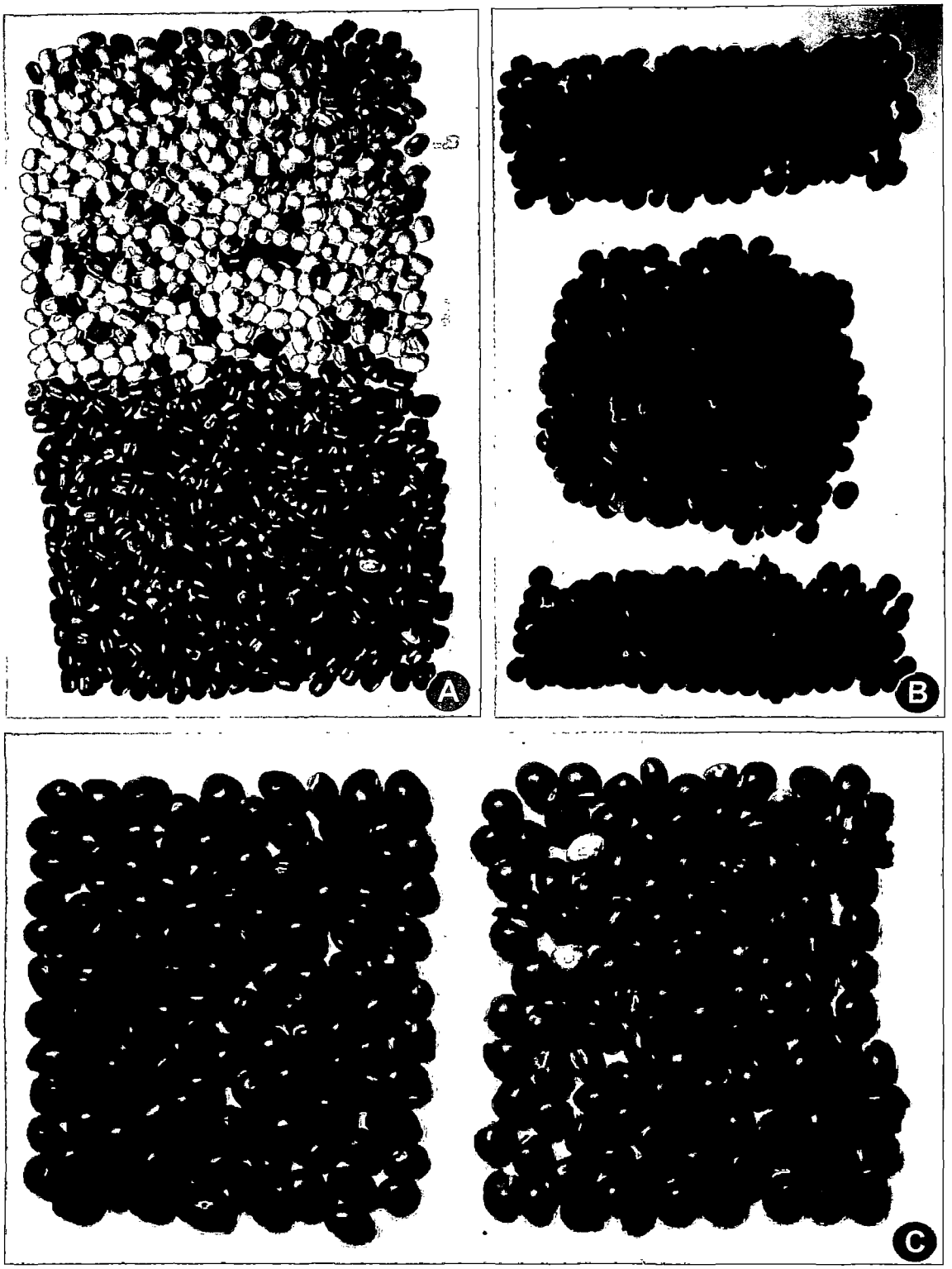

Plate-II: Seeds of different pulses.

A. *Vigna radiata* B1 = top; Pusa Baishakhi = bottom.
B. *Lens culinaris* Asha = top; Ranjan = Middle; Subrata = bottom.
C. *cajanas cajan* Rabi 20/105 = Left; ICPL 87 = Right.

fungi were isolated from seeds by different methods but finally Agar plate method as recommended by ISTA (1966) was selected and used for isolation. Several media were initially screened for isolation of fungi and finally Potato Dextrose Agar (PDA) was selected. PDA medium was prepared and poured into petriplates (9cm). Seeds were surface sterilized with 0.1% $Hgcl_2$ to remove surface contaminants for a period of 5 minutes. Following several washings with sterile distilled water, these were then put on the PDA plates @ 10 per plate. Observations were recorded after 24 and 48 hours and single colonies of fungi were isolated. These Isolated fungi were maintained on PDA with regular subculturings.

3.4. Identification of Isolates

Identification of fungi was done on the basis of morphological and microscopic studies in consultation with literature. For morphological studies observations regarding the colour and nature of hyphae, sporulation time, colour of spores, growth pattern etc. were recorded during different periods of growth. For microscopic studies, after sporulation, slides were prepared, stained with lactophenol-cotton blue and observed under bright field microscope. Type of hyphal structure, spores/ conidia, conidiophore etc. were noted. All these parameters were recorded and used for identification purposes.

3.5. Testing of viability

Seeds of the different pulses were surface sterilized with 0.1% $Hgcl_2$, washed with sterile distilled water and soaked in sterile distilled water overnight. Following this, seeds were taken out and placed on moist blotting paper 9cm petri plates which were previously sterilized. Seeds were allowed to germinate and the percent germination noted after a period of 4-5 days.

3.6. Determination of Percentage frequency

For the determination of the frequency of appearance of a particular fungal species, the number of times a particular species appeared, out of the

total observation was recorded. Percentage frequency was then calculated as follows.

$$\% \text{ Frequency} = \frac{\text{No of observatios in which a species appeared}}{\text{Total no. of observation}} \times 100$$

3.7. Determination of cultural characteristics of the isolated fungi

Detailed growth characteristics of two fungal species, which were selected was done. For this, the fungi were grown in Potato Dextrose Broth (PDB) for varying periods and their mycelia harvested at intervals of 2,4,6 and 8 days. Further, the fungi were also grown in different liquid and solid media- i.e. Richard's, Czapek Dox, Potato sucrose and Carrot juice. Fungal growth in each medium was determined after 6 days. In case of solid media, agar was added to each medium, poured into Petri plates (9cm), and inoculated with inoculum block. Growth was observed every 24h.

Composition of media:

Potato Sucrose Agar

Peeled potato	-	40.00 g
Sucrose	-	2.00 g.
Agar	-	2.00 g.
Distilled water	-	100 ml.

RA (Richards Agar)

KNO_3	-	1.00 g
KH_2PO_4	-	0.500 g
$MgSO_4$, $7H_2O$	-	0.25 g
Sucrose	-	3.00 g
Agar	-	2.00 g
Distilled water	-	100 ml

Elliot's Agar (E.A)

KH_2PO_4	-	0.136 g
$MgSO_4$, H_2O	-	0.050 g
Na_2CO_3	-	0.106 g
Dextrose	-	0.500 g
Asparagine	-	0.10 g
Agar	-	1.5 g
Distilled Water	-	100 ml

Czapek – Dox-agar (CDA)

$NaNO_3$	-	0.20 g
K_2HPO_4	-	0.10 g
KCl	-	0.05 g
$FeSO_4$, $7H_2O$	-	0.5 g
Sucrose	-	30.00 g
Agar	-	2.00 g
Distilled water	-	100 ml.

Carrot Juice Agar

Grated carrot	-	20.00 g
Agar	-	2.00 g
Distilled water	-	100 ml

Potato Dextrose Agar

Peeled potato	-	40 g
Dextrose	-	2 g
Agar	-	2 g
Distilled water	-	100 ml

3.8. Inoculation with selected fungi

3.8.1. Spore dusting

For determining the effect of specific storage fungi on particular seeds, 2g of seeds were taken separately in polythene bag and were mixed with spore of individual fungal species obtained from their culture (Purushotham *et. al.,* 1996). These were then shaken and kept in the laboratory for a period of 30 days prior to sampling.

For experimental purposes seeds were taken out from the spore suspension and washed to remove adhering spores.

3.8.2. Dipping in spore suspension

In studies where germinated seeds were taken, inoculation of the fungal species was done by preparation of a spore suspension and dipping seeds for a period of 48h. After this seeds were kept in petriplates and allowed to germinate, as mentioned above. In case of uninoculated control, seeds were soaked in sterile distilled water.

3.9. Soluble protein

3.9.1. Extraction

3.9.1.1. Mycelial protein

Mycelial protein was prepared following the method of Chakraborty *et al.* (1995). Initially the inoculum (6mm) disc containing mycelium) was transferred to 250ml Erlenmeyer flask each containing 50 ml of sterilized liquid Potato dextrose medium and incubated for 7 days at 30± 1^0C. For extraction of soluble protein, mycelial mats were harvested, washed with 0.2% Nacl and rewashed with sterile distilled water. Washed mycelia (25g fresh wt.), were homogenized with 0.05M sodium phosphate buffer pH (7.2) supplemented with 10mM sodium metabisulphite and 0.05mM magnesium chloride in mortar with pestle in the presence of sea sand. Cell homogenates were kept overnight at 4^0C. This was equilibrated to 100% saturated ammonium sulphate under constant stirring and kept overnight at 4^0C. After this period the mixture was centrifuged (1000 or pm) for 15 min at 4^0C and the precipitate was dissolved in 5ml 0.05M sodium phosphate buffer (pH 7.2). The preparation was dialyzed for 72h through cellulose tubing (Sigma Chemical Co. USA) against 1L of 0.005M sodium phosphate buffer (pH 7.2) with ten changes. Then the dialysed material was stored at – 20^0C and used as antigens for raising of antiserum and other experiments.

3.9.1.2. Seed Protein

Soluble proteins were extracted from healthy and treated seeds following the method of Chakraborty *et. al.* (1995). Seed tissues (1g) were homogenized with 0.05 M sodium phosphate buffer (pH 7.2) containing 10m M $Na_2S_2O_5$ and 0.5mM $Mgcl_2$, in mortar with pestle at 4^0C using sea sand. The homogenate was centrifuged at 4^0C for 20min at 10,000 r.p.m. and the supernatant was used as crude protein extract and immediately stored at – 20^0C for further use.

3.9.2. Estimation

Soluble proteins were estimated following the method as described by Lowry *et. al.* (1951). To 1ml protein sample (taking 10^{-1} or 10^{-2} dilution) 5 ml of alkaline reagent (0.5ml of 1% $Cuso_4$ and 0.5ml of 2% sodium potassium tartarate added to 50ml of 2% Na_2CO_3 in 0.1 (N) NaOH) was added and incubated for 15-20 min at room temperature. Then 0.5 ml of Folin ciocalteau reagent (diluted 1:1 with distilled water) was added and again incubated for 15 min. Absorbance values were measured at 700nm in a Systronics photoelectric colorimeter (Model 101) Quantity of protein was determined from standard curve made with bovine serum albumin (BSA).

3.9.3. SDS-PAGE analysis of total soluble protein

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for the detailed analysis of protein profile following the method of Sambrook *et. al.* (1989).

3.9.3.1. Preparation of stock solutions

The following stock solutions were prepared :

A. Acrylamide and N'N'. methylene bisacrylamide

A stock solution containing 29% Acrylamide and 1% bis acrylamide was prepared in warm water. As both of them are slowly denatured to acrylic and bis acrylic acid by alkali and light, the pH of the solution was kept below 7.0 and solution was prepared in diffuse light. Stock solution was filtered through whatman No. 1 filter paper and was kept in brown bottle, stored at 4^0C and used within one month.

B. Sodium Dodecyl sulphate (SDS)

A 10% stock solution of SDS was prepared in water and stored at room temperature.

C. Tris buffer :

(a) Lower gel buffer (1.5M Tris) 1.5M Tris buffer was prepared for resolving gel (pH adjusted to 8.8 with concentrated HCl and stored at 4^0C for use).

(b) Upper gel buffer (1.0M Tris). 1.0M Tris buffer was prepared for use in the stacking and loading buffer. (pH adjusted to 6.8 with HCl and stored at 4^0C).

D. Ammonium peroxidisulphate (APS):

Fresh 10% APS solution was prepared with distilled water each time before use.

E. Tris-Glycine electrophoresis buffer:

This is a running buffer and consists of 25mM Tris base, 250mM glycine (pH-8.3) and 0.1% SDS, A1X solution was made by dissolving 3.02 g Trisbase, 18.8g glycine and 10ml of 10% SDS in IL of distilled water.

F. SDS loading buffer

Tris buffer consists of 5mM Tris HCl (pH 6.8), 10mM β mercaptoethanol, 2% SDS, 0.1% bromophenol blue and 10% glycerol. A 1X .solution was made by dissolving 0.5ml of 1M Tris buffer (pH 6.8), 0.5ml of 14.4 M β mercaptoethanol, 2ml of 10% SDS, 10mg bromophenol blue and 1ml glycerol in 6.8 ml of distilled water.

3.9.3.2. Preparation of Gel

Slab (8 cm x 10cm) was prepared for the analysis of protein pattern by SDS-PAGE (Mini gel) For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. Resolving and stacking gels were prepared by mixing compounds in the following proportion.

Composition of solution for 10% resolving gel :

Constituents	Quantity
Distilled water	2.85 ml
30% acrylamide mix	2.55 ml
1.5M Tris (pH-8.8)	1.95 ml
10% SDS	0.075 ml
10% APS	0.075 ml
Temed	0.003 ml

Composition of solutions for 5% stacking gel :

Constituents	Quantity
Distilled water	2.1 ml
30% acrylamide mix	0.5 ml
1.5 M Tris (pH 6.8)	0.38 ml
10% SDS	0.03 ml
10% APS	0.03 ml
Temed	0.003 ml

After pouring the resolving gel solution, it was immediately over layered very gently with isobutanol and kept for polymerization for 2 hours. After polymerization of the resolving gel was complete overlay was poured off and washed with distilled water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and comb was inserted immediately, over layered with water and kept for polymerization. After polymerization the comb was removed and washed thoroughly. The gel

was then finally mounted in the electrophoresis apparatus. Tris-glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bent syringe.

3.9.3.3. Sample preparation

Sample (32µl) was prepared by mixing the sample protein with 1xSDS gel loading buffer (16µl) in cyclomixer. The samples were taken in Eppendorf tubes and floated in boiling water bath for 3 min to denature the protein sample. The samples were immediately loaded in a pre determined order into the bottom of the wells with a microliter syringe. Along with the samples, protein markers consisting of a mixture of six proteins ranging in molecular weight from high to low molecular weight (phosphorylase B-97.4, bovine serum albumin – 68, ovalbumin – 43, carbonic anhydrase-29, soybean trypsin inhibitor – 20 and Lysozyme – 14 kDa) was treated as the other samples and loaded in a separate well.

3.9.3.4. Electrophoresis

Electrophoresis was performed at constant 15mA current for a period of 3 hours until the dye front reached the bottom of the gel.

3.9.3.5. Fixing & Staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid: methanol : water (10:20:70) for overnight.

The staining solution was prepared by dissolving 250mg of coomassie brilliant blue (Sigma R 250) in 45 ml of methanol. After the stain was completely dissolved, 45 ml of water and 10 ml of glacial acetic acid were added. The prepared stain was filtered through What man No.1 filter paper.

The gel was removed from fixer and stained in this staining solution for 4 h at 37^0C with constant shaking at very low speed. After staining the gel was finally destained with distaining solution containing methanol, water and

acetic acid 4.5:4.5:1 at 40^0C with constant shaking until the back ground became clear.

3.10. Carbohydrates

3.10.1. Extraction of carbohydrate

For extraction of carbohydrate, method of Harborne (1973) was followed. Fresh tissue (1g each) was crushed with 95% ethanol in mortar with a pestle. The mixture was centrifuged for 20 min at 5000rpm in a table centrifuge and the supernatant was collected. Then the alcoholic fraction of the supernatant was evaporated off on a boiling water bath. Finally, the volumes were made upto a known volume with distilled water.

3.10.2. Estimation of carbohydrate content

3.10.2.1. Total sugar

Estimation of total sugar were done by following Anthrone method as described by Plummer (1978).

To 1ml of each test solution 4ml of Anthrone reagent was added and mixed thoroughly. Mixtures were placed in boiling water bath for 20 minutes. The test tubes were then cooled under running tap water and the absorbance was measured at 620nm in a Systronic Photoelectric colorimeter (Model, 101). Quantification was done from a standard curve of glucose.

3.11. Enzymes

3.11.1. Amylase

3.11.1.1. Extraction of Amylase

1g of fresh tissue was weighted out and crushed in a mortar with 10ml of ice cold citrate buffer (25mM, pH 5.0). The brei was centrifuged at 10,000 rpm for 10 minutes and supernatant was taken for analysis of starch degrading activity.

3.11.1.2. Assay of Amylase activity

0.1 ml of the extract was added to 5ml of starch solution and was mixed thoroughly. The mixture was incubated for 15 minutes at room temperature. After incubation 0.2 ml of iodine reagent was added to which 5ml of water was further added. The colour intensity was measured in a colorimeter at 670 nm against an appropriate blank. In case of blank 0.1 ml of buffer was added in place of the enzyme extract. The concentration of starch in case of blank, and enzyme treated extracts was determined from a standard curve of starch. Differences in these values gave the amount of starch hydrolysed by the enzyme. Activity was expressed as µg/mg starch hydrolysed by enzyme extract from 1g tissue per minute.

3.11.2. Protease

3.11.2.1. Extraction of Protease

Protease was extracted from seed samples following the method described by Jayaraman (1996). 1g of seed was weighted and then crushed with sodium phosphate buffer (.05M, pH 7.2) using mortar and pestle. The homogenate was centrifuged in a cooling centrifuge at 10,000 rpm for 10 minutes. The supernatant was collected and used as enzyme source.

3.11.2.2. Assay of Protease activity

Assay was performed on the basis of the amount of protein degraded by the enzyme, following the method suggested by Jayaraman (1996). Assay depended on quantifying of a protein solution by the enzyme. For this, initially (0.2ml of enzyme extract was taken to which 1ml of casein solution was added and incubated for 1hr. at 37^0C. But for blank there was no incubation. 1ml of 12% Trichloroaectic acid (TCA) was added to each tube and centrifuged for 15 mins at 10,000 rpm in cooling centrifuge (Remi-C-24 Model). The pellet was collected and to this pellet 2ml of extraction buffer was added. This formed the test solution for determining residual protein

afterenzyme action. The protein content was then quantified by Lowry's methd. 1ml of test solution (crude extract diluted 10^{-2} conc) was taken in each tube. But for control 1ml dH_2O was taken at the place of solution. To it 5ml of alkaline reagent was mixed thoroughly. The mixture was allowed to stand at room temperature for 15 mins. Addition of 0.5 ml folin ciocalteau diluted 1:1 with distilled water was done rapidly. Reaction mixtures were again allowed to stand at room temperature for 15 mins after mixing well. Finally absorbance values were noted in a systemic colorimeter at 720 nm. Concentration was calculated from a standard come of BSA.

3.12. Preparation of Antigen

3.12.1. Fungal Antigen

Preparation of mycelial antigen was done following the method described by Chakraborty *et. al.* (1995) described earlier in soluble protein extraction procedure.

3.12.2. Seed Antigen

Preparation of seed antigen was done following the method described by Chakraborty *et. al.* (1995) described earlier in soluble protein extraction procedure.

3.13. Polyclonal antibody preparation

3.13.1. Rabbits and their maintenance

Polyclonal antibody (i.e. antisera) for fungal were produced in New Zeland white male rabbits, Approximately 2kg of body weight of the rabbit is needed (Alba & Devay, 1985) for immunization. So, before immunization, the body weights of rabbits were recorded and were observed for at lest one week inside the cage maintained in Animal House (Antisera Reserve for Plant Pathogens), Dept. of Botany, University of North Bengal. Food given for rabbits were green grass, soaked gram seeds, green vegetables like cabbage, carrots (specially at the time of bleeding schedule). Rabbits were regularly fed in morning and evening providing proper washed and cleaned

utensils. 90-100g/ day gram seeds (soaked in water), alternately with green grass were given for each rabbit. Beside this they were given saline water after each bleeding for three consecutive days. Cages and floor were cleaned with antimicrobial agents every day in the morning for maintaining the hygienic condition.

3.13.2. Immunization

Following the method of Alba and DeVay (1985) and Chakraborty *et. al.* (1995) before immunization, normal sera were collected from rabbit. For raising antisera, intramuscular injections of 1ml antigens (1 mg/ml protein) emulsified in equal volume of Freunds, Complete adjuvant (Difco) were given to each rabbit 7 days after pre-immunization bleeding and repeating the doses at 7 days intervals for 2 consecutive week followed by Freund's incomplete adjuvant (Difco) at 7 days interval upto 10-14 consecutive weeks as required.

3.13.3. Bleeding

Blood samples were collected by marginal ear vein puncture. First bleeding was taken 3 days after sixth week of first immunization and subsequently seven times more every fortnight. During bleeding rabbit was placed on its back on a wooden board, fixed at a 60^0 angle. The neck of the rabbit was held tightly in the triangular gap at the edge of the board and the body was fixed in such a way that the rabbit could not move during the bleeding. The hairs were removed from the upper side of the ear with the help of a razor and disinfected with rectified spirit. Then the ear vein was irritated by xylene and an incision was made with the help of sharp sterile blade and blood samples (4-10ml) were collected in a sterile graduated glass tube. After collection, all precautionary measurers were taken to stop flow of the blood from the puncture. For clotting, the blood samples were kept at 37^0C for 1 h. and the then stored overnight at 4^0C. Then the clot was slightly loosened with sterile needle and antiserum was taken another sterile centrifuge tube and

clarified by centrifugation at 2000g for 10 min at room temperature. Finally antisera were stored at – 20^{0}C until required.

3.13.4. Purification of IgG

3.13.4.1. Precipitation

IgG was purified by Ion exchange chromatography on a DEAE cellulose column following the method of Clausen (1988). The crude antiserum (2ml) was diluted with two volumes of distilled water and then an equal volume of 4.0M ammonium sulphate was added. The pH was adjusted to 6.8 and the mixture was stirred for 16 h at 22^{0}C. Then it was centrifuged at 10,000 rpm for 1 h at 22^{0}C and precipitate was dissolved in 2 ml of 0.02M sodium phosphate buffer, pH 8:0.

3.13.4.2. Column preparation

Approximate 4g of DEAE cellulose (Sigma Co. USA) was suspended in distilled water overnight. The water was drained off and the gel was suspended in 0.005M sodium phosphate buffer, pH = 8.0 and the buffer washing was repeated for 6 times. The gel was then suspended in 0.02M sodium phosphate buffer, pH 8.0 and was applied to a column (2.6 cm wide, 30 cm high) and allowed to settle for 2 h. After the column material had settled, 25 ml of 0.02M sodium phosphate buffer, pH = 8.0 was applied to the gel material.

3.13.4.3. Fraction collection

2 ml ammonium sulphate precipitate was applied at the top of the column and the elution was performed at a constant pH and a molarity continuously changing from 0.02M – 0.3M. The initial elution buffer (1) was 0.02(M) sodium phosphate buffer pH = 8.0. The final elution buffer (2) was 0.3M sodium phosphate buffer pH = 8.0.

Buffer (1) was applied in a lower flask (or tank) in which one rubber tube connection from its bottom was supplying the column. Another connection from its top was connected to upper flask (or tank) containing

buffer (2). The buffer (2) had also connection to the open air. During the draining of buffer (1) to the column, buffer (2) was sucked into buffer (1), thereby producing a continuous rise in molarity. Finally 40x5 fractions each of 5ml were collected and the optical density values were recorded at 280nm by means of UV spectrophotometer (DIGISPEC-200G).

3.13.4.4. Estimation of IgG concentration

IgG concentration was estimated as described by Jayaraman (1996) Absorbance was taken for selected fractions at 280nm and 260nm and then concentration of IgG was calculated by the following formula : Protein concentration (mg/ml) = 1.55 x A 280 – 0.76 x A 260.

3.14. Immunodiffusion

3.14.1. Preparation of agarose slides

Glass slides (6cm x 6cm) were degreased in 90% (v/v) ethanol, ethanol: diethylether (1:1 v/v) and ether, then dried in hot air oven. After drying plates were sterilized inside the petridish each containing one plate. Agarose gel was prepared in 0.05M Tris-barbiturate buffer (pH-8.6). The buffer was heated within conical flask placed in a boiling water bath. 0.9% agarose was mixed to the hot buffer and boiled for the next 15min. The flask was repeatedly shaken thoroughly in order to prepare clear molten agarose and 0.1% (w/v) sodium azide (antibacterial agent) was added to it. For the preparation of agarose gel, the molten agarose was poured on sterilized glass slides (10ml / slides) in laminar flow chamber and kept 15 min for solidification. After that 3-7 wells were cut out with a sterilized cork borer (6/mm dia) at a distance of 1.5-2.0 cm from the central well and 2.0-2.5 from well to well (Peripheral).

3.14.2. Diffusion

Agar gel double diffusion tests were carried out using antigen and antiserum following the method of Ouchterlony (1976). The antigen and undiluted antisera (50µl (well) were pipetted directly inito the appropriate wells

in a laminar chamber. The diffusion was allowed to continue in a moist chamber for 72 h at 25^0C. Appearance of precipitin arc was recorded.

3.14.3. Washing staining and drying of slides

After immunodiffusion, the slides were initially washed with steriled distilled water and then with aqueous NaCl solution (0.9% NaCl and 0.1% NaN_2) for 72 h with 6 hourly changes to remove un reacted antigens and antisera widely dispersed in agarose gel. Then slides were stained with coomassie blue (R-250, Sigma 0.25mg coomassic blue dissolved in a solution of 45 methanol + 45 ml distilled water + 10 ml glacial acetic acid) for 10 min at room temperature. After staining, slides were washed in destaining solution (90ml of methanol : d H_2O (1:1) and 10ml acetic acid) with changes until the back ground become clear. Finally slides were washed with distilled water and dried in hot air oven for 3 h at 50^0C.

3.15. Enzyme linked immunosorbant assay (ELISA)

ELISA tests as out lined by Chakraborty *et. al.* (1996) was carried out using following buffers.

3.15.1. Preparation of buffers

Antigen coating buffer:

Carbonate-Bicarbonate buffer 0.05M pH 9.6.

Stocks:

A. Sodium carbonate – 5.30 g in 1000 ml distilled water.
B. Sodium bicarbonate – 4.2 g in 100ml distilled water.
C. 160 ml of stock A was mixed with 360ml of stock B and pH was adjusted to 9.6.

Phosphate Buffer Saline : 0.15M PBS pH 7.2

Stocks:

A. Sodium dihydrogen phosphate – 23.40 gm in 1000 ml dist. water.

B. Disodium hydrogen phosphate – 21.294 gm in 1000 ml dist. water.

280 ml of stock A was mixed with 720 ml of stock B and the pH was adjusted to 7.2. 0.8% NaCl and 0.02% KCl was added to the solution.

Phosphate buffer saline tween: (0.15M PBS Tween, pH 7.2): to 0.15M PBS, 0.05% Tween – 20 was added.

Blocking reagent (Tris buffer saline pH 8.0)

0.05M Tris, O.135M NaCl, 0.0027M Kcl

Tris – 0.657 gm

NaCl - 0.81 gm

KCl - 0.223 gm.

Distilled water was added to make up volume to 100 ml. then pH was adjusted to 8.0 and 0.05%. Tween 20 and 1% bovine albumin (BSA) were added.

Antisera dilution buffer (0.15M PBS – Tween, pH 7.2):

In 0.15M PBS–Tween, pH 7.2, 0.2% BSA, 0.02% Polyvinylpyrrolidone, 10,000 (pvpp, 10,000) and 0.03% Sodium azide (NaN_2) was added.

P. nitrophenylphosphate (Hi-media): 1mg/ml in substrate (1.0% [w/v] diethanolamine, 3m M NaN_3 pH 9.8).

3.15.2. Direct antigen coated (DAC) ELISA

ELISA was performed following the method as described by Chakraborty *et. al.* (1996). Seed and fungal antigens were diluted with coating buffer and the antigens were loaded (200µl/well) in 96 welled ELISA plate (costar EIA/RIA, strip plate USA) arranged in 12 rows in a (cassette) ELISA plate. After loading, the plate was incubated at 25^0C for 4 hrs. Then the plate was washed 4 times under running tap water and twice with PBS Tween and each time shaken to dry. Subsequently, 200µl of blocking reagent was added and incubated at 25^0C for 1hr. After incubation, the plate was washed as mentioned earlier. Purified IgG was diluted in antisera dilution

buffer and loaded (200µl/well) to each well and incubated at 4^0C overnight. After a further washing anti rabbit IgG goat antiserum labeled with Alkaline phosphatase (diluted, 10,000 times in PBS) was added to each well (200µl/well) and incubated at 37^0C for 2 hrs. The plate was washed, dried and loaded with 200µl of p-Nitro phenyl phosphate substrate in each well and kept in dark for 60 mins. Colour development was stopped by adding 50µl/well of 3N NaOH solution and the absorbance was determined in an ELISA Reader (Labsystem, Multiskan) at 405 mm. Absorbance values in wells not coated with antigens were considered as blanks.

3.16. Immunoblotting

Dot blot was performed following the method suggested by Lange *et. al.* (1989). Following buffers were used for dot-blot.

(i) Carbonate – bicarbonate (0.05 M, pH 9.6) coating buffer.

(ii) Tris buffer saline (10mM pH 7.4) with 0.9% NaCl and 0.05% Tween-20 for washing.

(iii) Blocking buffer 10% casein hydrolysate in 0.05M Tris, 0.5 NaCl, 0.5% Tween 20 pH = 10.3.

Nitrocellulose membrane (NCM; Millipore, H5SMO 5255, 7cm x 10cm, Pore size –0.45 µm Millipore corporation, Bedford) was first cut carefully into the required size and placed inside the template. 2µl of coating buffer (carbonate-bicarbonate buffer) was loaded in each well of the template over the NCM and kept for 25 min to dry.

Following this, 2µl of test samples (antigen samples) were loaded into the template wells over the NCM and kept for 3 h at room temperature. Template was removed and blocking of the NCM was done with 10% non fat dry milk (casein) prepared in TBS for 30 min. Previously raised PAb (IgG) (1:500) was added directly in the blocking solution and further incubated at 4^0C for overnight. The membrane was then washed several times in TBS-Tween-20 (pH 7.4). Enzymatic reactions were done by treating the NCM

membrane with Alkaline phosphatase conjugate (1:7500) for 2 h at 37^{0}C. This was followed by washing for 25 min in TBS-Tween. Substrate [1 tablet each of tris buffer and Fast red, Sigma Chemical) or NBT / BCIP tablet (Sigma) dissolved in 10ml double distilled water] was next added and colour development noted. Finally, reaction was stopped by floating the NCM in deionized water. Colour development was categorized on the intensity of the dots.

3.17. Fluorescence antibody staining and microscopy

Fluorescence antibody staining of mycelia, spores and seed sections was done following the method of Chakraborty and Saha (1994).

3.17.1. Mycelia

Fungal mycelia were grown in potato dextrose broth. After 4 days of inoculation young mycelia were taken out from the flask and kept in eppendrof tube. After washing with PBS (pH 7.2), mycelia were treated with normal sera or antisera diluted (3:10) with PBS (pH 7.2) and incubated for 1 h at room temperature. Then mycelia were washed thrice with PBS – tween (pH 7.2) as mentioned above and treated with goat antirabbit IgG (Whole molecule) conjugated with fluorescein isothiocyanate (Sigma) diluted (1:40) with PBS (pH-7.2) and incubated in dark for 45 min at room temperature. After incubation, mycelia were washed thrice in PBS (pH 7.2) and mounted in 10% glycerol. A cover glass was placed on mycelia and sealed. Then slides were observed and photographed under both phase contrast and uv-florescence condition using Leica Leitz Biomed microscope with fluorescence optics equipped with ultra-violet filter set I-3.

3.17.2. Spores

Fungal spores were collected from 12 days old culture and a suspension of this was prepared with PBS (pH 7.2), taken in micro-centrifuge tube, centrifuged at 3000g for 10 min and the PBS supernatant was discarded. Then 60µl of IgG diluted in PBS (1:1) was added to the spores and

incubated for 90 min at room temperature. After incubation, tubes were centrifuged at 3000g for 10 min and supernatant was discarded. The spores were rewashed 3 times with PBS – tween (pH-7.2) by centrifugation as before and 40µl of goat antirabbit IgG conjugated FITC (diluted 1:40 in PBS) was added and incubated in dark for 1 h. After the dark incubation excess FITC antisera was removed by repeated washing with PBS Tween (pH-7.2) and the spores mounted on glass slides in 10% glycerol jelly and observed under Leica microscope, equipped with I-3 uv- fluorescence filter. Photographs were taken as described before.

3.17.3. Cross section of seeds

Initially, cross sections of healthy and infected seeds (pulses) were cut and immersed in phosphate buffer saline (PBS, pH 7.2). These sections were treated with normal serum or antiserum diluted in PBS (1:40) and incubated for 1 h at room temperature. After incubation, sections were washed thrice with PBS-Tween (pH-7.2) for 15 min and transferred to 40µl of diluted (1:40) goat antiserum specific for rabbit globulins and conjugated with fluorescein isothiocyanate (FITC). The sections were incubated for 30 min in dark. After that sections were washed thrice with PBS-tween as mentioned above and then mounted on a grease free slide with 10% glycerol. Fluorescence of seed sections were observed using Leica Leitz Biomed microscope with fluorescence optics equipped with uv-filter set I-3. Tissue sections were photographed under both phase contrast and UV-fluorescent conditions.

3.18. Immunocytochemical staining

The combination of immuno-chemical and histochemical techniques is feasible for the development of a staining procedure capable of locating specific fungi in host tissue. Subsequent to location of CRA in pulse seed sections by fluorescence studies and detection of *A. niger* and *A. flavus* by ELISA and immunoblotting, immunocyto-chemical staining was performed to determine the specific location of *A. niger* and *A. flavus* in pulse seed tissues. For this the method of Young and Andrew (1990) was followed. In this

process the seed sections of infected seeds were made and incubated in 1% BSA solution for 15 min to prevent nonspecific binding of antibodies to seed tissue. The seed sections were rinsed (30s) three times with washing solution. Then sections were incubated for 1 hr at 37^0C on a rotary shaker in *A. niger* and *A. flavus* Pab at a dilution of 1:100. After incubation the seed segments were washed as above then incubated for 1 hr at 37^0C on a rotary shaker in goat antirabbit IgG 1:500 dilution in direct ELISA buffer containing 0.1% BSA) conguated with alkaline phosphatase (Sigma). After incubation, the seed sections were washed as before, incubated in α–naphtol-As-phosphate plus fast blue BB substrate solution for 40 minutes at 37^0C in the dark. Substrate solution consisted of 0.15 g α–naphthol-As-phosphate (Sigma) dissolved in 2.5 ml of N-N-dimethyl formamide (Sigma), which was added to 500ml of Tris buffer (17g of Tris in 500ml distilled water, pH-9.1). This solution was considered as stock solution. The staining solution prepared immediately before use, consisted of 1mg of fast blue (Himedia) and 5µl of 0.1M $Mgcl_2$ added per ml of stock solution and filtered through Whatman No.1 filter paper. After washing the sections in PBS, these were mounted in glycerol jelly and observed under bright field of the microscope (Leica Leitz Biomed) and photographs were taken in bright field.

EXPERIMENTAL

4.1. Isolation of seed storage fungi and their characterization

Fresh seeds of the three pulses i.e. *Cajanas cajan, Vigna radiata* and *Lens culinaris* obtained from the Pulses and oils seeds Research Centre Berhampore were stored upto 18 months under three conditions i.e.

(1) Laboratory condition with ambient temperature and humidity; (2) In the desiccator under controlled humidity and (3) in cold (approx 15^0C) with controlled humidity as described under materials and methods.

Seeds were taken out at three months intervals and fungi isolated from these seeds using standard procedures as described under materials and methods. *Cajanas cajan,* two varieties (ICPL 87 and Rabi 20/105) *Vigna radiata,* two varieties (B_1 and Pusa-Baishakhi) and *Lens culinaris,* three varieties (Asha, Ranjan and Subrata) were selected.

It was observed that a number of fungal colonies appeared in all cases within 7 days of placing on agar media (Plate-III, Figs. 1-3) No differences were observed among varieties (Plate IV) but period of storage, humidity and temperature had effect on the appearance of the colonies. Colonies of different fungal isolates were taken into pure culture and used for characterization. In each case 6 replicate plates were taken for each treatment (Plates V-VII).

Number of fungal isolates increased in all cases upto 9 months of storage after which a decline was observed. Among the three conditions of storage maximum fungal isolates were obtained under laboratory conditions of ambient temperature and humidity and the least in low temperature storage under low humidity. Results in between these two were obtained in case of storage under ambient temperature and low humidity (Tables 1-3) Analysis of variance of data revealed that in all cases significant differences were obtained between the periods of storage. For the appearance of colonies at different conditions, difference between ambient and the other two were

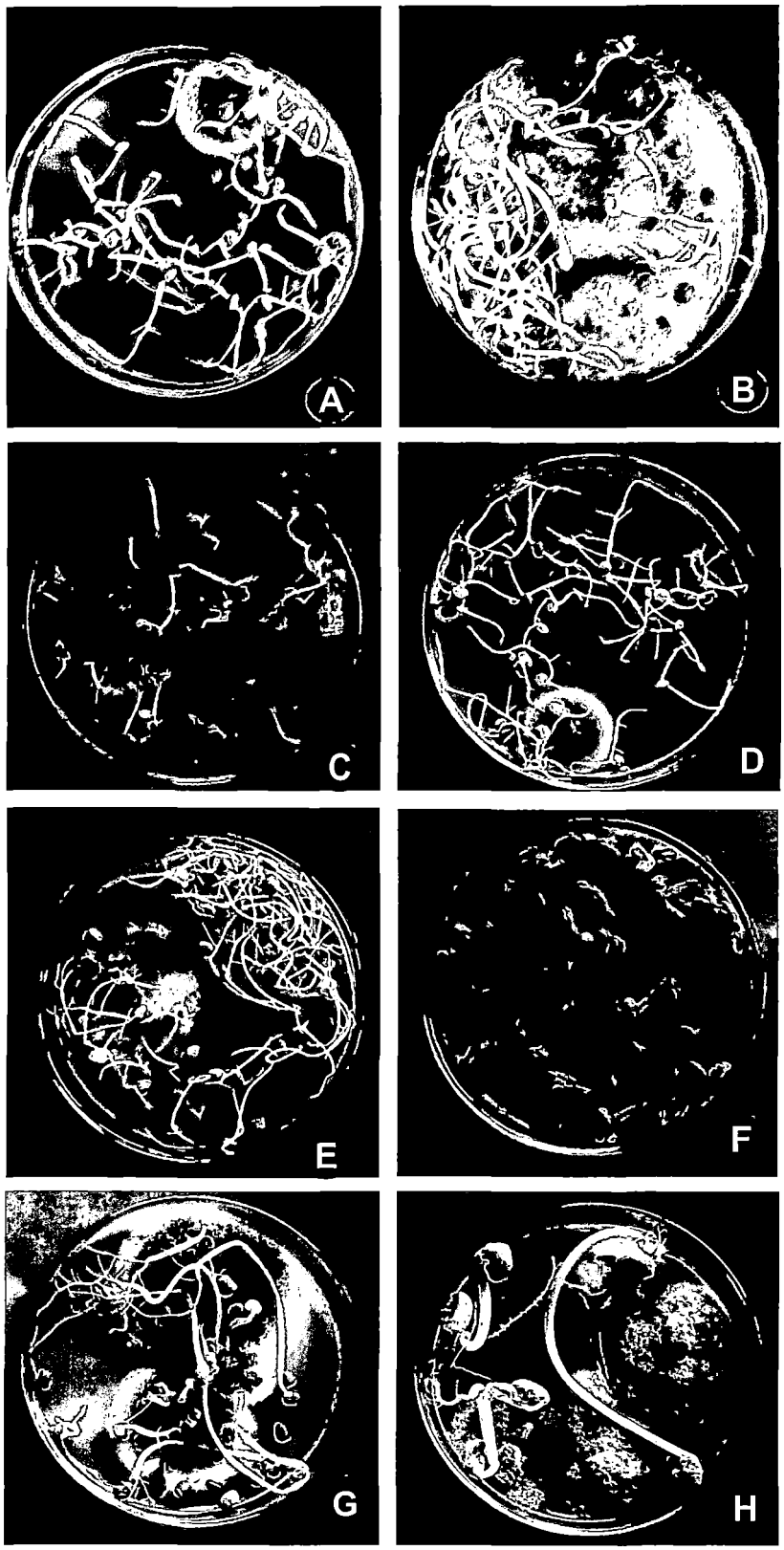

Plate-III: (Figs A-H): Various isolates from different pulses
A-E = *Lens culinaris*
F,G = *Vigna radiata*
H = *Cajanas cajan*

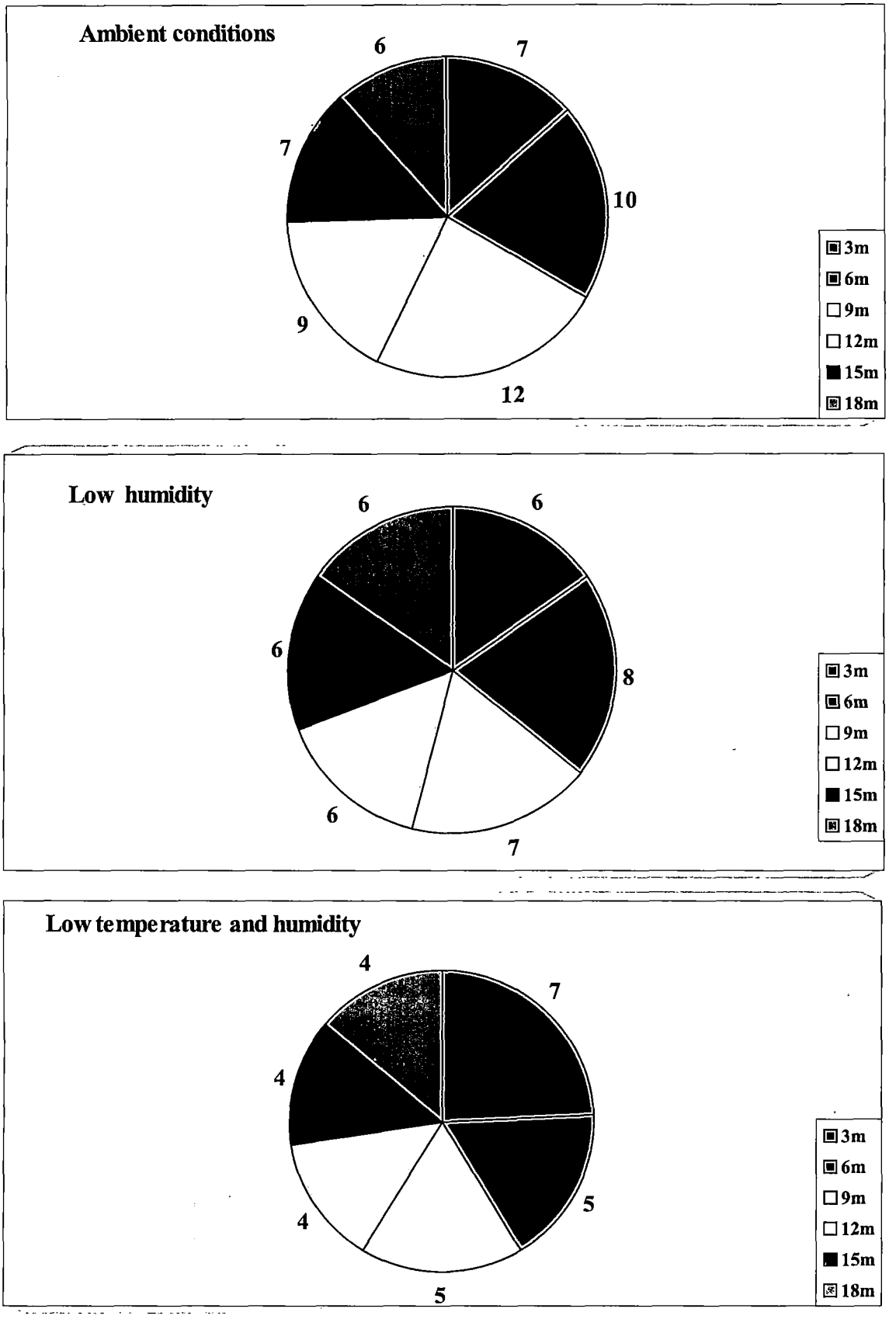

Fig.1: Isolation of fungi from seeds of *Cajanus cajan* stored under different conditions and period.

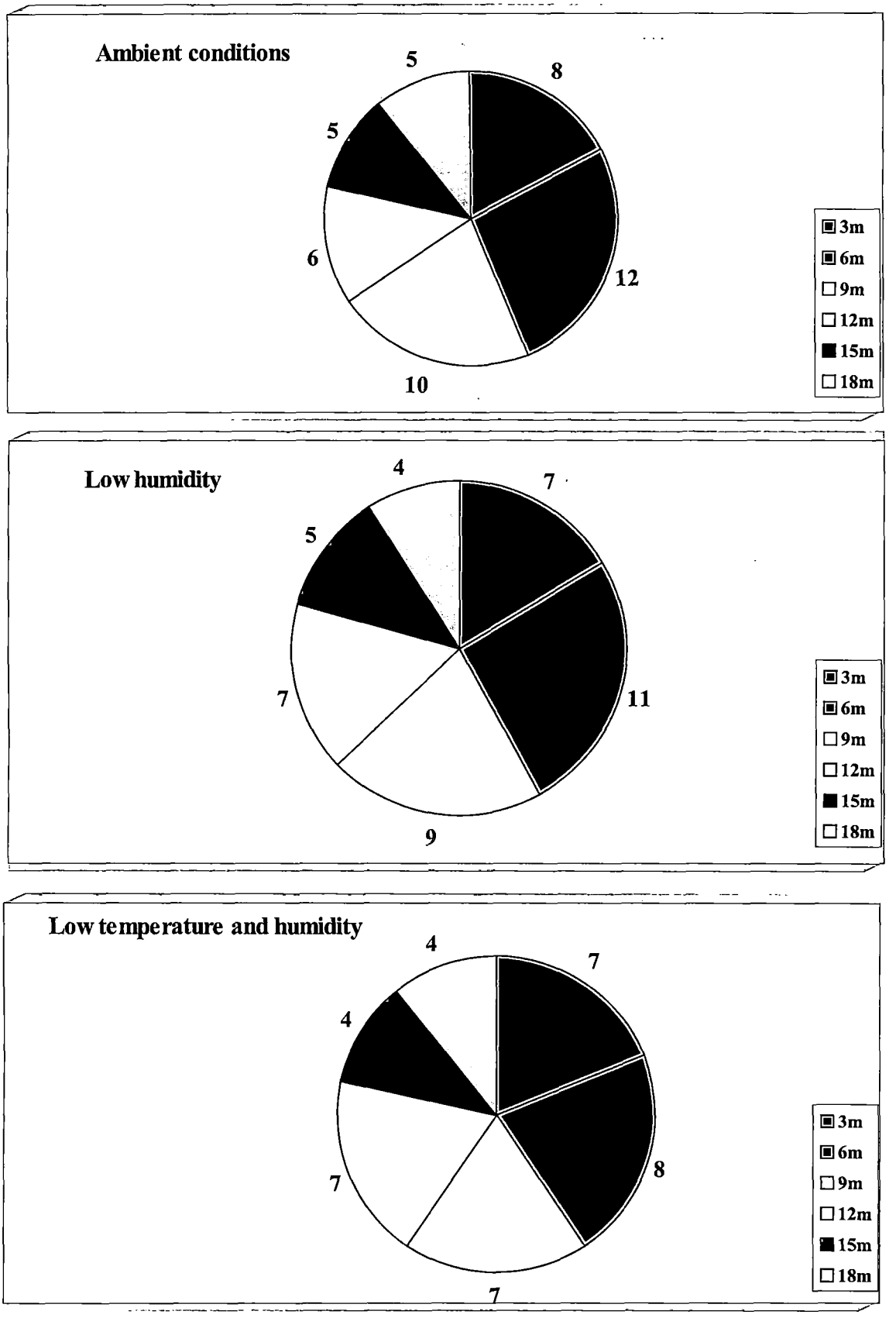

Fig.2: Isolation of fungi from seeds of *Vigna radiata* stored under different conditions and period.

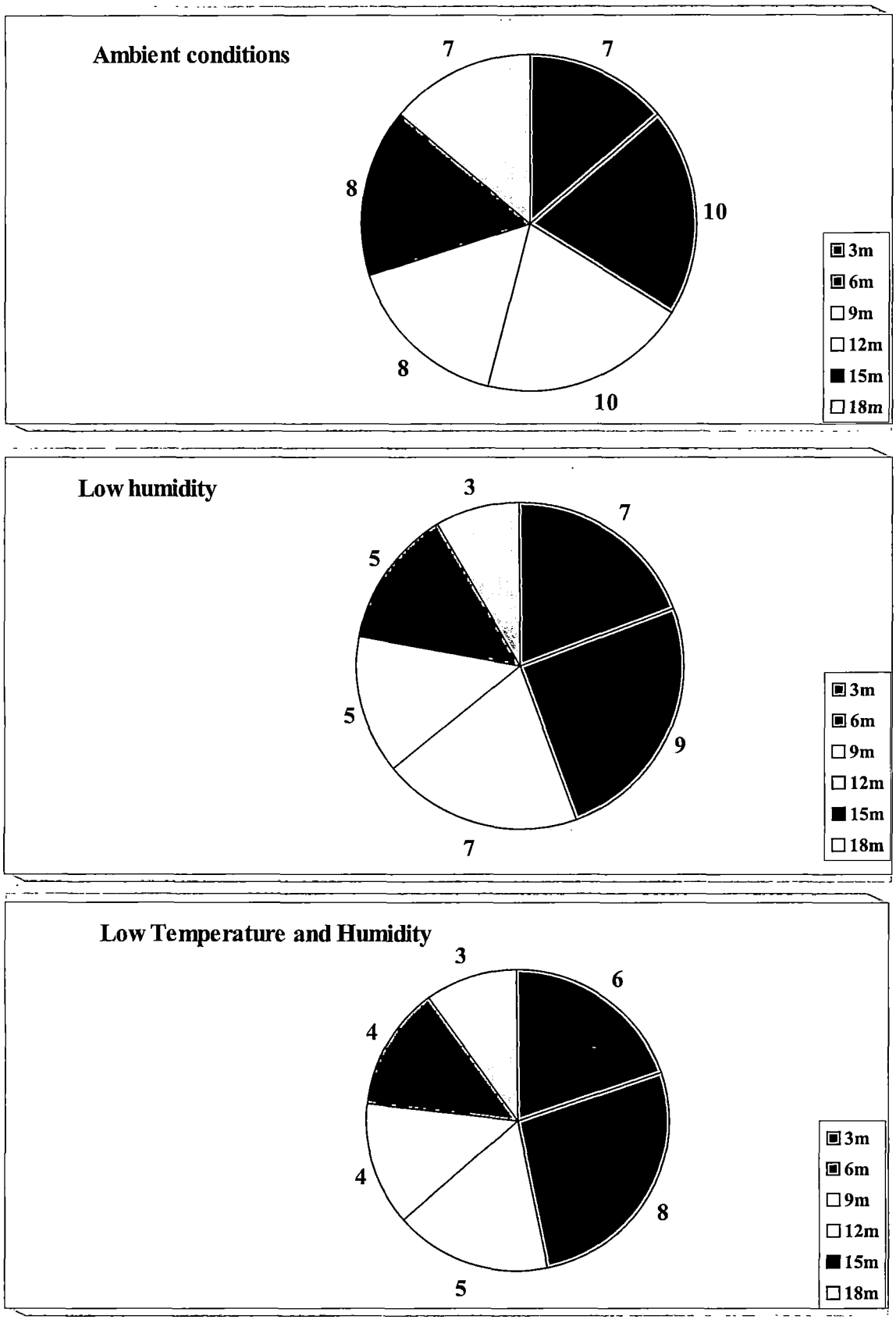

Fig.3: Isolation of fungi from seeds of *Lens culinaris* stored under different conditions and period.

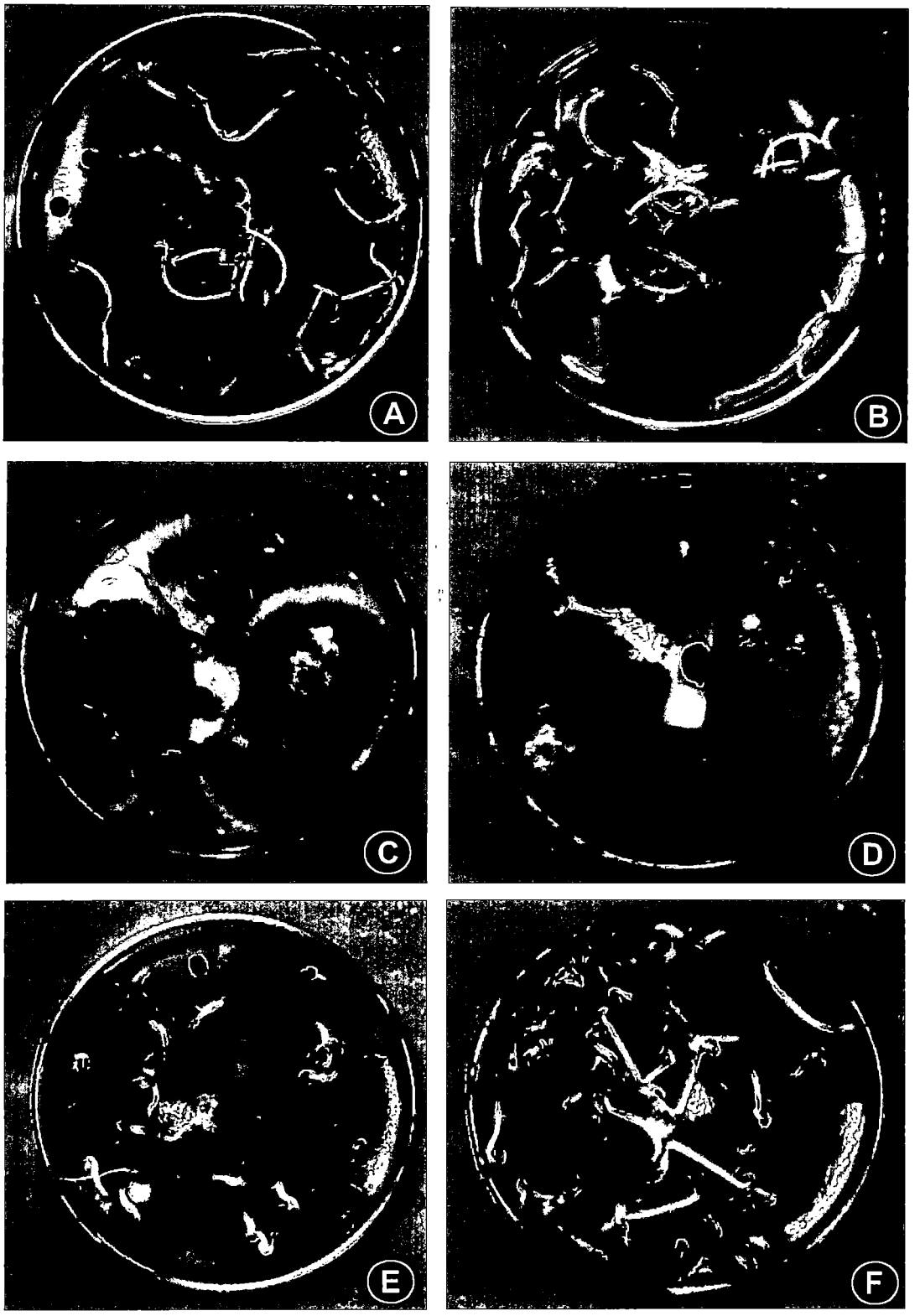

Plate-IV: (Figs : A-F) : *Aspergillus niger* and *Aspergillus flavus* (pre-dominant isolates) from different varieties of three selected pulses (i.e. *Lens culinaris* (A,B) *Vigna radiata* (E&F), *Cajanas cajan* (C&D)
A & B = *L. culinaris* (A = Subrata; B = Ranjan)
C & D= *C. cajan* (C = ICPL- 87, D = Rabi 20/105)
E & F= *V. radiata*; (E= Pusa Baishakhi F= B_1)

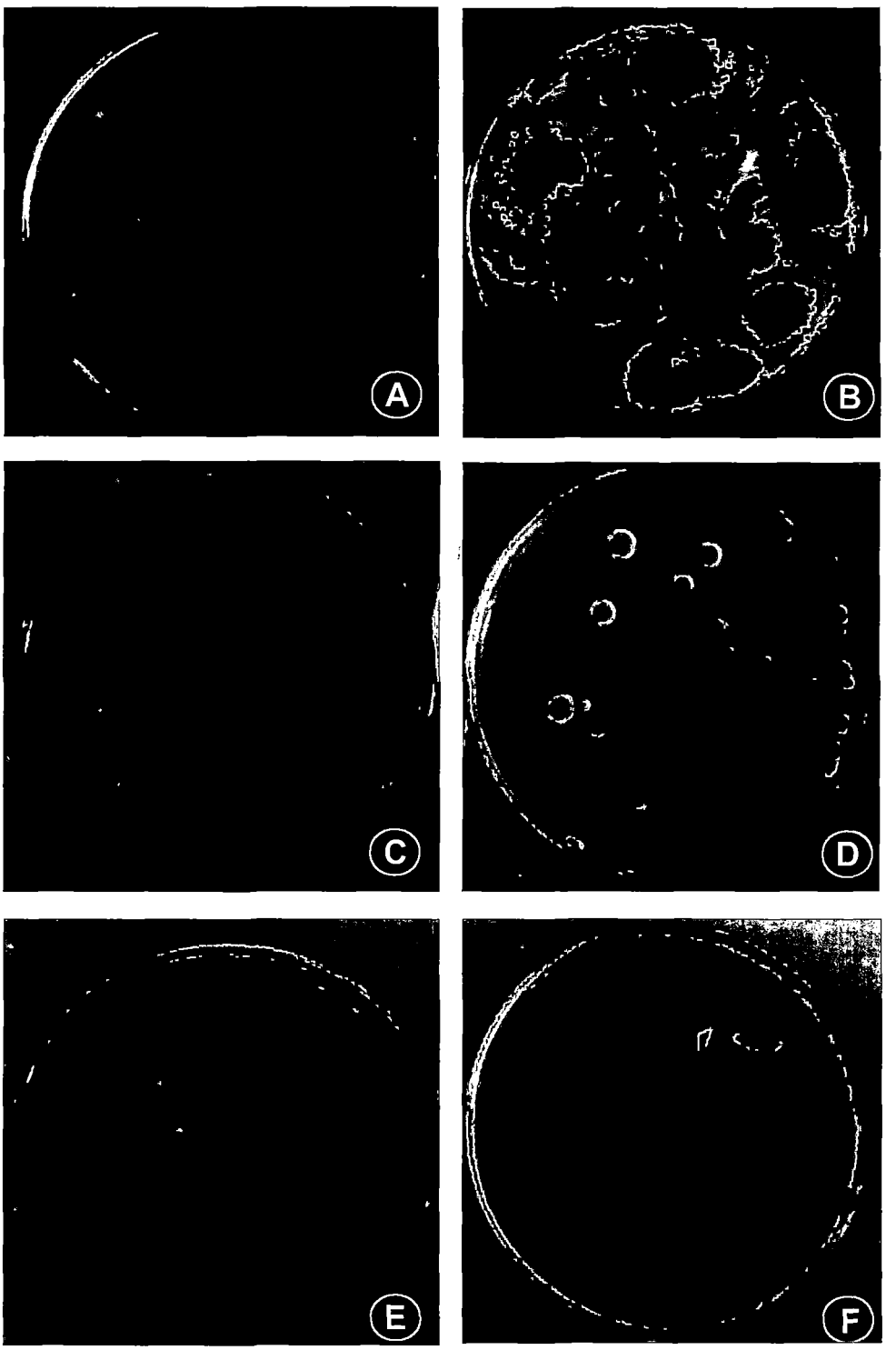

Plate-V: Fungal isolates from stored seeds grown on PDA

A. *Aspergillus niger* B. *Aspergillus terreus*
C. *Penicillium frequentaus* *D. Penicillium* sp.
E. *Aspergillus flavus* F. *Aspergillus fumigatus*

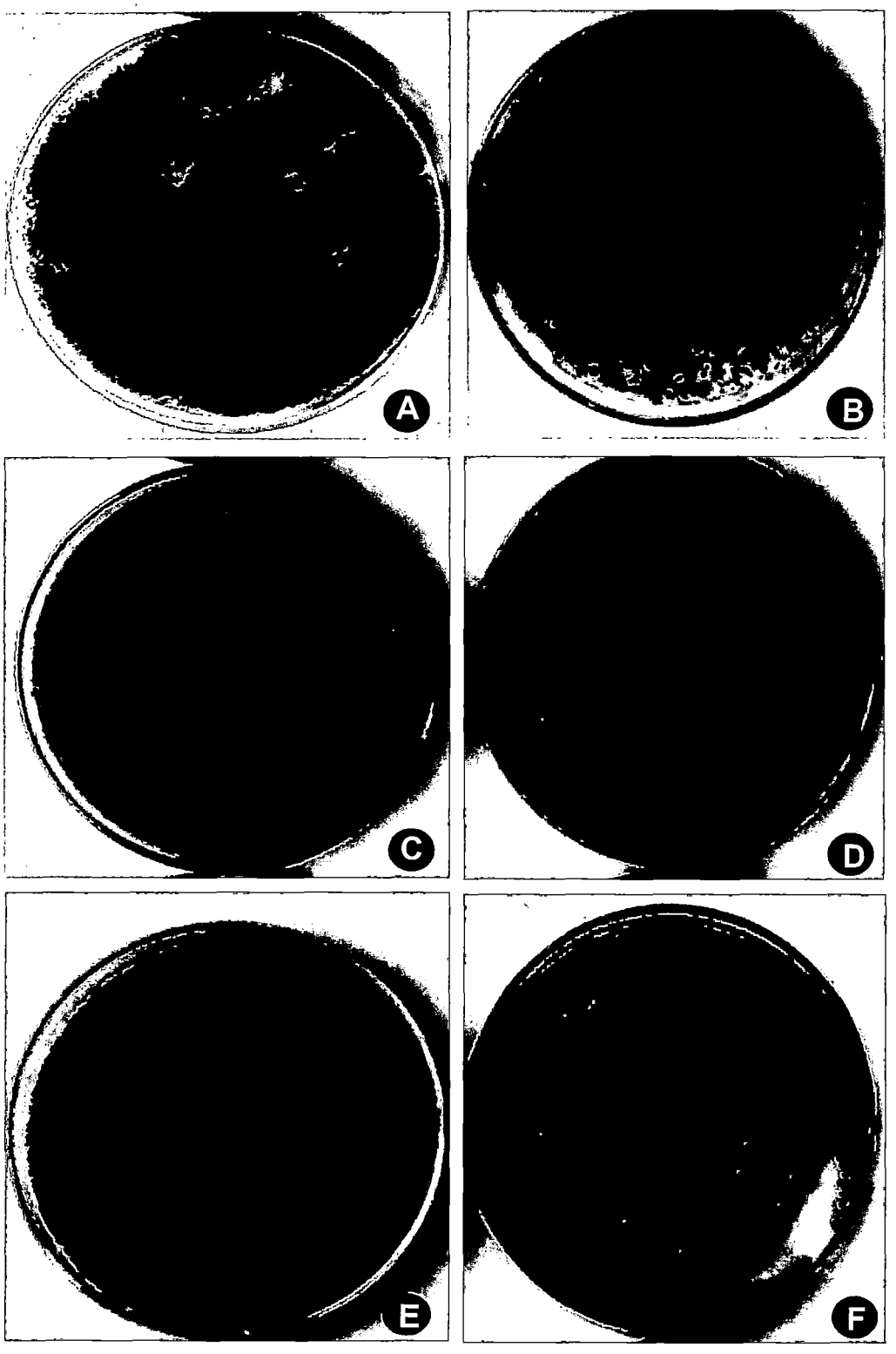

Plate-VI: (Fig. A-F) Fungal isolates from stored seeds grown on PDA
A. *Aspergillus niger* B. *Phoma* sp.
C. *Alternaria alternata* D. *Cochliobolus* sp.
E. *Penicillium* sp. F. *Colletotrichum* sp.

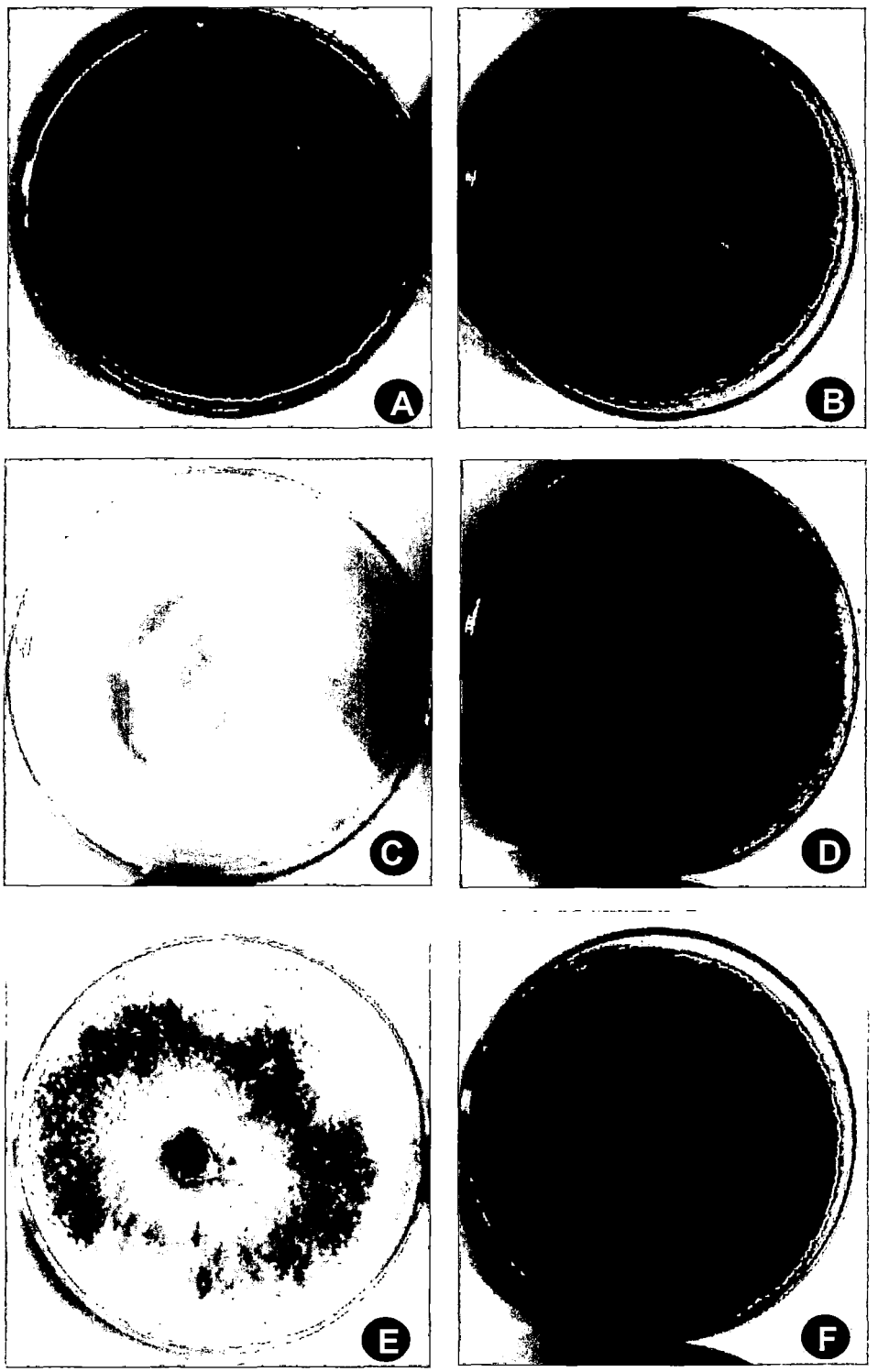

Plate-VII: (Fig. A-F): Fungal isolates from stored seeds grown on PDA
A. *Macrophomina phaseoline*. B. *Aspergillus veriecolor*
C. *Fusarium solani* D. *Rhizopus* sp.
E. *Fusarium oxysporum* F. *Curvularia* sp.

significant but no significant difference was obtained between treatments 2 & 3 (humidity and low temperature with low humidity).

Table 1: Isolation of fungi from stored seeds of *Cajanas cajan*

Period of Storage (month)	Average no. of fungal isolates*		
	Storage condition**		
	A	B	C
3	7 ± .5	6 ± .23	7 ± .11
6	10 ± .8	8 ± .5	5 ± .15
9	12 ± .72	7 ± .5	5 ± .23
12	9 ± .23	6 ± .23	4 ± .5
15	7 ± .23	6 ± .23	4 ± .5
18	6 ± .12	6 ± .2	4 ± .27

** A = Ambient; B = Low humidity; C = Low humidity + low temperature

* Average of 6 replicate plates / treatment.

± = Standard error.

Table 1 A: Analysis of variance of data presented in Table 1

Source	D.F	S.S	M.S	F	CD (5%)
Storage condition	2	40.444	20.222	9.3814	2.34292
Period of storage	5	14.278	2.856	1.3247	
Error	10	21.556	2.156		
Total	**17**	**76.278**			

Table 2: Isolation of fungi from stored seeds of *Lens culinaris*

Period of storage (Months)	Average no. of fungal isolates*		
	Storage condition**		
	A	B	C
3	7 ± .27	7.0 ± .5	5 ± .23
6	10 ± .32	9 ± .5	8 ± .45
9	10 ± .5	7 ± .28	5 ± .21
12	8.0 ± .48	5 ± .25	4 ± .72
15	8 ± 23	5 ± .23	4 ± .5
18	7.0 ± .17	3 ± .17	3 ± .5

*Average of 6 replicate plates / treatment

**A = Ambient, B = Low humidity, C = Low humidity + Low temperature

± = Standard error.

Table 2A: Analysis of variance of data presented in Table 2

Source	D.F.	S.S.	M.S.	F	CD (5%)
Storage condition	2	35.111	17.556	21.3514	1.447
Period of storage	5	39.111	7.822	9.5136	
Error	10	80.222	.822		
Total	**17**	**82.444**			

Table 3: Isolation of fungi from stored seed of *Vigna radiata.*

Period of storage (Months)	Average no. of fangal isolates*		
	Storage condition**		
	A	B	C
3	8 ± .23	7.0 ± .21	7 ± .21
6	12 ± .72	11 ± .45	8 ± .45
9	10 ± .72	9 ± .72	7 ± .23
12	6 ± .5	5 ± .23	4 ± .5
15	5 ± .5	5 ± .23	4 ± .5
18	5 ± .5	4 ± .23	4 ± .5

* Average of 6 replicate plates / treatment

**A = Ambient, B = Low humidity, C = Low humidity + Low temperature

Table 3A: Analysis of variance of data presented in Table 3.

Source	D.F.	S.S.	M.S.	F	CD (5%)
Storage condition	2	7.000	3.500	3.8889	1.51391
Period of storage	5	80.000	16.000	17.7778	
Error	10	9.000	.900		
Total	**17**	**96.000**			

4.1.1. Characterization and Identification

Morphological and microscopic examination of the different isolates revealed that a total of 26 fungal species were isolated, most of them appearing several times during the isolation procedure. The colony, characters, conidial and conidiophores nature, size etc have been tabulated in (Table 4) on the basis of the characters both morphological and

microscopic and by comparison with available literature, identifications were made (Plate VIII- XV).

Table 4: Characterization and identification of fungi from stored seeds

Mycelial character	Conidiophore	Nature & Size of Conidia	Name of Organism
Colonies reach 6cm dia in 7 days in PDA at 22-30^{0}C, light to dark brown.	Simple, straight or curved, 1-3 septate 50 µm long, 3-6 µm wide, golden brown.	Long branching chains, ovoid, obclavate, obpyri form with a short apical beak, smooth walled with 3-8 septa, each portion of lower part with 1-2 longitudinal septa,18-62x7-18 µm. (approx)	*Alternaira alternata*
Colonies fast growing on PDA at 25^{0}C, hyphae septate, branched, light brown becoming darker with age.	Simple, become geniculated by sympodial elongation.	Single, muriform, beaked, dark, 5-10 trans verse and a few longitudinal septa, 120-296 x 12-20 µm. (approx)	*Alternaria solani.*
Colonies reach 2-6cm dia. In 7 days on PDA, at 28^{0}C, yellow green.	Hyaline, 0.4-1.0 µm long and rough walled on large conidiophores a layer of medullae supports the phialides.	Conidial head radiating globose to subglobose, finely roughened to echinulate, 1-3 nucleate, 3.5 –4.5 µm, (approx)	*Aspergillus flavus*
Colonies reach 6-7 cm dia in 7 dyas at 24-26^{0}C on PDA, spread broadly thin, bluish green	Pigmented conidiophores with clavate vesicles arise from clearly differentiated thick walled foot cells.	Strictly columner conidial heads, conidia globose to subglobose, echinulate, 2.5-30µm. (approx)	*Aspergillus fumigatus*
In PDA colonies reach 2.5-6cm in 7 days, at 24-27^{0}C, powdery, black	Arise from long broad thick walled, mostly blackish, sometimes branched foot cells about 1.5-30mm.	Large radiating heads, mostly globose, irregularly roughened 4.0-5.5µm. (approx)	*Aspergillus niger*
Rapidly growth colonies in PDA reaching 3-3.5 cm dia in 7 days, cinnamon to orange brown or brown	Conidiophores normally 100-250µm long, smooth walled, hyaline, with hemispherical vesicles metulea present, conidial heads strictly columner	Conidia globose to slightly ellipsoidal smooth-walled, uninucleate, 1.8-2.4µm, (approx)	*Aspergillus terreus*

Mycelial character	Conidiophore	Nature & Size of Conidia	Name of Organism
Colonies reach 2-3 cm dia in 7 days, variable in colour, light yellowish to yellowish green.	Conidiophores colourless or yellowish, smooth walled, 500-700µm long vesicles elongate with metulae and phialides covering most of the surface. Conidial head radiating.	Conidia globose, echinulate, uninuleate, 2-3µm. (approx)	*Aspergillus variecolor*
Mycelia usually hyaline to subhyline, hyphae septate, branched, white becoming blackish gray with age.	Brown, 150-550 µm long, 4-8µm broad, unbranched or sparingly branched, producing conidia through apical pore.	Straight or curved with 3-9 septa, septa always atansverse, fusioid 12-100x6-20µm. (approx)	*Bipolaris carbonum*
Fast growing colonies, reach 5.0cm in 7 days at 20^0C in PDA olivaceous black.	Erect, strainght, olivaceous brown but later becoming black.	Ellipsoidal, gradually tapering towards the ends, straight on slightly curved, dark brown, smoth walled, glossy, 6-9 distoseptate, 60-100x18-23µm. (approx)	*Cochliobolus sativus*
Colonies with a daily radial increment of 5-7mm, reaching approximately 5cm dia in 7 days at 26^0C in PDA variable colonies white in colour.	Conidia appearing scantily on solitary phialides but normally in orange sporodochia, basal stromatic cushion covered with dense layer of cylindrical, slightly tapering phialides upto 20µm long.	Cylindrical with a rounded apex, slightly trancated base, hyaline, filled with granular cytoplasm forming orange-red shiny masses 9-24x3-4.5µm. (approx)	*Clletotrichum gloeosporioides*
Colonis fast growing form compact, black stromata on PDA at 25^0C brown in colour	Erect, pigmented, geniculated form sympodial elongations.	Olive brown, curved ellipsoidal, 3-septate, round at the apex slightly accuminate at the base, the middle septum below the certre and the third cell strongly curved, 20-30x9-15µm (approx)	*Curvularia lunata*
Fast growing colonies on PDA at 25^0C, often forming stromata, black.	Pigmented, erect conidio phores	Conidia predominantly 4 sepetate, the central cell distinctly geniculate, tapering gradually towards each and 18-37x8-14µm. (approx)	*Curvularia geniculatus*

Mycelial character	Conidiophore	Nature & Size of Conidia	Name of Organism
Colonies fast growing reaching 6.5cm dia in 7 days at 25^{0}C on PDA, aerial mycelium spares to abundant, then floccose, delicate, white with purple or violet tinge.	Simple, lateral phialides short, sparsely branched.	Microconidia never in chain, mostly O-septate, ellipsoidal to cylindrical, 5-12 x2.3-3.5µm. Macroconidia fusiform, modetrately curved pointed at both ends basal cells pedicellate, 27-46x3.0-4.5µm.	*Fusarium oxysporum*
Fast growing colonies 6-8cm dia in 7 days at 25^{0}C aerial mycelium but abundant cream to puff conidial slime formed in sporodochia.	Short, branched conidiophores 8-16x2-4µm. (approx)	Macroconidia abundant, chlamydospores borne singly, sometime in pairs, in terminal, lateral, hyaline, smooth walled 6-10µm.	*Fusarium solani*
Colonies reaching 4-7 cm dia in 7days at 25^{0}C on PDA, velvety, dark green reverse variable in colour, typically yellow-brown	Conidiophores 100-200µm long in compact columns.	Globose to subglobose smooth walled, sometimes finely roughened 3.0-3.5µm	*Penicillium* sp.
Colonies reaching 4-0-6.0cm dia on PDA at 20^{0}C. irregularly lobed, whitish to olivaccous grey.		Oblong. sometimes two-celled4.0-8.5x2.0-3.0□m.	*Phoma exigua*
Very fast growing colonies on PDA, often over 2cm high stolons, hyaline to brown, 13-20µm wide abundantly branched rhizoides (300-350µm long) whorls of sporangiophores produced terminally, mycelia reddish gray brown in colour	Sporangiophores pale to dark brown, straight, mostly 1.5-3mm tall, 20-25µm wide.	Sporangiospores subglobose, biconical to oval, ridged, mostly 4nucleate, 7-12x6-8.5µm.	*Rhizopus stolonifer*
At 20^{0}C, colonies reach 15mm high, buff to smoke-gray in the dark and smoke-gray in the light.	Sporangiophores 14 (–17) µm wide, some tall, some short	Sporangia at first hyaline, later becoming brownish.Columellae ovoid or oval. Sporangiophores broad oval to subglobose 5.5-8.5x4-7µm, greyish.	*Mucor* sp.

Mycelial character	Conidiophore	Nature & Size of Conidia	Name of Organism
Colonies reaching 1.5 cm dim. in seven days at 28^{0}C, Isabelline to olivaceous brown, fluffy reverse fuscous-black.	Aleurioconidia formed in sympodial succession on scarcely differentiated and deusely septate conidiophores.	Aleuroconidia, brown smooth-walled, provided with a longithdinal germslit, mostly sessile, ellipsoidal to pyriform.	*Mammaria sp.*
Colonies reaching 1.4cm dim in five days on PDA at 25^{0}C covered with a dark powdery bloom of conidial theses, reverse uncoloured	Conidiophores simple or irregularly branched, usually about 100µm but to 1000µm long and 3-6µm wide, hyaline at the base and dark olivaceous, bearing clusters of 4-10 phialides	Phialides ovate or ellipsoidal, hyaline, later becoming olivaceous 9-14x4-6µm. Conidia aggregated in slimy messes, ellipsoidal at first hyaline, at maturity dark livaceous-gray.	*Stachybotrys sp.*
Colonies reaching about 4cm dim in five days at 20^{0}C, yellow green myceliaum.	Phialoconidia are abundantly produced.	Ascomata are black subglobose, to ovate 350-500x230-40µm. Ascospores dark olivaceous brown.	*Chaetomium sp.*
Colonies reaching 2.5-3.5 cm diam in ten days at 20^{0}C, olive green, often covered with a pale grey aerial mycelium.	Conidiophores straight often geniculate.	Conidia usually in rather short chains ellipsoidal, mid brown to olivacous brown.	*Cladosporium* sp.
Colonies reaching >6.00 cm dim in ten days at 20^{0}C, yellow, orange or brown.	Sporodochia visible as black dots. 100-200µm diam.	Conidia globose to pyriform mostly 15-25µm diam with a funnel shaped base	*Epicoccum*, sp.
Pinkish, dusty colonies	Erect unbranched conidiophores	2-celled thick walled conidia with truncate bases.	*Trichothecium* sp.

The presence and absence of the identified species in different pulses were recorded and has been presented in the Table 5.

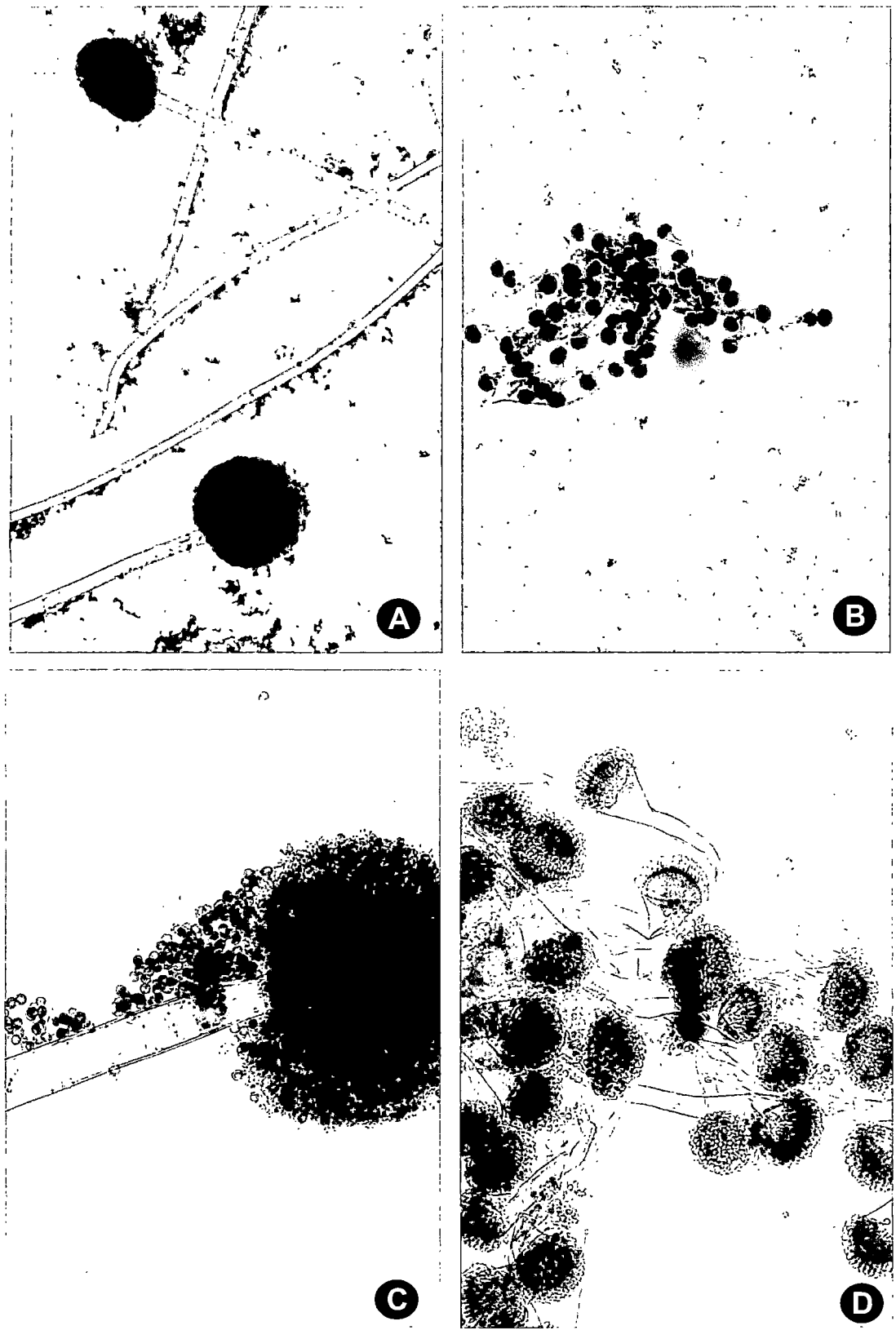

Plate-VIII: Hyphae, conidiophores & conidia of *Aspergillus niger* (A & C) and *Aspergillus flavus* (B & D), A & B x 10; C & D x 45

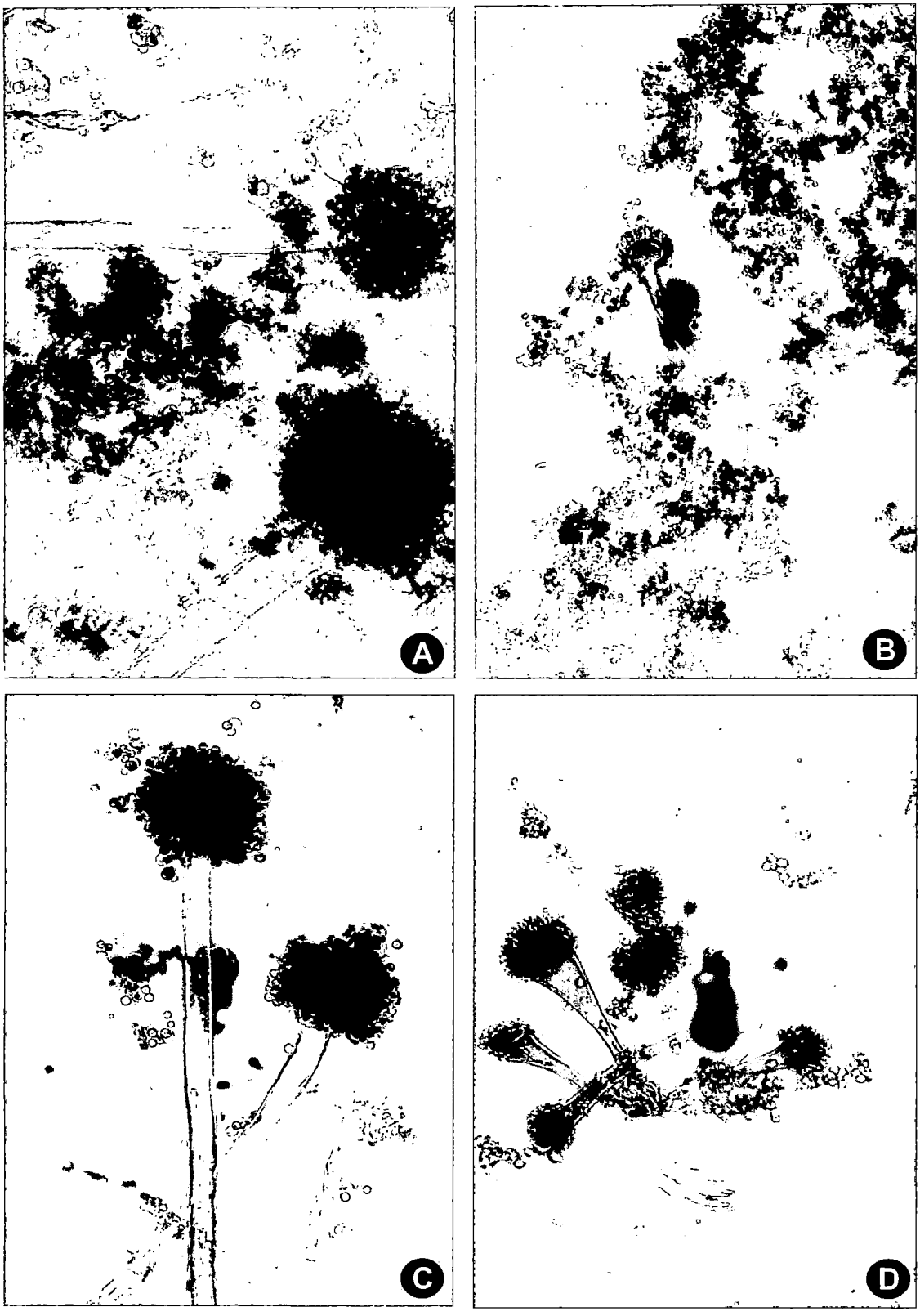

Plate-IX: Figs A-D. Hyphae, conidiophores and conidia of
A : *Aspergillus variecolor* (x45);
B : *Aspergillus* sp. (10)
C : *Aspergillus fumigatus* (x45)
D : *Aspergillus fumigatus* (x10)

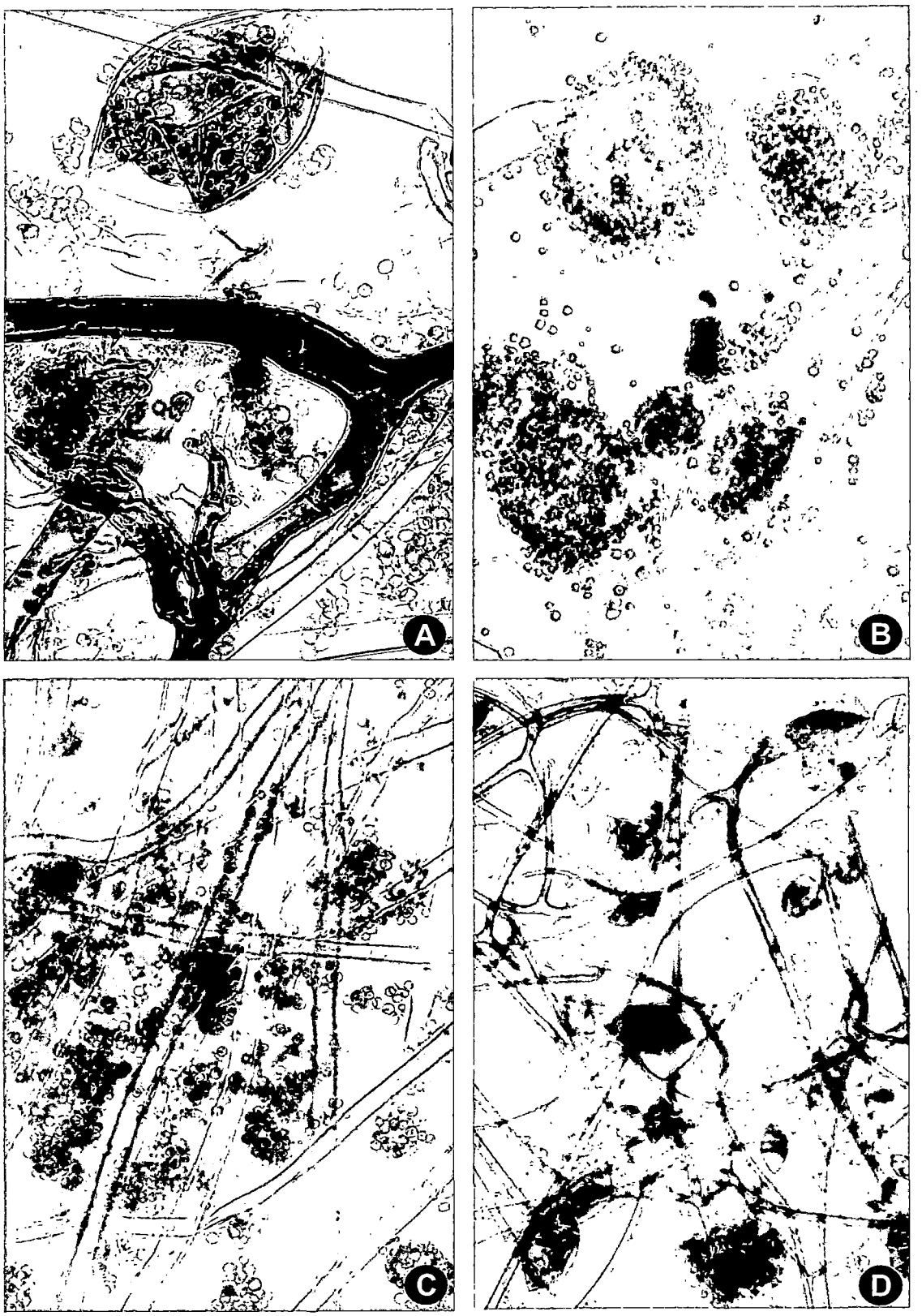

Plate-X: Hyphae, conidiophore and conidia of *Rhizopus* (A&D) & *Mucor* (B&C) C&Dx10, A&B x 45.

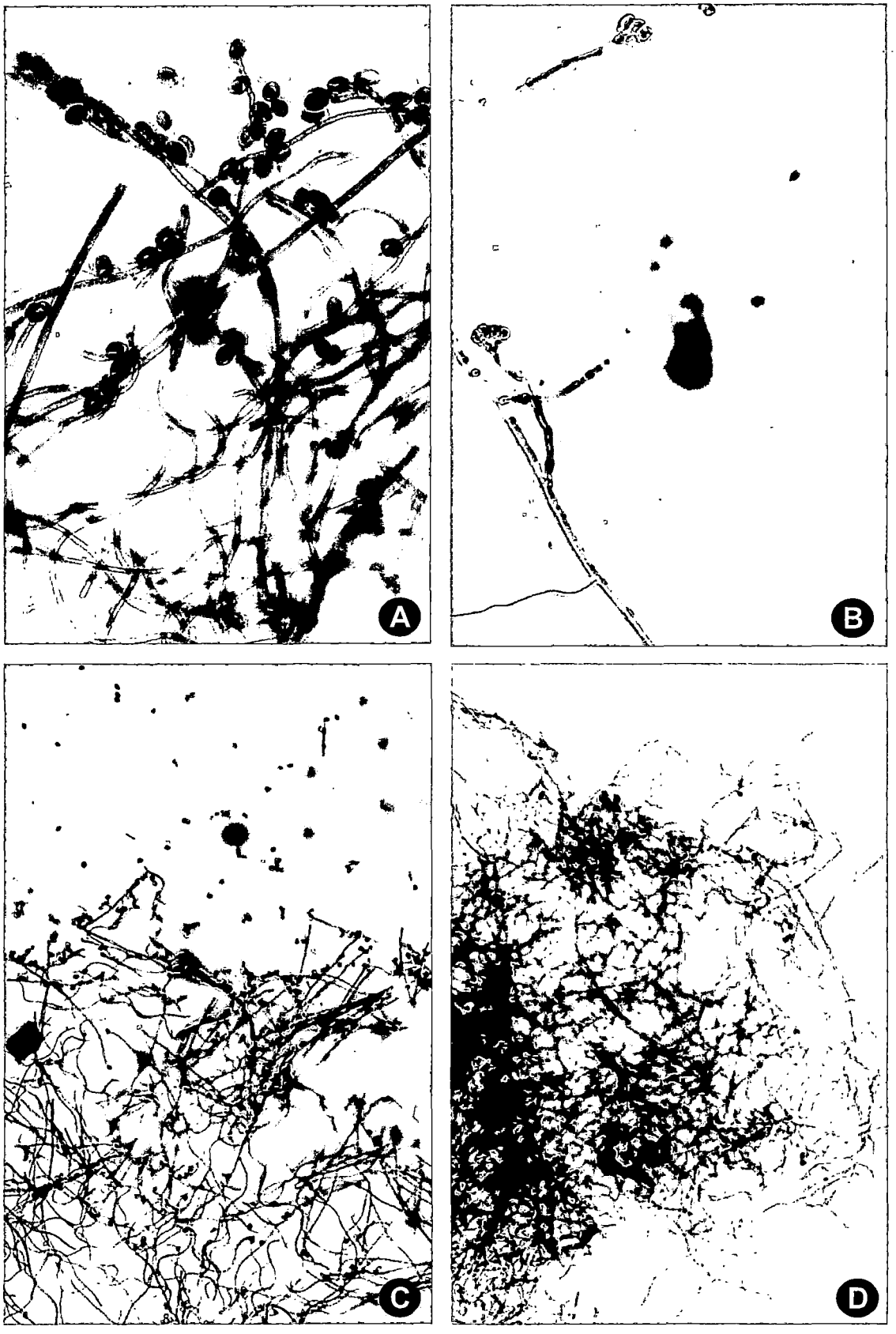

Plate-XI: Hyphae and conidia of *Mammaria* sp. (A) *Stachybotrys* (B) and *Fusarium* sp. (C-D) x 10

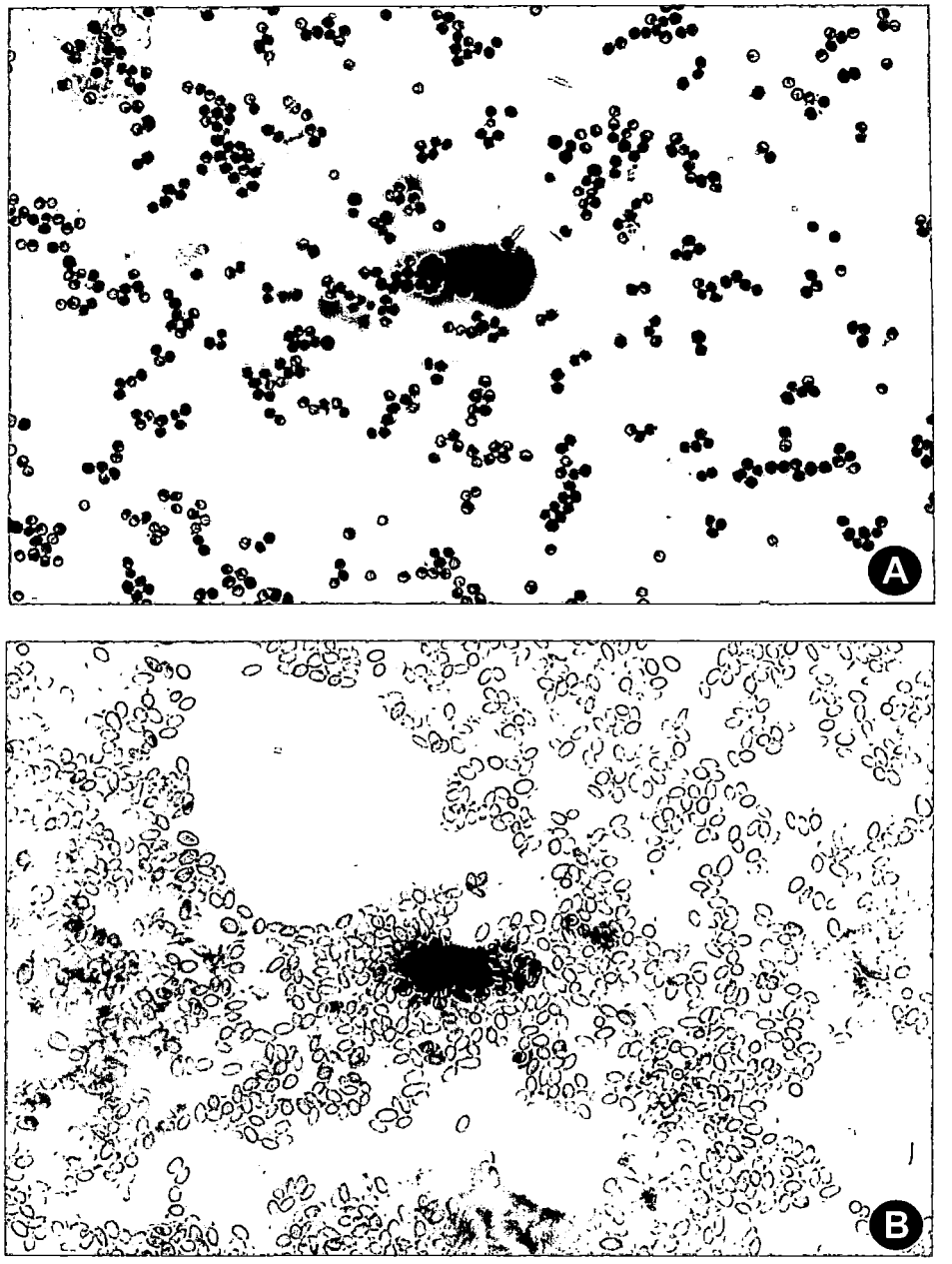

Plate-XII: (Fig. A&B)
Spores of *Penicillium* (A) and *Colletotrichum* (B) x 10

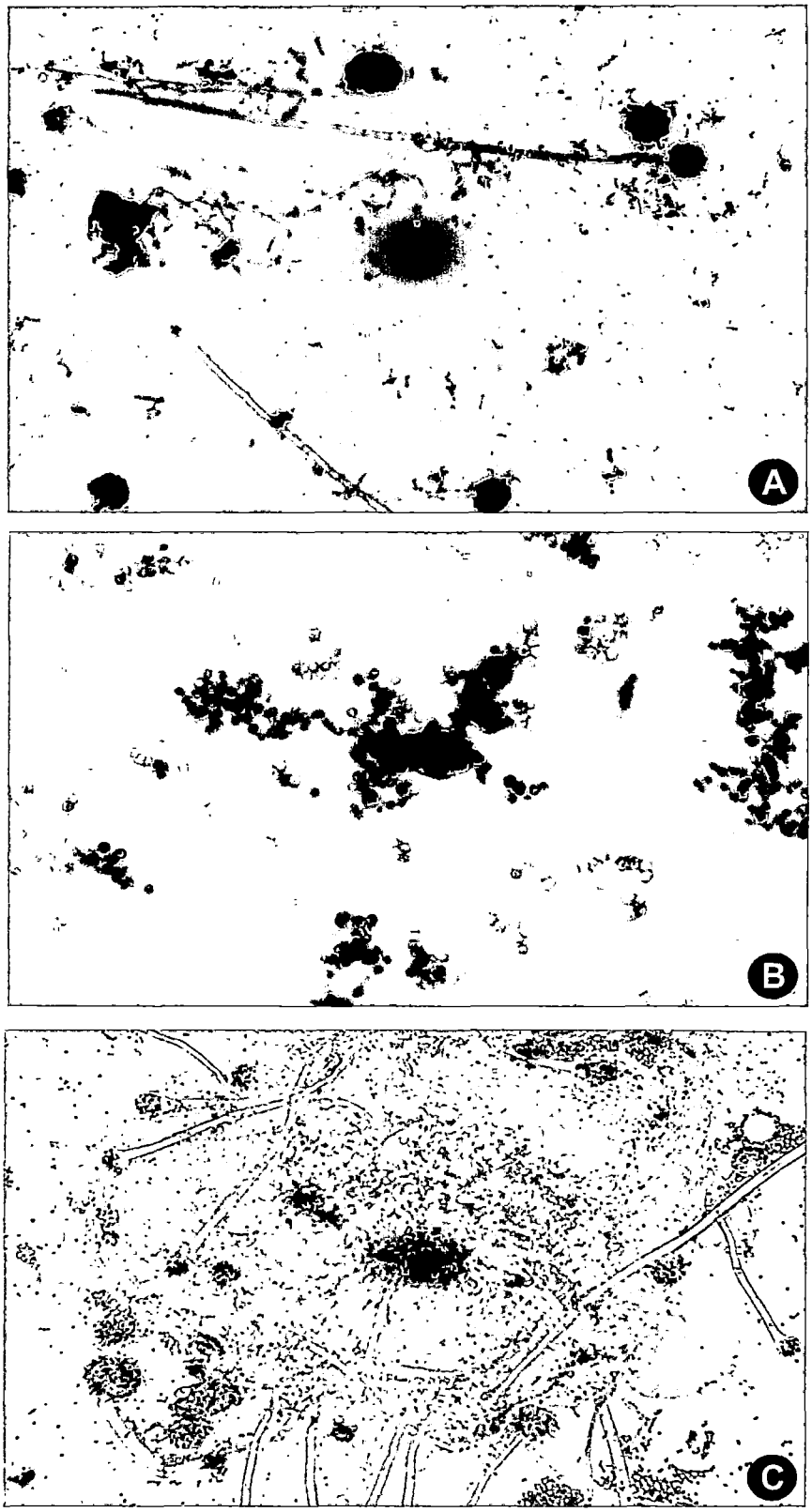

Plate-XIII: (Fig. A-C) Hyphae, conidia and conidiophores of various sp. of *Aspergillus.*
A = *A. terreus*,B=*A. tamerii*, C= A.sp.,A,B.&Cx10

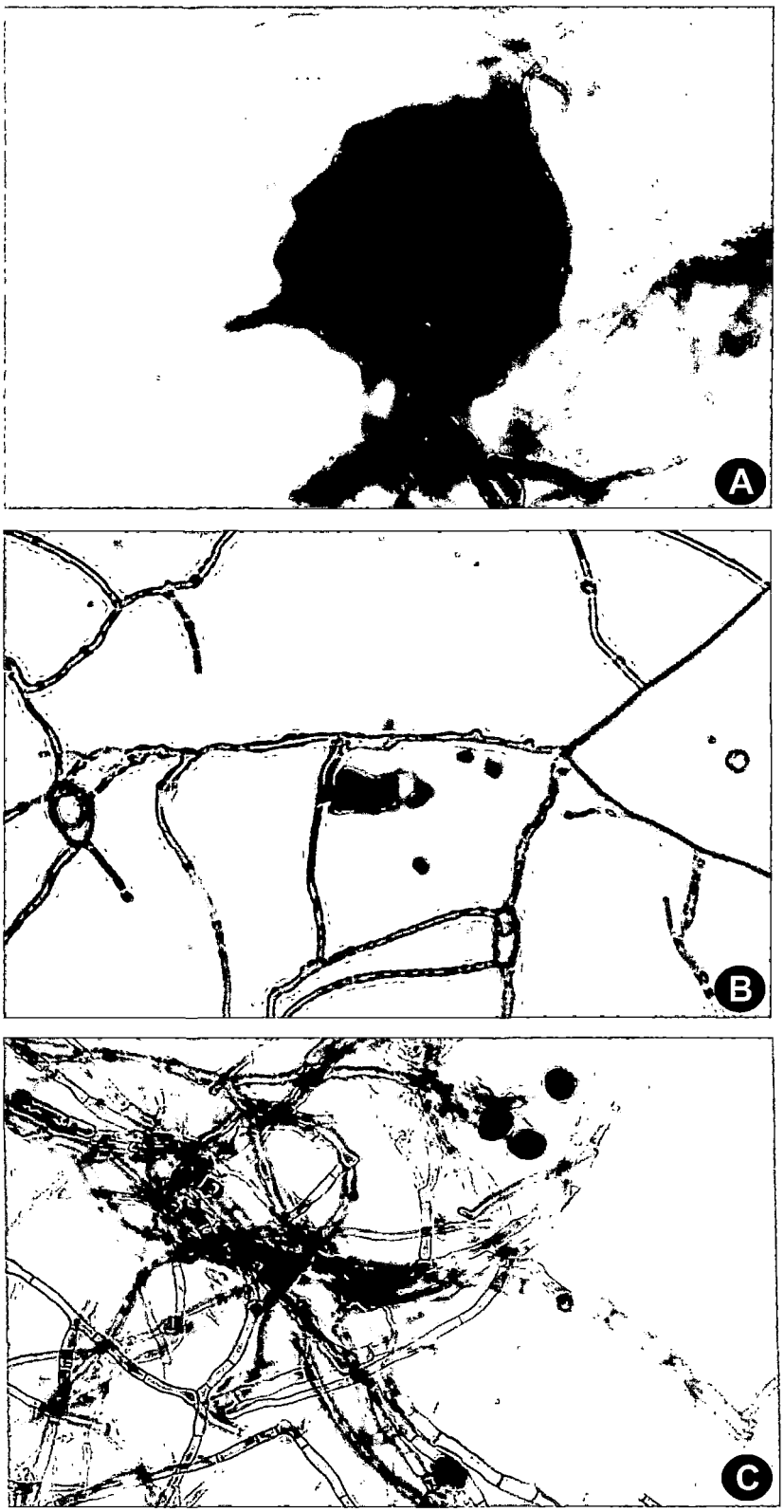

Plate-XIV: (Fig. A-C)

A: *Macrophomina phaseolina* (x45)
B: Isolate VR-10 (x10)
C: Isolate LC-7 (x10)

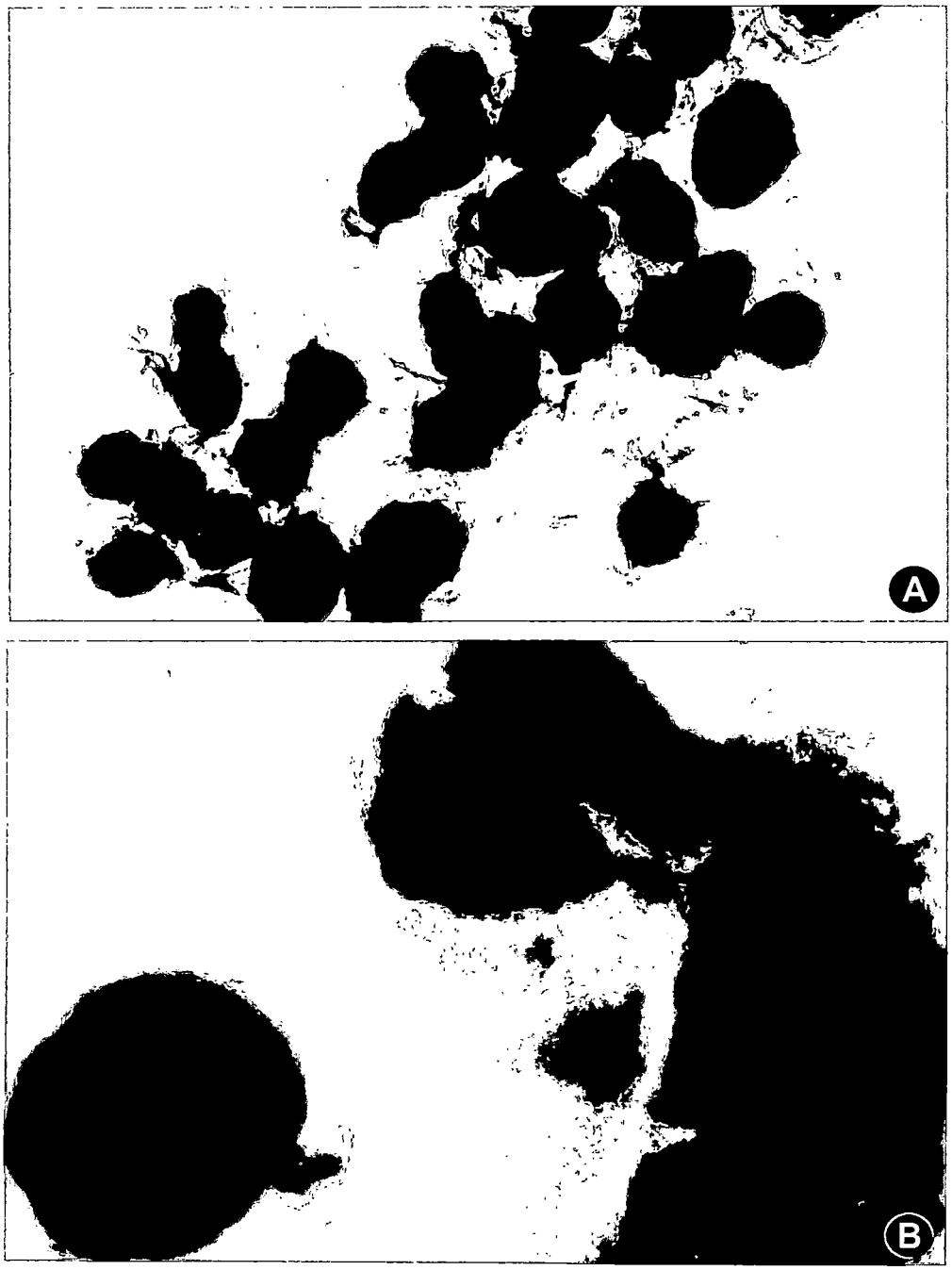

Plate-XV: (Fig. A&B)
Phoma sp. A(x10), B (x45).

Table 5: Association of fungal species in the stored seeds

Fungal species	*Lens*			*Vigna*		*Cajanas*	
	Asha	Rajan	Subrata	B_1	Pusa	IC PL	Rabi 20/105
1. *Mucor mucata*	+	–	+	+	+	+	+
2. *Alternaria alternata*	–	+	–	–	+	–	+
3. *Alternaria solani*	+	–	–	+	–	+	
4. *Aspergillus flavus*	+	++	++	+	+	+	
5. *Aspergillus fumigatus*	+	+	+	+	+	+	+
6. *Aspergillus niger*	+	+	+	+	+	+	+
7. *Aspergillus terreus*		+		+	+		+
8. *Aspergillus veriecolour*	+	+	+	+	+	+	+
9. *Bipolaris carbonum*	+		+	+		+	+
10. *Cochliobolus sativus*		+		+	+	+	
11. *Colletotrichum gloeosporioides*		++		+			+
12. *Curvularia lunata*	+		+		+		+
13. *Curvularia geniculatus*	+	+		+	+	+	
14. *Fusarium oxysporum*		+			+		+
15. *Fusarium solani*	+	+		+	+		+
16. *Aspergillus tamarii*		+	+	+	+	+	+
17. *Penicilliumoxalicum* sp.	+	+	+	+	+	+	+
18. *Stachybotrys* sp.	+	–	–	+	–	–	+
19. *Phoma exigua*	+	+		+		+	
20. *Rhizopus stolonifer*	+	+	+	+	+	+	+
21. *Chaeotomium globosum*	+		+		+		+
22. *Cladosporium herbarum*		+	+	+		+	
23. *Epicoccum* sp.	+		+		+	+	
24. *Mammaria* sp.		+	+		+	+	
25. *Trichothecium* sp.	+		+	+			+
26. *Macrophomina* sp.	+	+		+	+		+

+ = Present; – = Absent

4.1.2. Selection of consistently associated fungi

In order to determine the fungi most consistently appearing during storage the frequency of appearance was calculated by determining the no of times a particular fungal species appear using the following formula described in Materials and Methods.

Results presented in Table 6 revealed that *Aspergillus flavus* and *Aspergillus niger* 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% (Table-6, Figs. 4 and 5).

Table 6: Frequency of appearance of fungal species isolated from seeds

Sl. No.	*Fungal Species*	% Frequency		
		Cajanas	*Vigna*	*Lens*
1.	*Aspergillus flavus*	81	83	80
2.	*Aspergillus fumigatus*	60	57	61
3.	*Aspergillus niger*	90	87	89
4.	*Aspergillus terreus*	40	32	41
5.	*Aspergillus variecolor*	30	37	41
6.	*Aspergilus tamarii*	25	20	24
7.	*Chaetomium* sp.	17	0	12
8.	*Cladosporium* sp.	15	18	20
9.	*Epicoccum* sp.	12	17	0
10.	*Mammaria* sp.	5	0	0
11.	*Trichothecim* sp.	15	10	12
12.	*Bipolaris carbonum*	9	7	10
13.	*Cochliobolus sativus*	5	8	5
14.	*Colletotrichum gloeosprioides*	5	2	2
15.	*Curvularia lunata*	10	7	5
16.	*Curvularia geniculatus*	12	18	10
17.	*Phoma exigua*	5	2	7
18.	*Alternaria solani*	7	11	8
19,	*Penicillium* sp.	58	55	63
20.	*Stachybotrys* sp.	5	7	0
21.	*Rhizopus stoloniforme*	46	48	39
22.	*Mucor mucata*	37	41	45
23.	*Fusarium oxysporum*	10	5	7
24.	*Fusarium solani*	10	17	15
25.	*Macrophomina phaseolina*	21	20	15
26.	*Alternaria alternata*	10	17	13

% Frequency calculated on the basis of 25 observations.

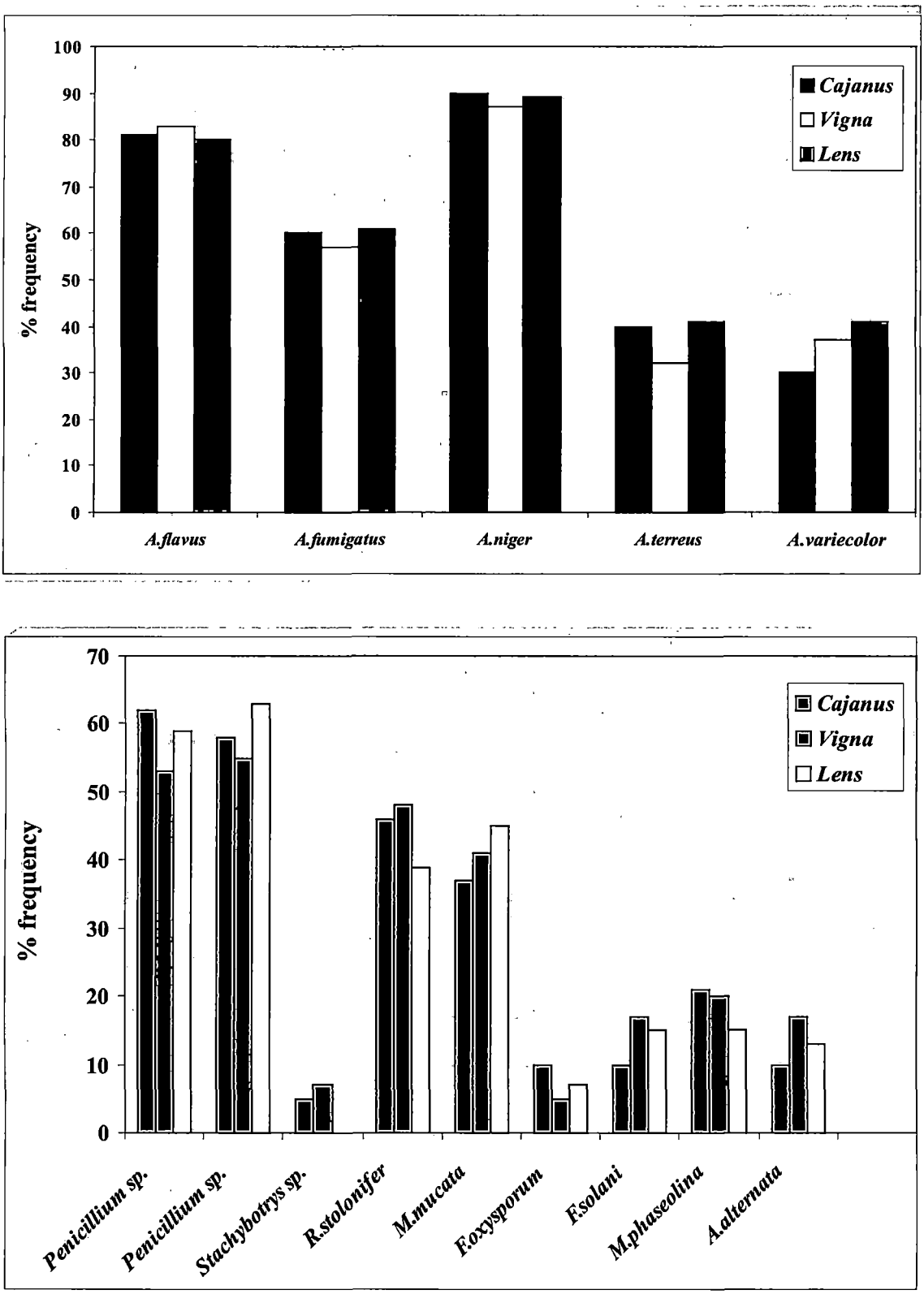

Fig.4: Frequency of appearance of different fungi during storage of the different seeds

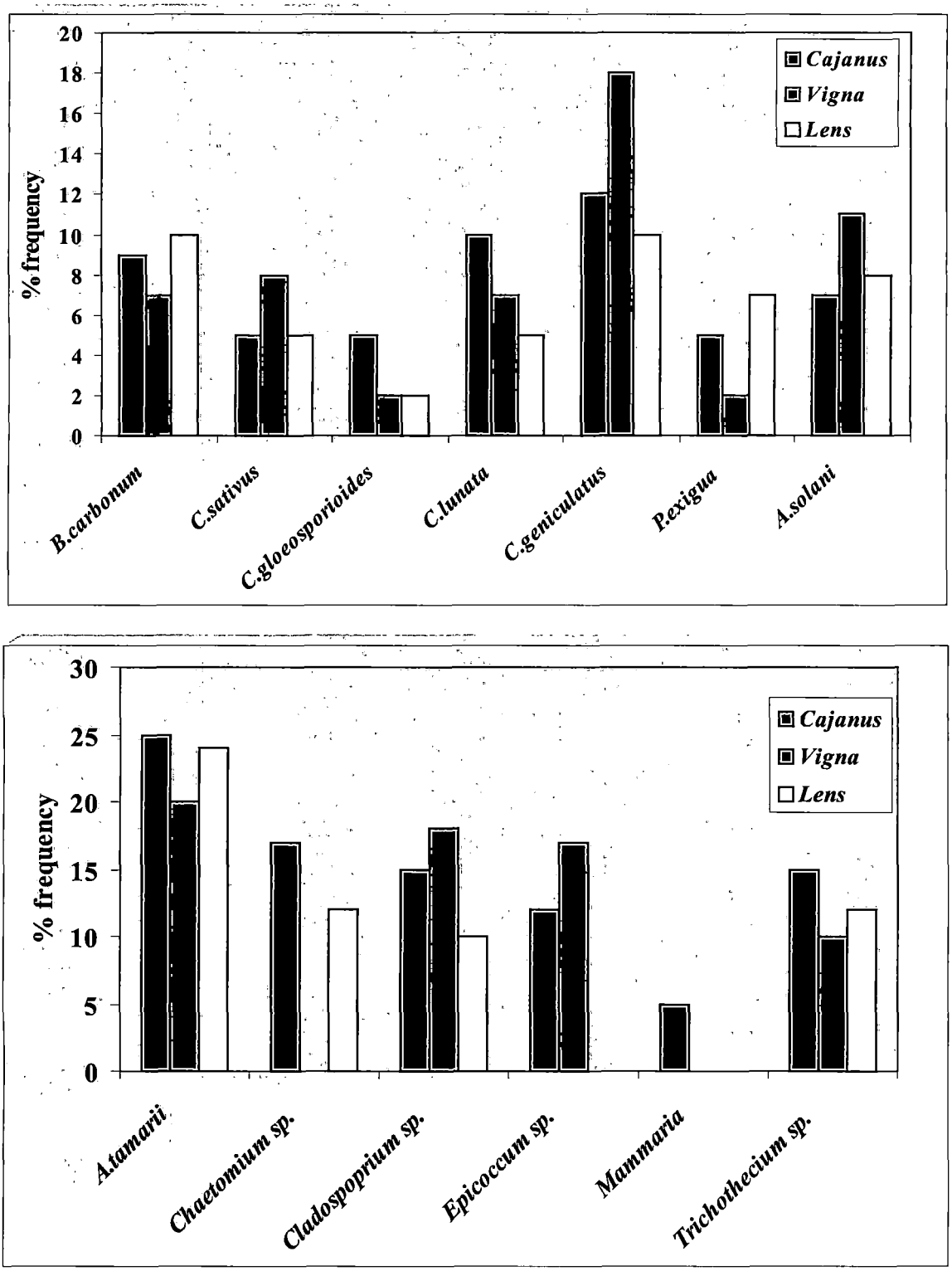

Fig.5: Frequency of appearance of different fungi during storage of the different seeds.

Based on these results *A. niger* and *A. flavus* were selected for further studies.

4.2. Seed Viability

All seeds were taken out and tested for viability. Percent germination of seeds were calculated in each case on the basis of 50 seeds in each case. It was observed that in all cases viability was maintained to a great degree even upto 18 months of storage under low temperature and low moisture. In *Cajanas cajan* total viability was lost from 12 months onwards when stored under normal storage condition. However when stored in low temperature and humidity 80-90% germination was observed even after 18 months of storage (Table 7&7A, Fig. 6).

Table 7: Germination of seeds of *Cajanas cajan* after different storage periods.

Period of Storage (Months)	% of germination**		
	Storage condition*		
	A	B	C
3	100. ± 1.78	100 + 1.18	100 ± 1.18
6	65 ± .72	70 ± .72	100 ± .72
9	27 ± .45	62 ± .48	90 ± .45
12	0	50 ± .5	90 ± .72
15	0	0	85 ± .72
18	0	0	85 ± .72

* A = Ambient, B = Low humidity, C = Low humidity + Low temperature, ** Average of 50 seeds / treatment.

Table 7A: Analysis of variance of data presented in Table 7

Source	D.F.	S.S.	M.S.	F	CD (5%)
Period of storage	2	11314.330	5657.167	11.7166	49.58
Storage condition	5	11905.830	2381.167	4.9317	–
Error	10	4828.334	482.833	–	–
Total	**17**	**28048.500**	–	–	–

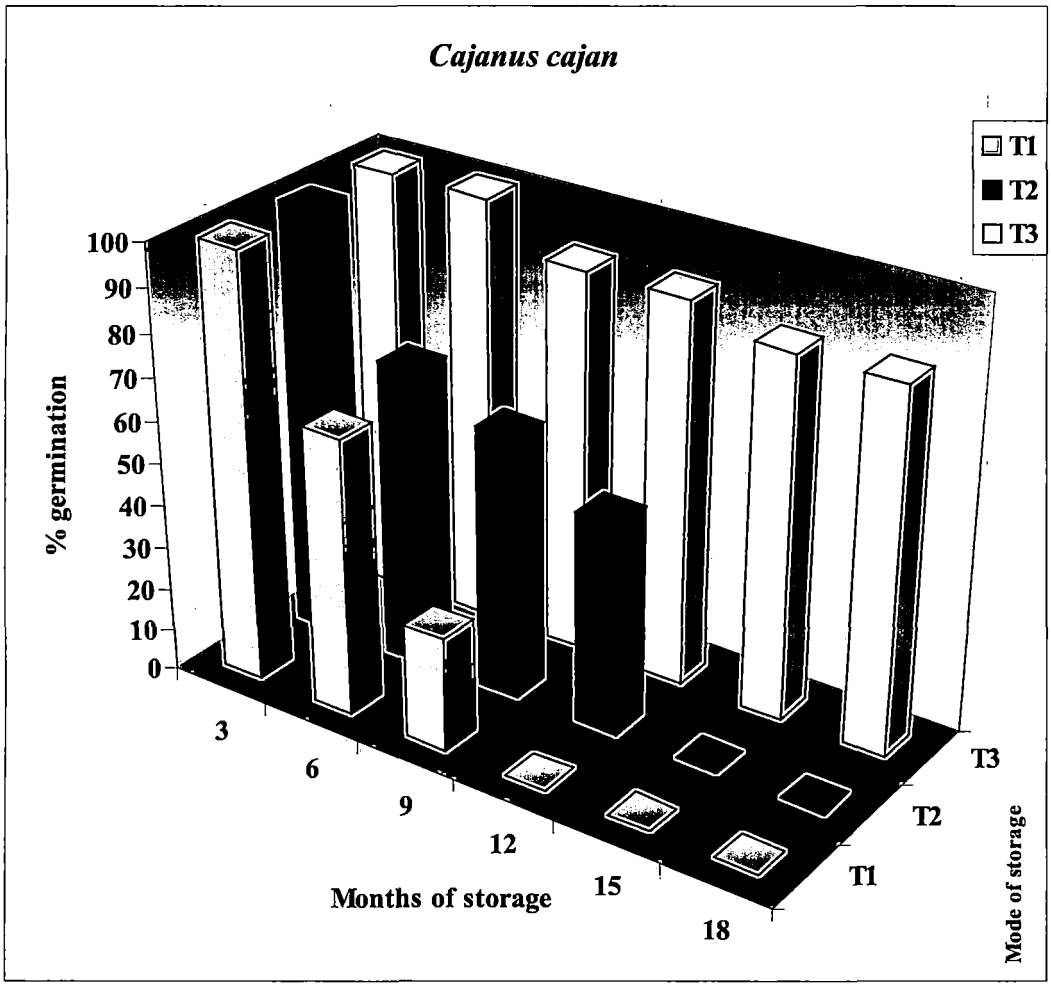

Fig.6: Germination percentage of *Cajanus cajan* seeds stored for different periods under varying conditions. T1=Ambient; T2=Low humidity; T3=Low temperature and humidity.

In case of *Vigna radiata* no germination was observed after 15 months of storage under ambient conditions; however storage under low humidity and temperature increased % of germination and viability was retained even after 18 months of storage (Table 8 & 8A, Fig. 7).

Table 8: Germination of seeds of *Vigna radiata* after different storage periods.

Period of Storage (Months)	% of germination**		
	Storage condition*		
	A	B	C
3	100. ± 1.18	100 + 1.18	100 ± 1.18
6	85 ± .72	80 ± .72	90 ± .72
9	65 ± .5	65 ± .5	90 ± .72
12	20 ± .2	35 ± .5	75 ± .45
15	0	20 ± .15	67 ± .48
18	0	23 ± .15	67 ± .48

* = A = Ambient, B = Low humidity, C = Low humidity + Low temperature,
** = Average of 50 seeds / treatment.

Table 8A : Analysis of variance of data presented in Table 8

Source	D.F	S.S	M.S	F	CD (5%)
Period of storage	2	4767.406	2383.703	10.7814	33.556
Storage condition	5	6912.740	1382.548	6.2532	–
Error	10	2210.949	221.094	–	–
Total	**17**	**13891.090**	–	–	–

L. culinaris was most resistant to deterioration as viability was lost only after 18 months of storage under ambient conditions and germination of upto 90% was obtained when stored under low temperature and humidity (Table 9&9A, Fig. 8).

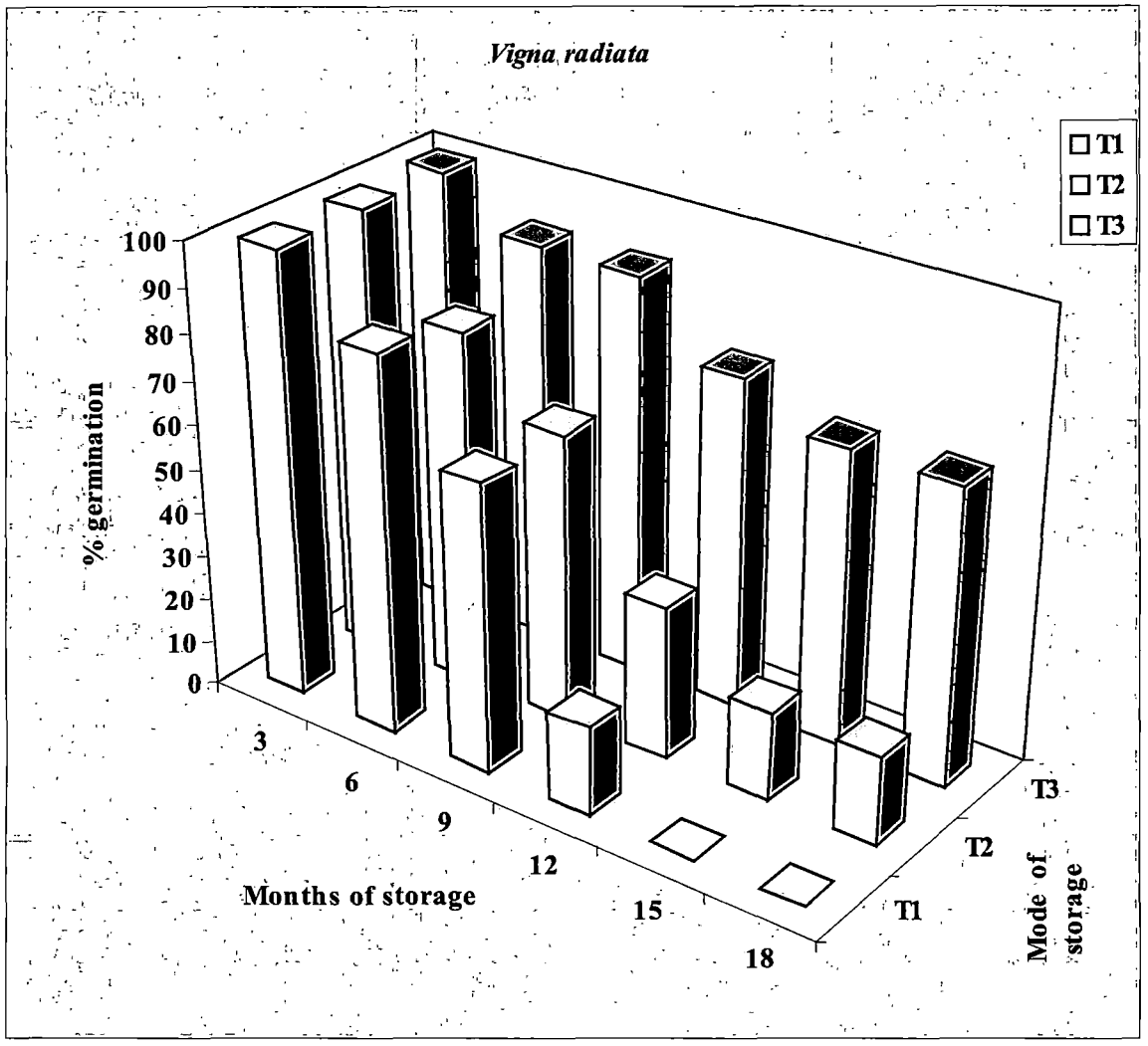

Fig.7: Germination percentage of *Vigna radiata* seeds stored for different periods under varying conditions. T1=Ambient; T2=Low humidity; T3=Low temperature and humidity.

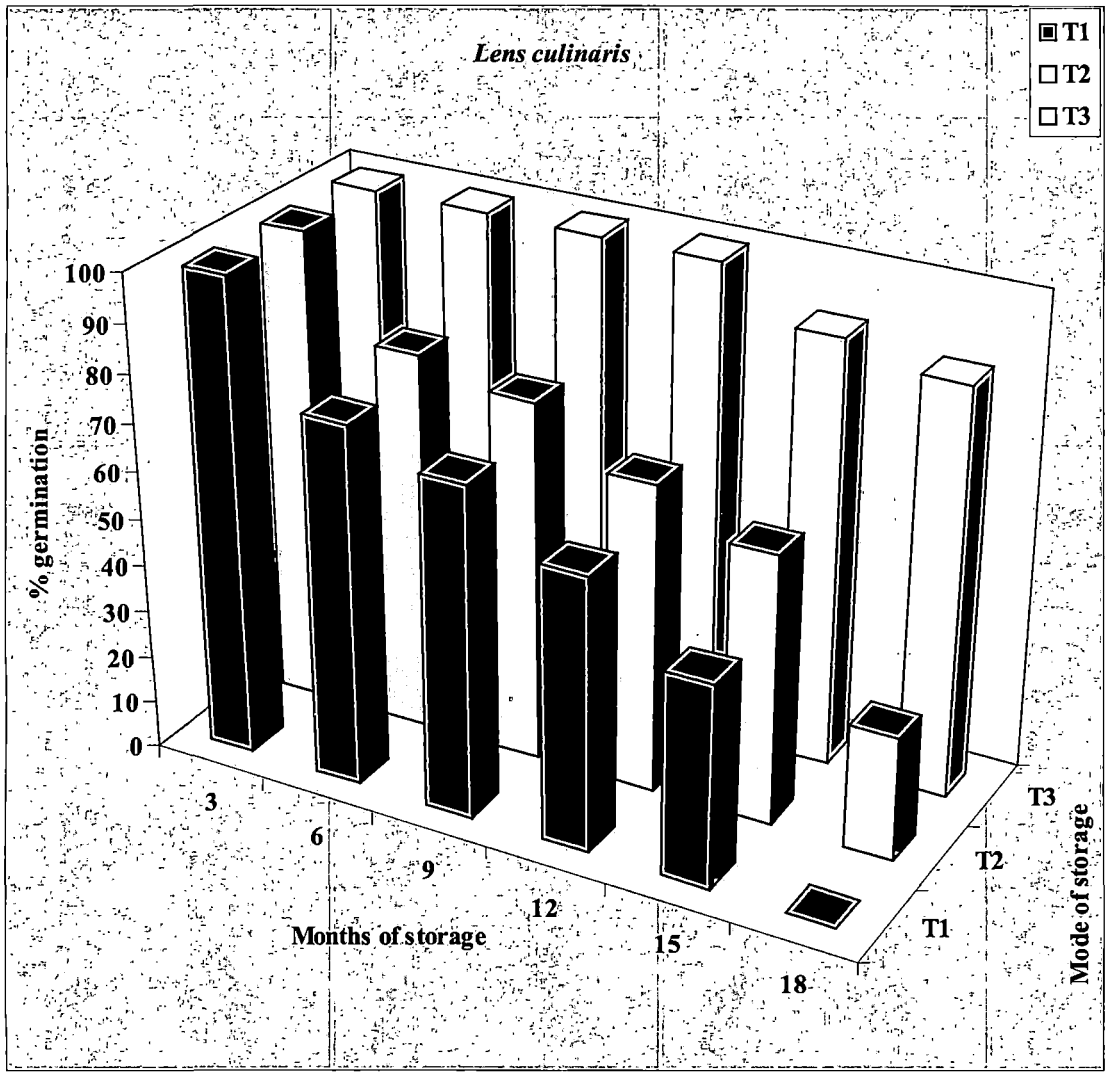

Fig.8: Germination percentage of seeds of *Lens culinaris* stored under different conditions for varying periods. T1=Ambient; T2=Low humidity; T3=Low temperature and humidity.

Table 9: Germination of seeds of *Lens culinaris* after different storage periods.

Period of Storage (Months)	% of germination**		
	Storage condition*		
	A	B	C
3	100. ± .72	100 + .85	100 ± .85
6	76 ± .72	76 ± .82	100 ± .72
9	70 ± .5	80 ± .5	100 ± .72
12	57.6 ± .45	65 ± .45	100 ± .72
15	43.3 ± .23	57 ± .5	90 ± .85
18	0	26 ± .23	86.6 ± .72

* A = Ambient, B = Low humidity, C = Low humidity + Low temperature, ** Average of 50 seeds / treatment.

Table 9A : Analysis of variance of data presented in Table 9

Source	D.F	S.S	M.S	F	CD (5%)
Period of storage	2	4400.694	2200.347	8.9836	35.319
Storage condition	5	13593.400	2718.680	11.0998	–
Error	10	2449.306	244.931	–	–
Total	**17**	**28048.500**	–	–	–

Analysis of variance of data in all three seed germinations revealed that significant differences in germination was present between the different periods of storage but not among the modes of storage.

4.3. Cultural characteristics of *A. niger* and *A. flavus*

After selection of *A. niger* and *A. flavus* for further experimental purposes detailed studies on their cultural characteristics were made. Growth of these fungi in different media as well as during different time interval were noted.

4.3.1. Incubation period

In order to determine the optimum incubation period the fungi were grown for different time intervals in both potato dextrose broth (PDB) and potato dextrose agar (PDA). For liquid medium 50 ml of medium was taken in each 250ml Ehrlenmyer flask, autoclaved and was inoculated with 4mm agar block containing in noculum of *A. niger* and *A. flavus*. Flasks were incubated at 28^0C for 2, 4, 6 and 8 days after which mycelia were harvested, dried at 60^0C for 96 hr. and weighed. Results revealed that significant growth was obtained from 2 days onwards and maximum growth occurred at 6 days of incubation (Table 10) In 8 days mycelia weight decreased from 0.69 g (6 days) to 0.40 g (6 days) (in *A. niger*) and from 0.58 g (6 days) to 0.34 gm (in *A. flavus).* Sporulation was observed from 2 days onwards. Of the two, growth of *A. niger* was greater than *A. flavus*.

Table 10: Growth of *Aspergillus niger* and *A. flavus* at different incubation periods.

Incubation Period (days)	Average dry wt. of mycelia (g)*	
	Aspergillus niger	***Aspergillus flavu***
2	0·44 ± ·12	0·37 ± ·18
4	0·67 ± ·23	0·47 ± ·22
6	0·69 ± ·29	0·58 ± ·27
8	0·40 ± ·09	0·34 ± ·05

* Average 3 replicates. ± = Standard error.

In soild medium (PDA) within 2 days the petridishes were covered with spores in both case. Mycelial growth was suppressed and was not visible.

4.3.2. Different media

Growth of *A. niger* and *A. flavus* was also determined in different media-both liquid and solid. The tested media were potato dextrose broth and Agar (PDB and PDA). Potato sucrose broth and Agar (PSB and PSA), Elliot's broth and Agar (EB and EA), Czapek – Dox broth and Agar (CDB and CDA),

Richard's broth and Agar (RB and RA), Carrot Juice broth and Agar (CJB and CJA).

In case of liquid medium mycilial dry weight was taken after 6 days. Results (Table 11) revealed that maximum growth and sporulation occurred in PDB and least in EB. In PDB *A. niger* had an average mycelial dry weight of 0.69g and *A. flavus* of 0.58 g, whereas in EB the dry weight of mycelium was 0.02 g in both cases.

Table 11: Growth and sporulation of *Aspergillus niger* and *Aspergillus flavus* in different media (after 6 days of growth).

Medium*	Average dry wt of mycelia**		Sporulation***	
	A. niger	***A. flavus***	***A. niger***	***A. flavus***
PDB	·69 ± ·12	·58 ± ·12	++++	++++
PSB	·57 ± ·03	·52 ± ·05	+++	+++
CDB	·42 ± ·12	·38 ± ·12	++	++
CJB	·06 ± ·005	·05 ± ·005	+	+
RB	·04 ± ·001	·04 ± ·004	+	+
EB	·02 ± ·005	·02 ± ·005	–	–

** Average of 3 replicates ; ± = Standard error.

*** ++++ = Profuse sporulation; +++ = High sporulation; ++ = moderate sporulation; + = low sporulation,

* PDB = Potato dextrose broth, PSB = Potato sucrose broth, CDB = Czapek Dox broth, CJB = Carrot juice broth, EB + Elliot's broth, RB = Richard's broth.

In both solid and liquid medium profuse sporulation was observed in PDA/PDB. Growth rate in EA was very slow and no sporulation was observed sporulation in CDA, CJA and RA were also lower than PDA and PSA (Plate XVI).

On the basis of these studies, PDA and PDB were selected for further experiments and incubation periods of 6 days was also selected.

4.4. Changes in seed protein induced by storage fungi

During seed storage the macromolecular components are likely to be affected by the storage fungi. Proteins are important component of pulses

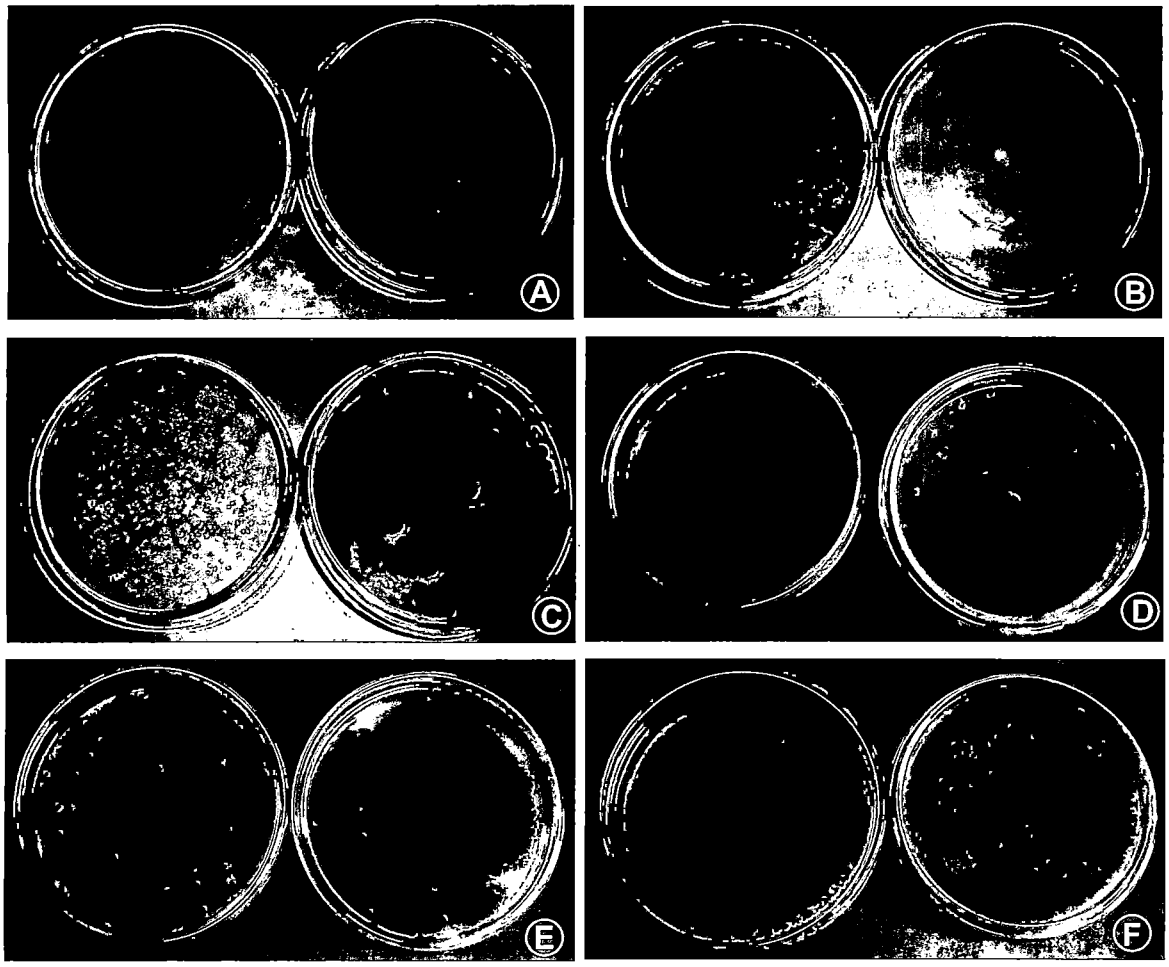

Plate - XVI : (Fig. A-F) Growth and sporulation of *Aspergillus niger* (black) and *Aspergillus flavus* (green) in different media. In PDA (A), CDA (B), PSA (C), CJA (D), RA (E), EA (F).

and hence experiments are carried out to determine the effect of storage fungi on both quantity and types of proteins.

Analysis of protein pattern was done by SDS-PAGE.

4.4.1. Protein content

Protein contents were estimated from stored seeds as well as seeds inoculated with selected fungi.

4.4.1.1. Stored seeds

Seeds of all three pulses were taken out from storage under ambient condition after a period of 12 months. Protein contents of both fresh seeds as well as those from storage were determined. Among the three pulses *Vigna radiata* had the highest protein content (ranging from approx 250-300mg/ gm tissue) followed by *Lens culinaris* (100-140mg / gm tissue) and least in *Cajanas cajan* (85-95 mg/gm tissues). In all cases there was significant reduction in protein content (Table 12) with the decreases being more than 50% which was statistically highly significant.

Table 12: Protein content of Fresh & Stored seeds different pulses.

Seeds	Variety	Protein content (mg / gm tissue)**	
		Fresh seeds	Stored seeds*
Cajanas cajan	ICPL-87	85.0 ± 1·18	21.0 ± 0·5
	Rabi 20/105	95·0 ± ·72	28.0 ± 0·5
Vigna radiata	B_1	261.0 ± ·72	115.0 ± 1·18
	Pusa Baisakhi	308.0 ± ·47	164.0 ± 0·72
Lens culinaris	Rajan	140.0 ± ·72	65.0 ± 0·47
	Subrata	100.0 ± ·54	42·5 ± 0·23
	Asha	128·5 ± ·23	40.0 ± 0·54

* Storage period –12 months,

** Average of 3 replicates.

± = Standard error.

Difference between fresh and stored seeds in all cases significant at P=0.01 in Students 't' test.

4.4.1.2. Artificially inoculated

In order to determine the effect of the two selected storage fungi i.e. *Aspergillus niger* and *Aspergillus flavus* on protein deterioration, fresh seeds were taken and inoculated with the fungal spores separately as described materials and methods. During sampling seeds were taken out, washed thoroughly and used for estimation of protein content. Un inoculated seeds were taken as control. In both cases significant decrease in protein content was observe. (Table 13, Fig. 9). In *Lens culinaris, Aspergillus flavus* showed greater effect while in *Cajanas cajan, Aspergillus niger* caused greater deterioration.

Table 13: Protein content of control and artificially innoculated seeds different pulses.

Seeds	Variety	Protein content (mg / gm tissue)*		
		Control	Treated	
			Aspergillus niger	***Aspergillus flavus***
Cajanas cajan	ICPL-87	80.0 ± 0·23	23.0 ± 0·23	40.0 ± 0·23
	Rabi 20/105	100.0 ± 1·18	42.0 ± 0·54	49.0 ± 0·23
Vigna radiata	B_1	190.0 ± 1·18	60.0 ± 0·23	97·5 ± 0·54
	Pusa Baisakhi	267·5 ± 1·18	109.0 ± 1·18	95.0 ± 0·5
Lens culinaris	Rajan	145.0 ± 0·72	68·25 ± 0·54	33·6 ± 0·47
	Subrata	145.0 ± 1·18	62.0 ± 0·54	41·6 ± 0·23
	Asha	116.0 ± 1·18	94.0 ± 0·72	32.0 ± 0·47

* Average of 3 replicates.
Difference between fresh and stored seeds in all cases significant at P=0.01 in Students 't' test ; ± = Standard Error

4.4.2. Protein pattern

In order to determine the effect of storage fungi on protein patterns SDS PAGE analysis was carried out with freshly harvested seeds, stored seeds as well as artificially inoculated seeds of three pulses.

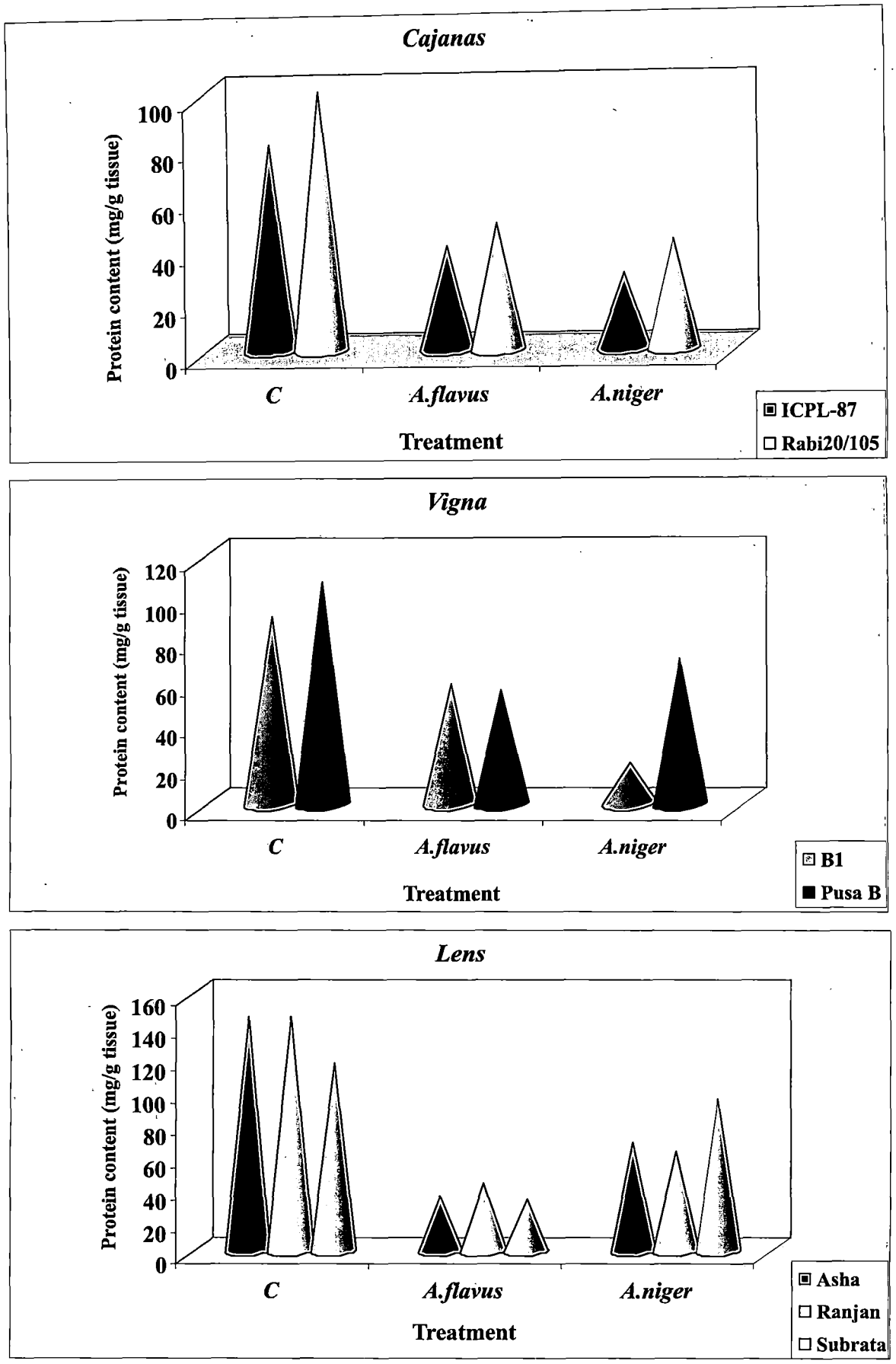

Fig. 9: Protein content of fresh seeds and seeds artificially inoculated with *A.flavus* and *A.niger*.

Results (Plates XVII and XVIII) revealed that protein bands decreased in number and intensity with storage as well as artificial inoculation in all cases. The number of bands and the approximate molecular weights as determined from the gels are depicted (Tables 14-16).

Table 14: SDS-PAGE analysis of proteins from fresh and stored seeds of *L. culinaris*

Source of Protein		No. of bands	Approx molecular Wts. (kd)
Asha	Fresh	18	97,84, 82,81,83, 66, 60, 55, 52, 45, 38, 36, 34, 29, 24, 20, 14·2, 8·5
	Stored	10	66, 60, 55, 52, 45, 38, 36, 29, 24, 20
Ranjan	Fresh	18	66, 60, 62, 55, 54, 52, 45, 38, 34, 30, 20, 27, 24, 22, 20, 18, 16, 8·5
	Stores	9	66, 60, 55, 45, 38, 20, 18, 16, 8·5
Subrata	Fresh	18	62, 58, 55, 50, 45, 40, 38, 34, 36, 32, 30, 29, 27, 25, 20, 18, 12, 8·5
	Stored	10	62, 50, 55, 45, 29, 27, 25, 20, 18, 12

Table 15: SDS-PAGE analysis of proteins from fresh and artificially inoculated seeds (Inoculated with *A. niger)*

Source of Protein		No. of bands	Approx molecular Wts. (kd)
C. cajan (Rabi 20/105)	Control	25	97, 92, 90, 86, 84, 70, 68, 66, 63, 60, 58, 55, 50, 45, 42, 44, 40, 38, 26, 32, 34, 29, 24, 27, 20
	Treated	17	97, 90, 84, 70, 55, 52, 45, 29, 27, 26, 24, 20, 14·2, 12, 10, 9, 8·5
V. radiata (B_1)	Control	38	99, 97, 95, 92, 86, 84, 80, 78, 72, 68, 66, 62, 60, 58, 56, 55, 50, 48, 45, 42, 40, 38, 36, 32, 30, 29, 24, 22, 21 20, 18, 16, 15, 14·2, 12, 11, 10, 8·5
	Treated	8	90, 86, 66, 52, 55, 38, 29, 18
L. culinaris (Asha)	Control	32	99, 96, 93, 84, 82, 80, 78, 72, 67, 66, 62, 61, 58, 55, 52, 50, 45, 42, 40, 38, 36, 32, 30, 28, 24, 22, 20, 18, 14·2, 12, 10, 8·5
	Treated	13	95, 93, 84, 80, 78, 66, 55, 45, 38, 36, 29, 20, 14·2.

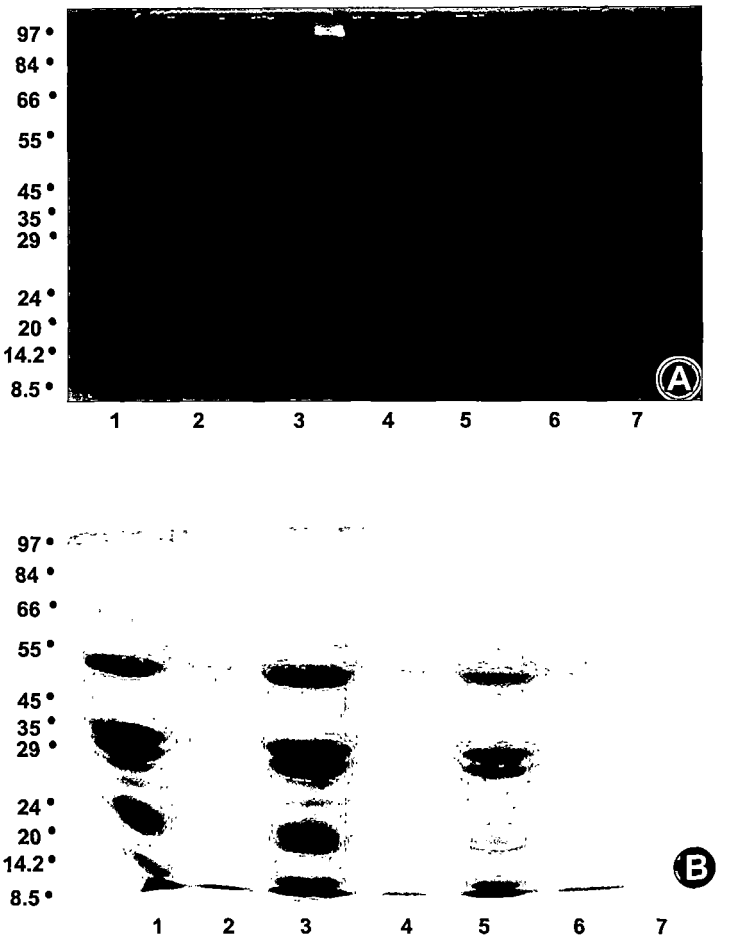

Plate-XVII: (Fig. A&B) : SDS-PAGE analysis of fungal (Fig. A) and seed (Fig. B) proteins.
Fig. A: Lanes 1-3: antigens of *A. niger ;* Lanes 4-6: antigens of *A.flavus*; Lane 7 Marker.
Fig. B: Lanes 1& 2 : *L. culinaris* variety Asha; Lanes 3 & 4: *L. culinaris* variety Ranjan; Lanes 5 & 6: *L. culinaris* variety Subrata; Lanes 1,3 & 5 Fresh seeds and Lanes 2, 4 & 6 stored seeds.

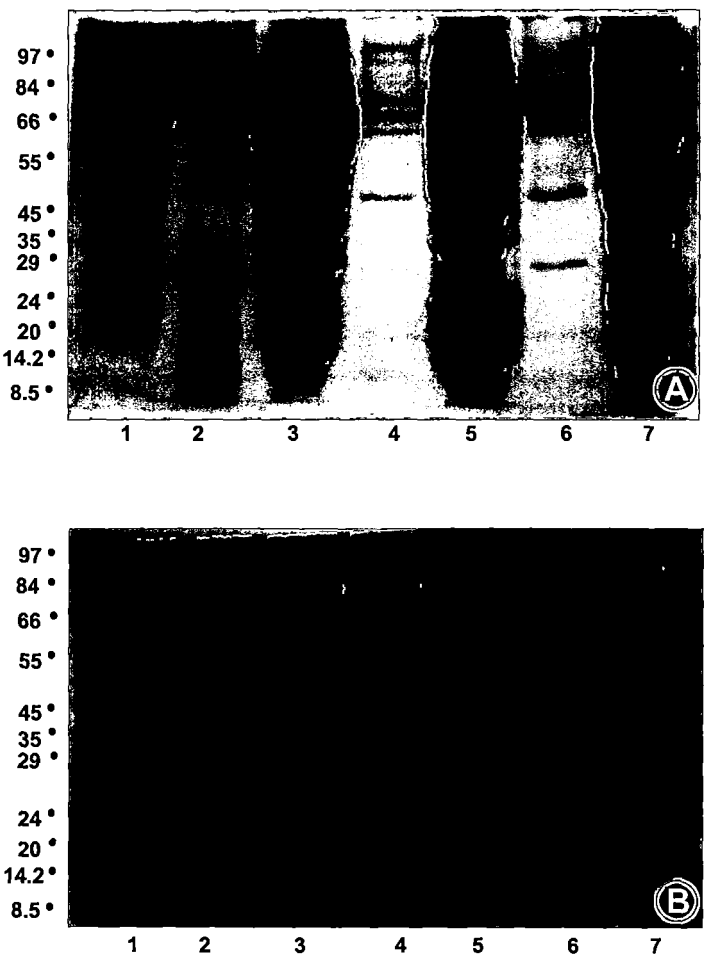

Plate-XVIII: (Fig. A&B): SDS-PAGE analysis of seed proteins

Fig. A: Lanes 1&2: *C.cajan*:3&4: *L..culinaris*; 5,6 &7 *V. radiata*; 1,3,5 &7: Healthy seeds; 2,4&6: Inoculated with *A. niger.*

Fig. B: Lanes 1&2: *C.cajanas*, 3&4: *L. culinaris*; 5,6 & 7: *V. radiata;* 1,3,5&7: Healthy seeds 2,4&6: Inoculated with *A. Flavus.*

Table 16: SDS-PAGE analysis of proteins from fresh and artificially inoculated seeds (Innoculated with *A. flavus*

Protein		No. of bands	Approx molecular Wts. (kd)
C. cajan (Rabi 20/105)	Control	25	97, 92, 90, 86, 84, 72, 68, 66, 63, 60, 58, 55, 50, 45, 42, 44, 40, 38, 36, 32, 34, 29, 24, 14·2, 20
	Treated	9	66, 55, 45, 42, 44, 29, 24, 20, 14·2,
V. radiata (B_1)	Control	38	99, 97, 95, 92, 86, 84, 80, 78, 72, 68, 66, 62, 60, 58, 56, 55, 50, 48, 45, 42, 40, 38, 36, 32, 30, 29, 24, 22, 21 20, 18, 16, 15, 14·2, 12, 11, 10, 8·5
	Treated	5	45, 38, 24, 14·2, 8·5
L. culinaris (Asha)	Control	32	99, 96, 93, 84, 82, 80, 78, 72, 67, 66, 62, 61, 58, 55, 52, 50, 45, 42, 40, 38, 36, 32, 30, 28, 24, 22, 20, 18, 14·2, 12, 10, 8·5
	Treated	7	55, 36, 28, 24, 22, 20, 12

4.5. Changes in carbohydrates induced by storage fungi

Since carbohydrates are also present in all seeds specially as total sugar, these were estimated to determine the extent of deterioration.

4.5.1 Stored seeds

There was an overall decrease in total sugars following storage but it was not significant in all cases (Table 17).

Table 17: Total carbohydrate content of Fresh and Stored seeds of different pulses.

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

* Storage period –12 months, ** 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.

4.5.2. Artificially inoculated

Innoculation with *A. flavus* and *A. niger* led to decrease in total sugar contents, with *A. flavus* causing more deterioration. In *V. radiata* and *L. culinaris,* the reduction caused by the two fungi were not greatly different. In *C. cajan* however the total sugar content was drastically reduced by *A. flavus* but not so significantly by *A. niger* (Table 18, Fig. 10).

Table 18: Total carbohydrate contents of control and artificially inoculated seeds of different pulses.

Seeds	Variety	Total sugar content* (mg/gm tissue)		
		Control	Treated	
			Aspergillus niger	***Aspergillus flavus***
Cajanas cajan	ICPL-87	44.0 ± 0·72	40·8 ± 0·54	12.0 ± 0·23
	Rabi 20/105	48.0 ± 0·54	43.0 ± 0·72	16.0 ± 0·23
Vigna radiata	B_1	55·0 ± 0·54	41.0 ± 0·23	36·5 ± 0·23
	Pusa Baisakhi	60·4 ± 0·72	40.0 ± 0·54	38.0 ± 0·47
Lens culinaris	Rajan	95.0 ± 1·18	60.0 ± 0·72	50.0 ± 0·72
	Subrata	73.0 ± 0·54	38.0 ± 0·23	35.0 ± 0·23
	Asha	93.0 ± 0·72	51.0 ± 0·54	50.0 ± 0·72

[a] Different with control significant at P = 0·05 in students 't' test. In all other cases, different with control significant at P = 0·01

* Average of 3 replicates; ± = Standard error.

4.6. 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 as described in materials and methods and were allowed to germinate. Uninoculated seeds were also allowed to germinate. Protease and amylase activities were estimated from the germinated seedlings. Sampling was done after 24, 48 and 72 hours of

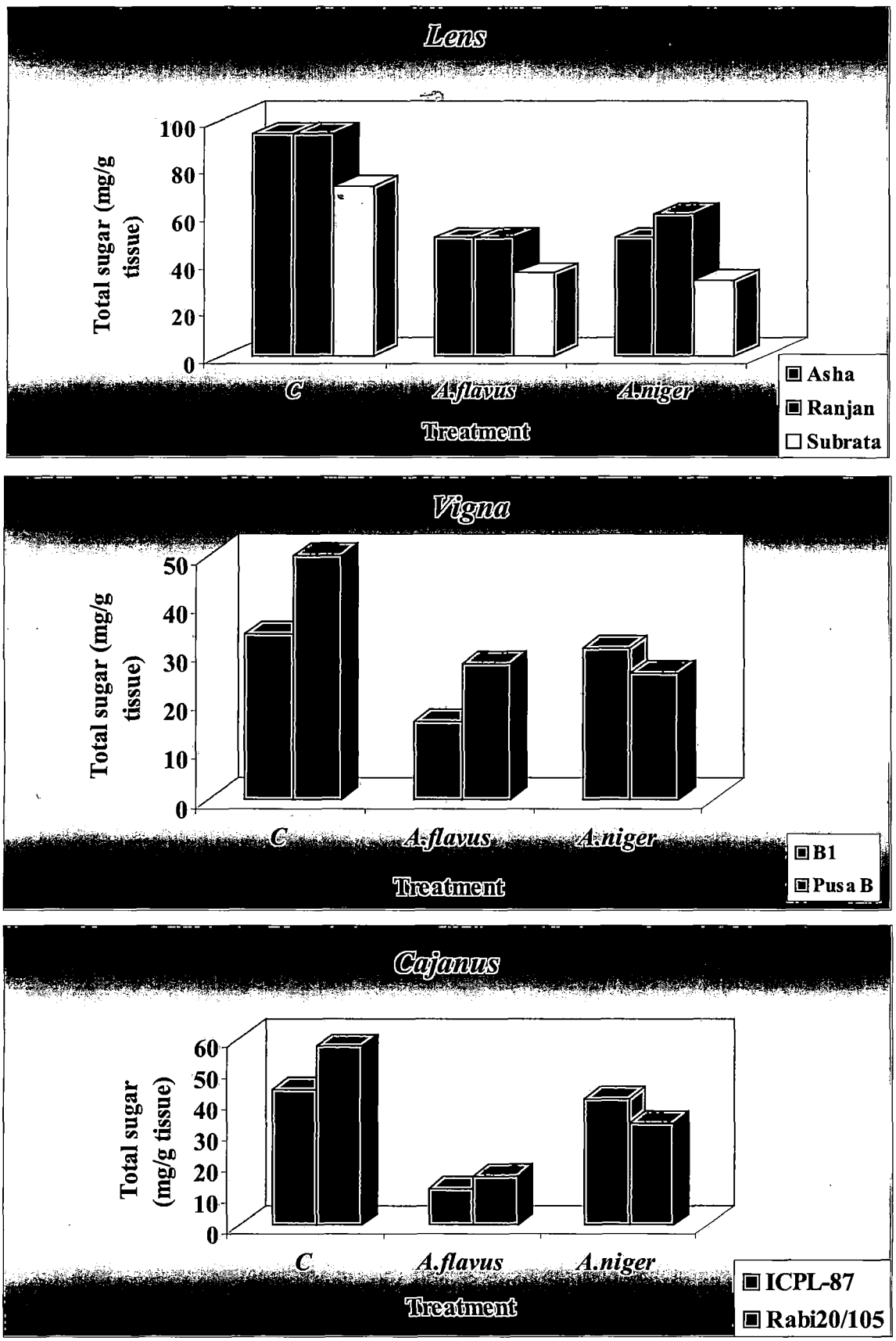

Fig.10: Total sugar content of fresh seeds and seeds artificially inoculated with *A.flavus* and *A.niger*.

seed plating in case of *V. radiata, C. cajan, L. culinaris* respectively. Difference in timing was based in the speed of germination of different seeds.

4.6.1. Protease

Protease activity was assayed in case of stored seeds as well as artificially inoculated one. General decline in activity was obtained in all cases, though it was more significant when seeds were stored for prolonged period. Artificial inoculation did not lead to significant decreases in activity (Tables 19 & 20, Fig. 11).

Table 19: Protease activity of fresh and stored pulses seeds

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

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

Table 20: Protease activity of control and artificially inoculated pulses seeds

Seeds	Variety	Protease activity (µg protein hydrolyzed / min/gm tissue)*		
		Control	Treated	
			Aspergillus niger	*Aspergillus flavus*
Cajanas cajan	ICPL-87	125.0 ± 0·54	120.0 ± 0·47	103.0 ± 0·47
	Rabi 20/105	155.0 ± 0·54	137.0 ± 0·47	143.0 ± 0·47
Vigna radiata	B_1	180.0 ± 0·5	137.0 ± 0·72	120.0 ± 0·72
	Pusa Baisakhi	120.0 ± 0·5	100.0 ± 0·72	67.0 ± 0·72
Lens culinaris	Rajan	292.0 ± 0·72	275.0 ± 0·5	210.0 ± 0·47
	Subrata	278.0 ± 0·72	230.0 ± 0·5	210.0 ± 0·47
	Asha	250.0 ± 0·72	230.0 ± 0·5	210.0 ± 0·47

* Average of 3 replicates; ± = Standard error.

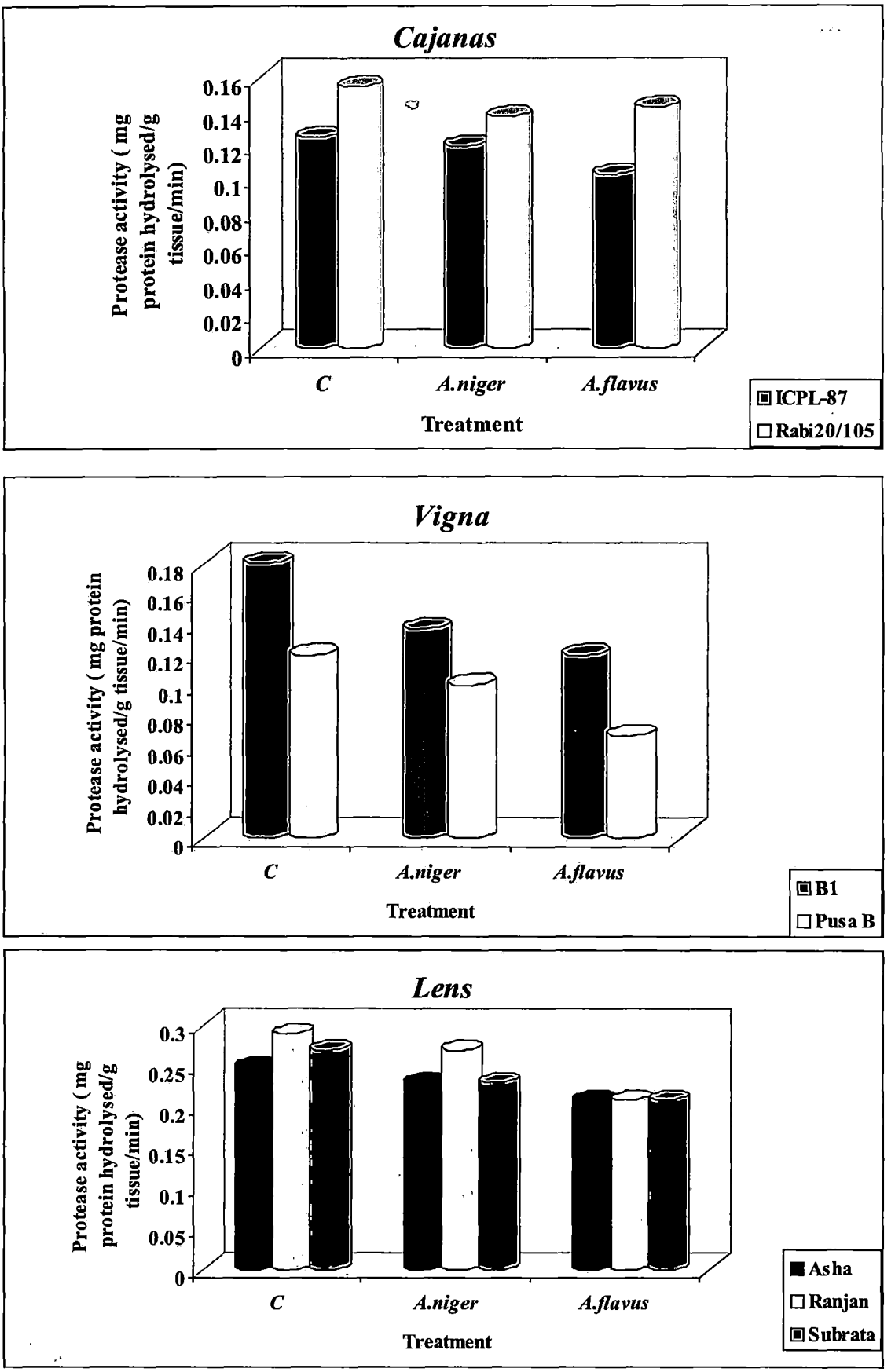

Fig.11: Protease activity of seeds artificially inoculated with *A.flavus* and *A.niger*

4.6.2. Amylase

Similar results were also obtained in case of amylase activity with the general trend being decrease in activity. Significant decrease was obtained from stored seeds (Tables 21 & 22, Fig. 12).

Table 21: Amylase activity of fresh and stored seeds of different pulses.

Seeds	Variety	Amylase activity µg starch hydrolyzed / min / g of tissue)**	
		Fresh seeds	Stored seeds*
Cajanas cajan	ICPL-87	208.0 ± 1·18	98.0 ± 0·72
	Rabi 20/105	308.0 ± 1·18	132.0 ± 0·72
Vigna radiata	B_1	258.0 ± 0·72	102.0 ± 0·54
	Pusa Baisakhi	133.0 ± 0·72	89.0 ± 0·54
Lens culinaris	Rajan	116.0 ± 0·54	67.0 ± 0·47
	Subrata	75.0 ± 0·54	38.0 ± 0·42
	Asha	175. ± 0·54	78.0 ± 0·47

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

Table 22: Amylase activity of control & artificially inoculated pulses seed

Seeds	Variety	Amylase activity (µg starch hydrolyzed / min/gm tissue)*		
		Control	Treated	
			Aspergillus niger	*Aspergillus flavus*
Cajanas cajan	ICPL-87	208.0 ± 1·18	166.0 ± 0·72	160.0 ± 0·72
	Rabi 20/105	288.0 ± 1·18	208.0 ± 1·18	233.0 ± 1·18
Vigna radiata	B_1	258.0 ± 0·72	133.0 ± 0·72	153.0 ± 0·72
	Pusa Baisakhi	233.0 ± 1·18	83.0 ± 0·72	103.0 ± 0·72
Lens culinaris	Rajan	116.0 ± 0·41	50.0 ± 0·23	80.0 ± 0·54
	Subrata	125.0 ± 0·54	65.0 ± 0·23	89.0 ± 0·72
	Asha	175.0 ± 0·47	75.0 ± 0·23	95.0 ± 0·34

* Average of 3 replicates; ± = Standard error.

4.7. Optimization of PAbs raised against against *A. niger* and *A. flavus*

Polyclonal Antibodies (PAbs) were raised against *A. niger* and *A. flavus* for detecting the presence of each fungi in seeds even in minute

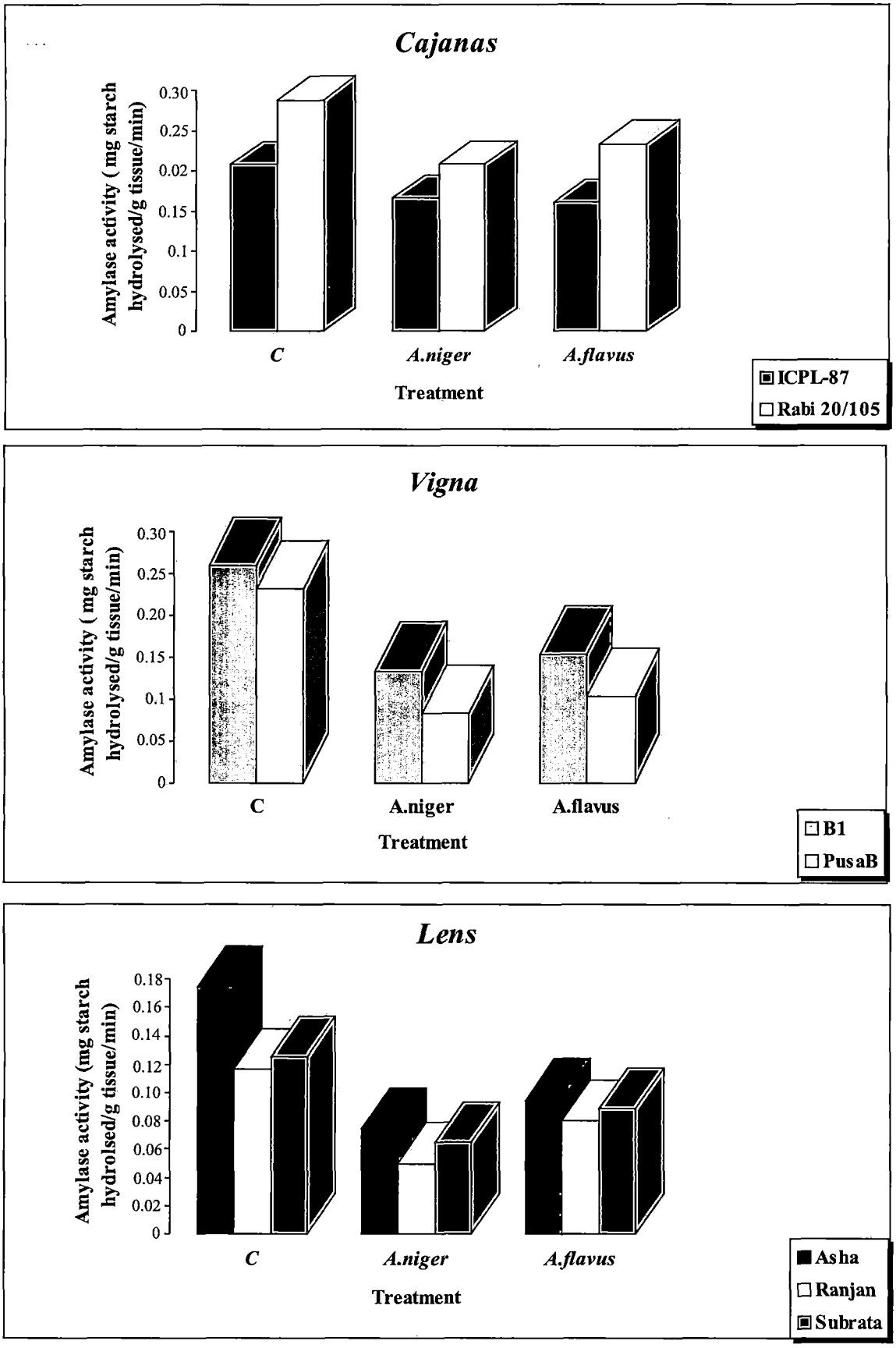

Fig.12: Amylase activity of seeds artificially inoculated with *A.flavus* and *A.niger.*

quantities. Intially before proceding with raising of PAbs, the antigen preparation were analysed by SDS-PAGE and the protein pattern determined. *A. niger* had 17 bands are *A. flavus* had 11 bands. In the beginning effectiveness of antigen preparations from the fungi for raising PAbs were checked by homologous cross reactions following Agar gel double diffusion test enzyme linked immunosorbant assay (ELISA) and also by dot-blot assay.

4.7.1. Immunodiffusion

PAbs raised against fungal extract immunogens with 5 bleedings were used for immunodiffusion tests. Results have been presented in Table 23 and Plate XIX. Precipitation reactions were observed from 1st to 5th bleedings though in the 1st bleeding it was found to be weak. 5th bleedings also showed strong reaction. In case of strong reactions, in the 5th bleed 3 sharp arc appeared while in case of weak reactions 1 faint arc was visible. Results of immunodiffusion showed 5th bleed PAb to have the highest titre value in case of *A. flavus* and *A. niger* also.

Table 23: Agar gel double diffusion tests of PAbs of *A. niger* and *A. flavus* with homologous antigens

PAb (Bleed)	Precipitation reaction	
	Aspergillus niger	***Aspergillus flavus***
1st	±	±
2nd	+	+
3rd	+	+
4th	+	+
5th	+	+

Reaction in each case homologous.

± = week; + = Strong precipitation reaction.

In all cases, PAbs were applied to the central wells and the homologous antigens to the peripheral wells.

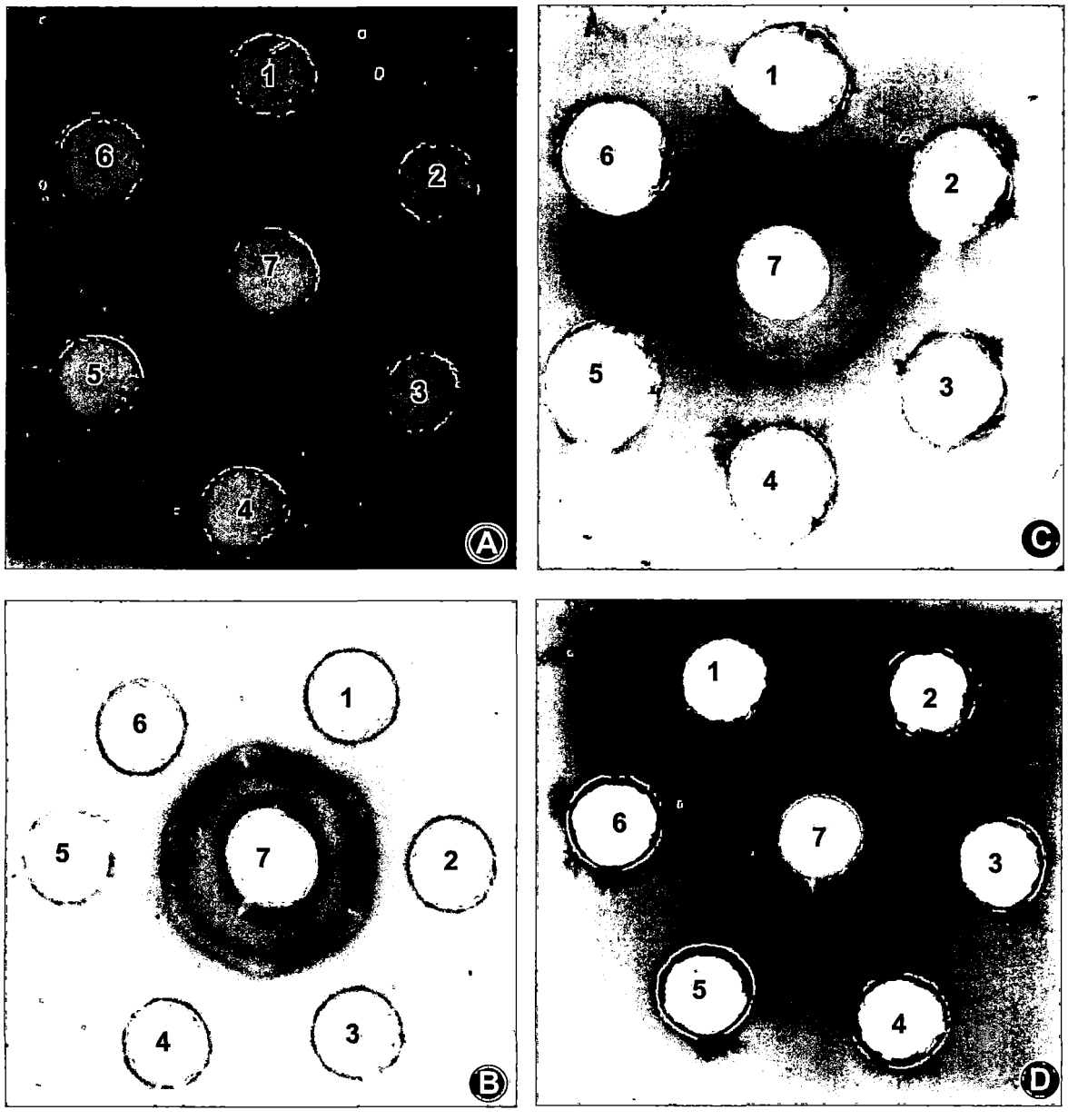

Plate - XIX : Agar gel double diffusion test with PAb of *Aspergillus flavus* (A & B) and *Aspergillus niger* (C & D). Peripheral wells (1-6) were loaded with fungal antigens and central wells were loaded with PAb (5th bleeding A & 2nd bleeding B) of *Aspergillus flavus* and PAb (5th bleeding-C and 2nd bleeding-D) of *Aspergillus niger.*

4.7.2. Enzyme Linked Immunosorbant Assay (ELISA)

One of the most sensitive methods for detection of organism within a tissue is enzyme linked immunosorbant assay (ELISA). In ELISA using polyclonal antibodies, these PAbs are allowed to react with antigens and their reactivity measured by methods employing tagging of enzymes to IgG. These enzymes react with substrate and colour intensity of the product is measured. Higher absorbance values indicate higher antigen antibody reaction.

Since ELISA depends on a number. of factors and these vary from system to system it was considered necessary to optimize various conditions before further tests. Hence initially a number of experiments was performed for optimization.

4.7.2.1. Different bleedings

IgG obtained from PAbs of 1st to 5th bleed were tested in homologous reactions, keeping the concentration of IgG and antigen at 10µg/ml. It was observed that absorbance values were high from 1st bleed onwards in case of both *A. niger* and *A. flavus* (Table 24 & 25). However highest A_{405} readings were obtained in case of 5th bleed and lowest in 1st bleed.

Table 24: ELISA reaction of the antigen of *A. niger* reacted with homologous PAbs from different bleeds.

Different bleed	Absorbance at 405 nm			
	Exp-1	Exp-2	Exp-3	Mean
1st	1·973	1·890	1·921	1·928
2nd	2·257	2·420	2·259	2·312
3rd	2·562	2·533	2·432	2·509
4th	2·950	2·950	2·873	2·924
5th	3·132	3·002	3·112	3·082

Antigen concentration = 10 µg / ml

IgG concentration = 10 µg / ml.

Table 25: ELISA reaction of the antigen of *A. flavus* reacted with homologous PAbs from different bleeds.

Different bleed	Absorbance at 405 nm			
	Exp-1	Exp-2	Exp-3	Mean
1st	2·001	2·105	2·115	2·073
2nd	2·660	2·532	2·671	2·621
3rd	2·878	2·750	2·785	2·804
4th	2·978	3·101	3·001	3·029
5th	3·281	3·275	3·270	3·275

Antigen concentration = 10 µg / ml
IgG concentration = 10 µg / ml.

4.7.2.2. Antisera dilution

In these experiments PAbs from 5th bleed were selected and doubling dilutions of IgG were done from concentration of 40 µg/ml to 1.25 µg/ml. Antigen concentration was kept fixed at 10 µg/ml. Highest ELISA values in both *A. niger* and *A. flavus* PAbs were obtained at 40 µg/ml with reduction in absorbance values with dilution. Even at a concentration of 1.25 µg/ml. A_{405} values as high as 1.2-1.3 (Tables 26 & 27 Fig. 13).

Table 26: ELISA reaction of the antigen of *A. niger* reacted with homologous PAbs of different concentration

IgG Conc. (µg/ml.)	Absorbance at 405 nm			
	Exp-1	Exp-2	Exp-3	Mean
40	3·263	3·223	3·206	3·230
20	3·146	3·103	3·101	3·116
10	2·562	2·533	2·432	2·509
5	2·275	2·278	2·263	2·272
2·5	1·501	1·513	1·497	1·503
1·25	1·20	1·212	1·201	1·201

Antigen concentration = 10 µg / ml; Bleed of PAbs = 5th

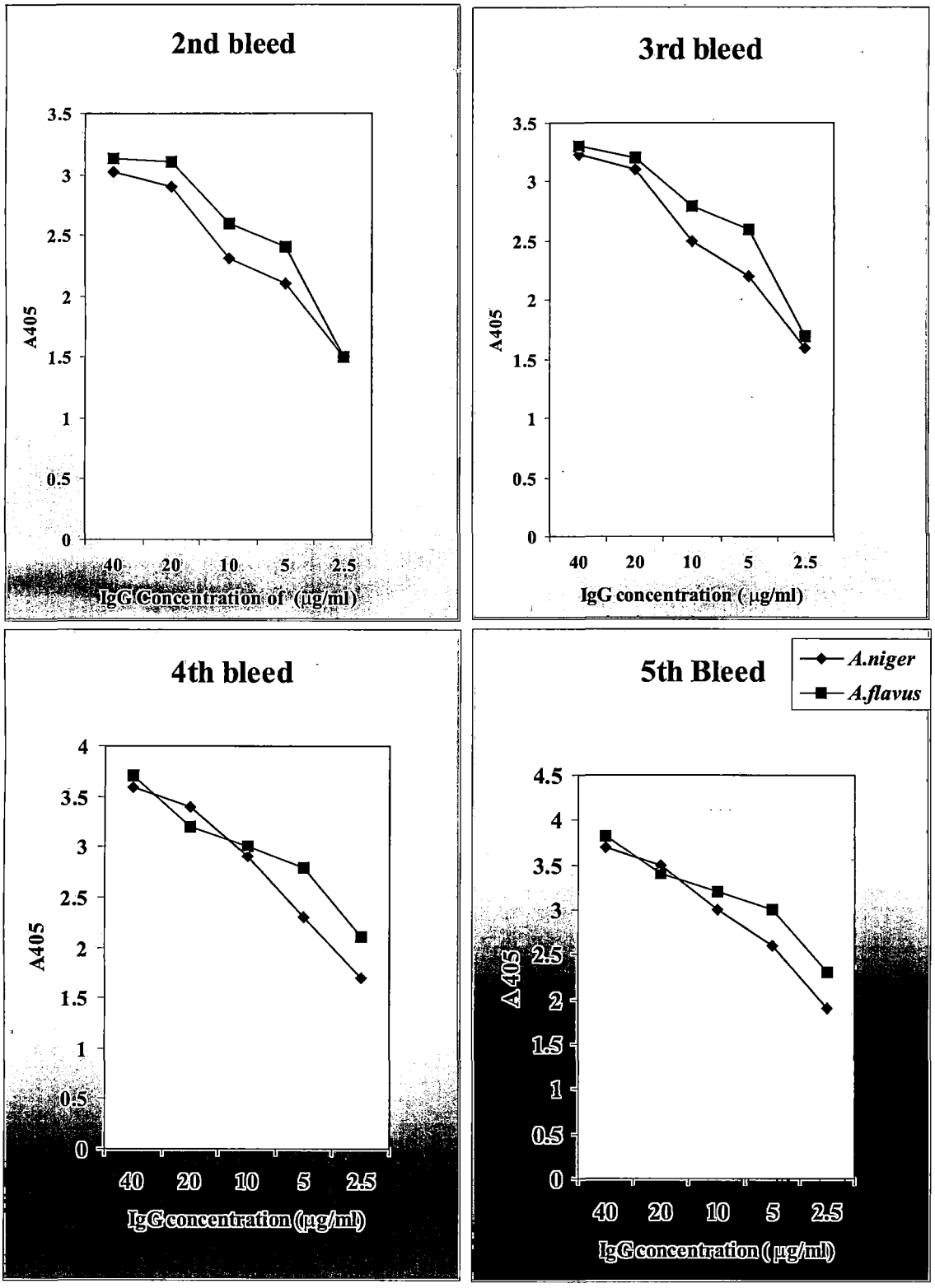

Fig. 13: ELISA values of homologous reactions of antigens of *A. niger* and *A. flavus* with different concentrations of PAbs from different bleeds.

Table 27: ELISA reaction of the antigen of *A. flavus* reacted with homologous PAbs of different concentration

IgG Conc. (µg/ml.)	Absorbance at 405 nm			
	Exp-1	Exp-2	Exp-3	Mean
40	3·505	3·515	3·588	3·536
20	3·482	3·445	3·440	3·455
10	3·281	3·275	3·270	3·275
5	2·949	2·934	2·938	2·943
2·5	2·758	2·751	2·760	2·756
1·25	1·30	1·29	1·25	1·27

Antigen concentration = 10 µg / ml
Bleed of PAbs = 5th

4.7.2.3. Antigen concentration

In this case antigens were diluted by doubling dilution from 40-1.25 µg/ml keeping the bleed constant (5th bleed) and IgG concentration at 10 µg/ml. In this case also decrease in A_{405} values were obtained with increase in dilution (Table 28 & 29, Fig. 14).

Table 28: ELISA reaction of different concentration of antigen of *A. niger* reacted with homologous PAb*

Antigen Conc. (µg/ml.)	Absorbance at 405 nm			
	Exp-1	Exp-2	Exp-3	Mean
40	3·330	3·218	3·521	3·356
20	2·862	2·735	2·886	2·827
10	2·562	2·533	2·432	2·509
5	2·357	2·400	2·325	2·360
2·5	1·987	1·993	1·970	1·983
1·25	1·782	1·703	1·729	1·738

Antigen concentration = 10 µg / ml; Bleed of PAbs = 5th

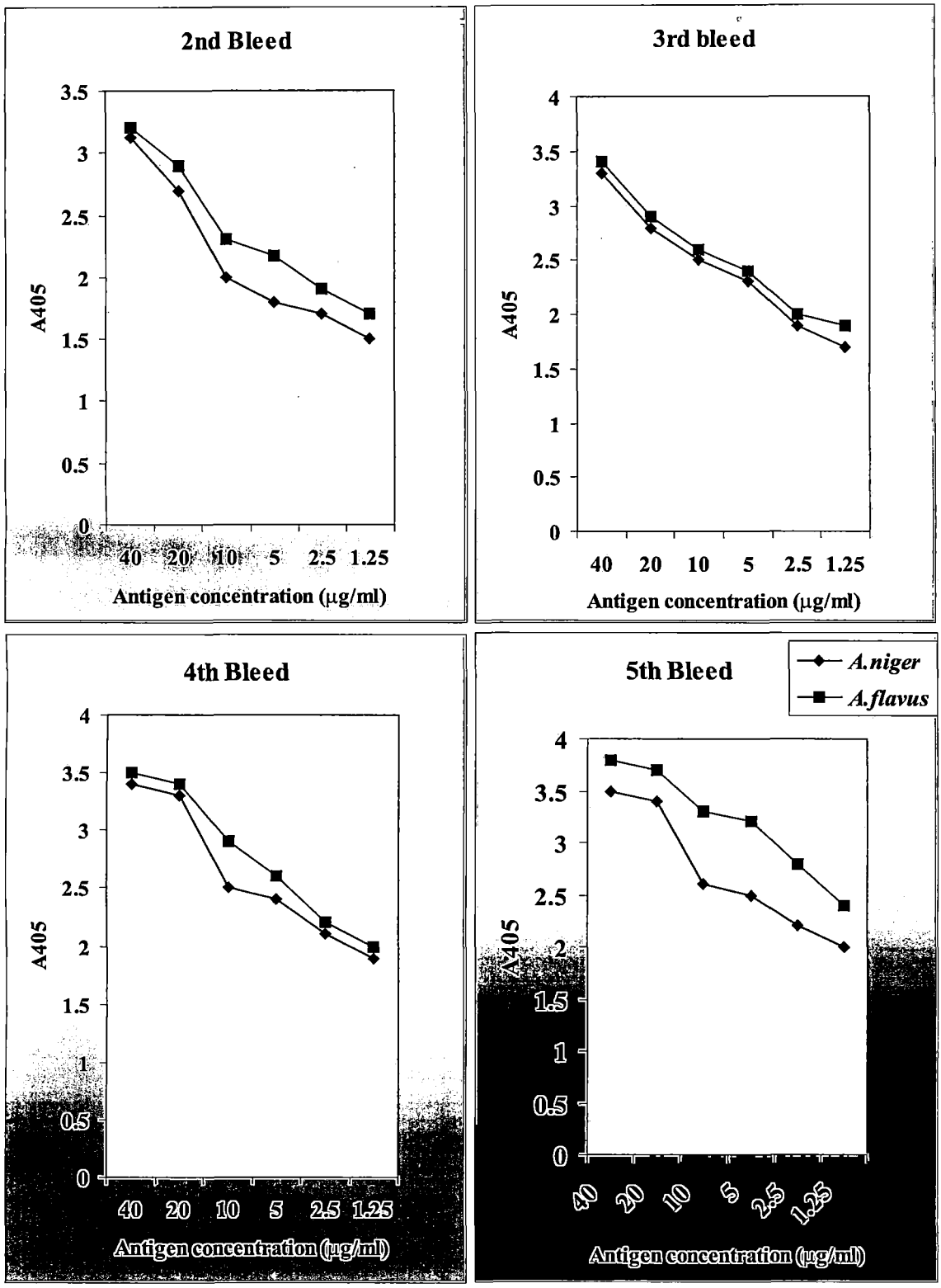

Fig.14: ELISA values of different concentrations of *A.flavus* and *A.niger* antigens reacted with homologous PAbs from different bleeds.

Table 29: ELISA reaction of the different concentration of antigen of *A. flavus* reacted with homologous PAb

Antigen Conc. (µg/ml.)	Absorbance at 405 nm			
	Exp-1	Exp-2	Exp-3	Mean
40	3·826	3·826	3·670	3·774
20	3·755	3·723	3·710	3·729
10	3·281	3·275	3·270	3·273
5	3·205	3·315	3·228	3·249
2·5	2·929	2·876	2·881	2·895
1·25	2·412	2·526	2·530	2·489

IgG Concentration = 10 µg / ml; Bleed of PAbs = 5th

4.7.3. Dot Blot

Immunoblotting or dot blot is a simple specific and relatively easy technique for detection of viruses, fungi etc. in soil, seeds or plant tissues. Being an immunological technique it depends on the relationship between antigen and antibody which is detected enzymaticaty on a nitrocellulose membrane. The method is qualitative and depends on visual estimation of colour intensity.

In the present study initially antigens of *A. niger* and *A. flavus* were allowed to react with their homologous PAbs from different bleedings. Positive reaction as indicated by the appearance of pink coloured dots was evident from 1st bleed onwards. However colour intensity in the 1st bleed was much lighter as compared to the 5th bleed (Table 30).

Table 30: Dot Blot analysis of fungal antigens reacted with homologous PAbs.

PAb	Bleed				
	1st	2nd	3rd	4th	5th
Aspergillus niger	+	++	+++	+++	++++
Aspergillus flavus	+	++	+++	+++	++++

+ = Light pink; ++ = Pink; +++ = Dark pink ; ++++ = Deep pink.

4.8. Immunodetection of fungi in stored seeds

It is often difficult to identify the presence of storage fungi in seeds unless laborious isolation techniques are used. Immunological techniques making use of PAbs raised against specific fungi or their products can be used to detect these fungi in stored seeds. This detection is very specific and even very early stages of attack can be detected.

In the present study detection of *A. niger* and *A. flavus,* the two most commonly occurring storage fungi was carried out by two immunodetection methods i.e. DAC-ELISA and Dot Blot. In both cases IgG obtained from PAbs raised against these two fungi were used.

For these tests stored seeds of the three pulses were obtained from thirteen different locations of West Bengal and Sikkim. i.e. from Raiganj, Malda, Rohini, Siliguri, Matigara, Bagdogra, Jalpaiguri, Berhampore, Burdwan, Bidhannagar (Kolkata), Khidirpur (Kolkata) and Gangtok (Sikkim). Antigens were prepared from these seeds and tested against PAbs of *A. niger* and *A. flavus* by ELISA and Dot-Blot.

4.8.1. ELISA

DAC-ELISA tests as mentioned above revealed that among the two fungi *A. niger* was most prevalent in all the three pluses i.e. *V. radiata, C. cajan* and *L. culinaris*. Highest absorbance values were obtained when seed antigens of *V. radiata* tested against PAb raised against *A. niger*. A_{405} values in this case ranged from 2.4-2.8. Seeds from different localities fell within this range (Table 31, Fig. 15).

Table 31: ELISA reaction of antigens from seeds of *Vigna radiata* collected from different sources reacted with PAbs of *A.niger.*

Sample*	Absorbance at 405 nm			
	Exp-1	Exp-2	Exp-3	Mean
V1	2·72	2·78	2·78	2·76
V2	2·38	2·36	2·44	2·40
V3	2·28	2·32	2·29	2·31
V4	2·44	2·48	2·48	2·46
V5	2·27	2·31	2·29	2·29
V6	1·80	2·22	2·10	2·00
V7	1·90	2·22	2·10	2·10
V8	1·73	1·93	2·40	2·00
V9	1·90	2·27	2·25	2·20
V10	2·45	2·48	2·35	2·40
V11	2·22	1·90	2·10	2·10
V12	2·01	2·42	2·18	2·20
V13	2·38	2·37	2·49	2·40
A. niger	3·38	3·17	3·27	3·30

*Sample, 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

In case of *L. culinaris* A_{405} values were lesser than *V. radiata* but still greater than 1.000 (Tables 32, Fig. 15). Source variation in this case was lesser and fell within 1.12-1.8. In case of *C. cajan* values were still lesser and were in the range of 0.4 - 0.9 (Table 33, Fig. 15).

Table 32: ELISA reaction of antigens from seeds of *Lens culinaris* collected from different sources reacted with PAbs of *A.niger.*

Sample*	Absorbance at 405 nm			
	Exp-1	Exp-2	Exp-3	Mean
L1	1·18	1·19	1·24	1·20
L2	1·50	1·48	1·49	1·49
L3	1·25	1·25	1·32	1·28
L4	1·09	1·06	·97	1·03
L5	1·17	1·06	1·09	1·11
L6	1·25	1·27	1·35	1·29
L7	1·22	1·23	1·15	1·20
L8	1·13	118	1·25	1·19
L9	1·51	1·52	1·55	1·53
L10	·93	·94	·95	·94
L11	1·18	1·16	1·06	1·13
L12	·95	1·00	1·05	1·00
L13	1·26	1·16	1·18	1·20
A. niger	3·38	3·17	3·27	3·30

*Sample, 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

Table 33: ELISA reaction of antigens from seeds of *Cajanas cajan* collected from different sources reacted with PAb of *A. niger.*

Sample*	Absorbance at 405 nm			
	Exp-1	Exp-2	Exp-3	Mean
C1	0·79	0·84	0·82	0·81
C2	0·72	0·73	0·69	0·71
C3	0·48	0·40	0·44	0·44
C4	0·52	0·59	0·60	0·57
C5	0·52	0·49	0·54	0·51
C6	0·32	0·39	0·38	0·37
C7	0·79	0·73	0·84	0·78
C8	0·53	0·58	0·60	0·56
C9	0·87	0·89	0·91	0·89
C10	0·44	0·39	0·40	0·42
C11	0·48	0·48	0·42	0·46
C12	0·49	0·51	0·53	0·51
C13	0·70	0·65	0·68	0·67
A. niger	3·38	3·17	3·27	3·30

*Sample, 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

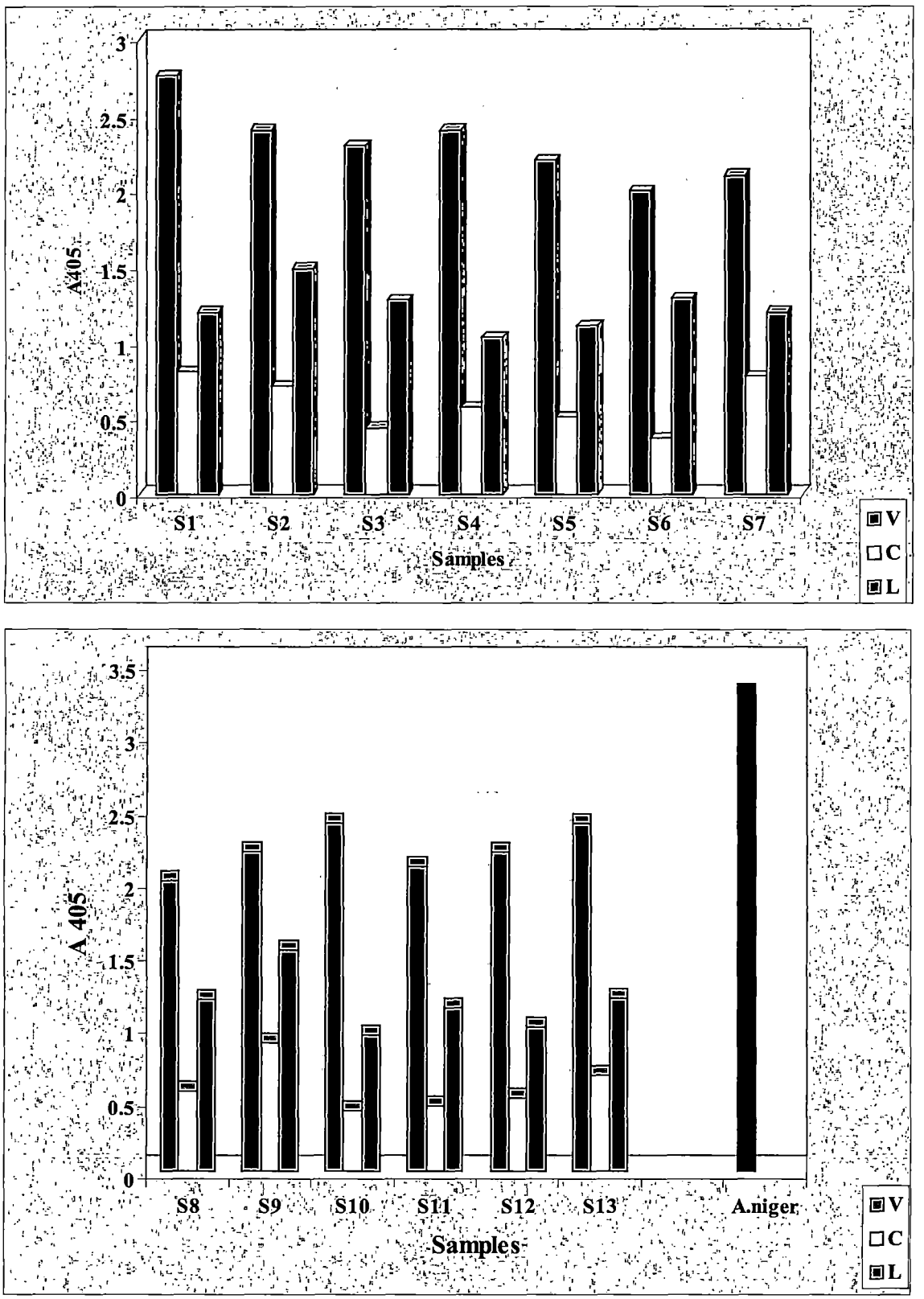

Fig.15: ELISA values of heterologous reactions of antigens from seeds collected from different localities with PAb of *A.niger* V= *V.radiata*; C=*C.cajan*; L= *L. culinaris*.

ELISA tests reveal that in the stored seeds *A. flavus* was much lesser than *A. niger*. In all three seeds A_{405} values were the range 0.2-0.4 in case of *C. cajan* (Table 34, Fig. 16). 1.00-1.30 in case of *V. radiata* (Table 35 Fig. 16), and 0.6-0.8 in case of *L. culinaris* (Table 36, Fig. 16).

Table 34: ELISA reaction of antigens from seeds of *Cajanas cajan* collected different sources reacted with PAbs of *A. flavus*.

Sample*	Absorbance at 405 nm			
	Exp-1	Exp-2	Exp-3	Mean
C1	0·18	0·22	0·26	0·22
C2	0·30	0·29	0·31	0·30
C3	0·23	0·23	0·21	0·23
C4	0·26	0·31	0·30	0·29
C5	0·38	0·33	0·37	0·36
C6	0·30	0·31	0·32	0·31
C7	0·37	0·34	0·31	0·34
C8	0·23	0·17	0·28	0·22
C9	0·32	0·34	0·24	0·30
C10	0·27	0·23	0·28	0·26
C11	0·30	0·31	0.32	0·31
C12	0·26	0·30	0·28	0·28
C13	0·23	0·28	0·24	0·25
A. flavus	3·78	3·82	3·80	3·8

*Sample, 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.

Table 35: ELISA reaction of seed antigens from *V. radiata* reacted with PAb of *Aspergillus flavus.*

Sample*	Absorbance at 405 nm			
	Exp-1	Exp-2	Exp-3	Mean
V1	1·31	1·29	1·33	1·31
V2	1·39	1·32	1·36	1·35
V3	1·08	1·40	1·40	1·20
V4	1·29	1·31	1·21	1·27
V5	1·32	1·30	1·25	1·29
V6	1·19	1·27	1·29	2·25
V7	0·87	0·88	0·95	0·90
V8	1·39	1·36	1·41	1·34
V9	1·27	1·20	1·22	1·23
V10	1·31	1·34	1·22	1·29
V11	1·08	1·13	1·12	1·10
V12	0·93	1·00	1·01	0·98
V13	0·97	1·03	1·00	1·00
A. flavus	3·78	3·82	3·80	3·8

*Sample, 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.

Table 36: ELISA reaction of antigens from seeds of *Lens culinaris* collected different sources reacted with PAbs of *A. flavus.*

Sample*	Absorbance at 405 nm			
	Exp-1	Exp-2	Exp-3	Mean
L1	0·60	0·62	0·64	0·62
L2	0·66	0·67	0·65	0·66
L3	0·72	0·73	0·77	0·75
L4	0·83	0·83	0·80	0·82
L5	0·52	0·53	0·54	0·53
L6	0·73	0·80	0·70	0·74
L7	0·68	0·70	0·66	0·68
L8	0·80	0·79	0·73	0·75
L9	0·84	0·82	0·80	0·82
L10	0·65	0·68	0·58	0·63
L11	0·74	0·73	0·70	0·72
L12	0·57	0·58	0·59	0·58
L13	0·63	0·69	0·67	0·67
A. flavus	3·78	3·82	3·80	3·8

*Sample, 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= Bidhanagar (Kolkata); 11= Khidirpur (Kolkata); 12= Behala (Kolkata); 13= Gangtok

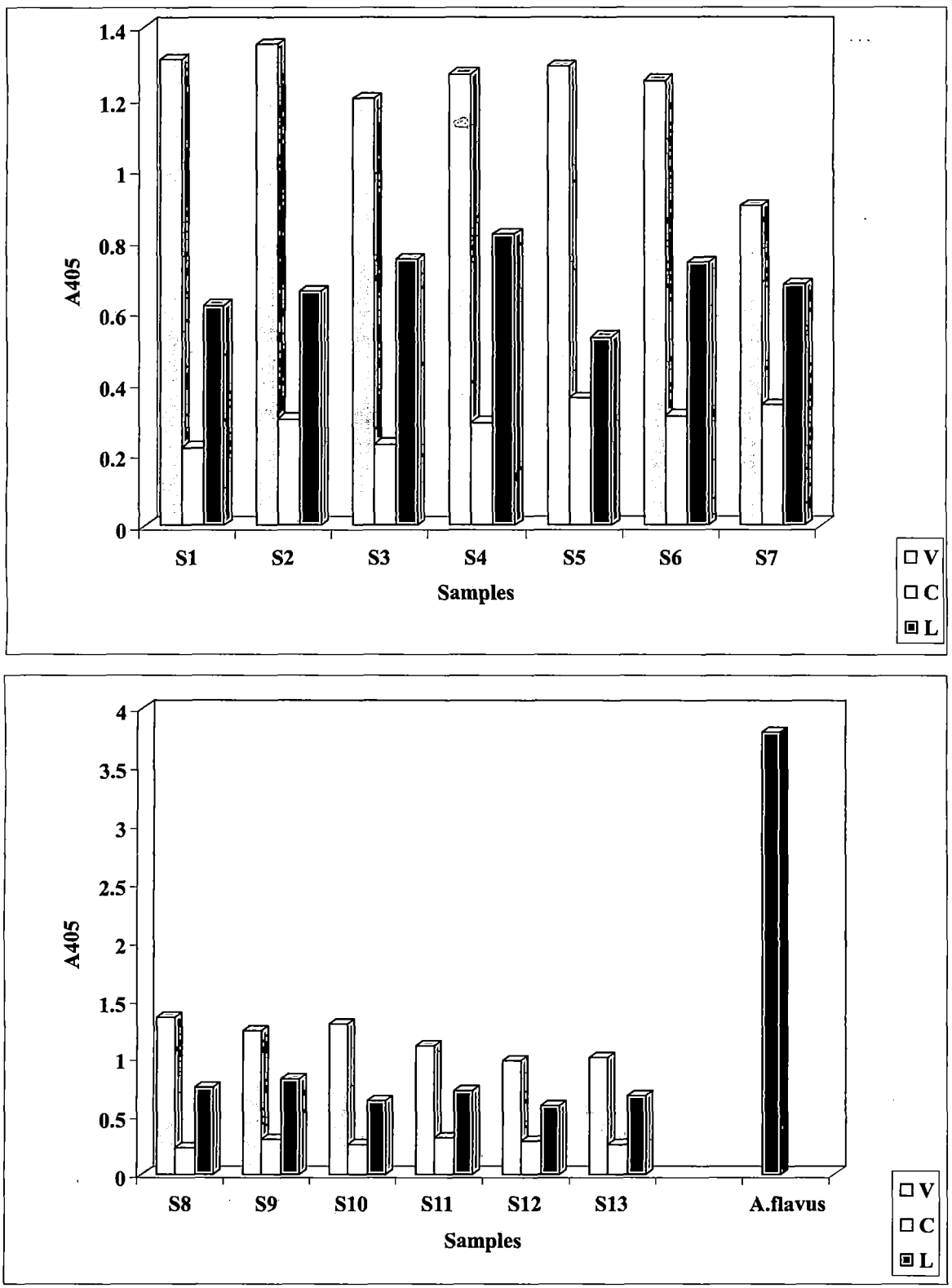

Fig.16: ELISA vallues of heterologous reactions of antigens from seeds collected from different localities with PAb of *A.flavus* V= *V.radiata*; C= *C.cajan;* L= *L. culinaris.*

4.8.2. Dot-Blot

In this case colour intensity of reaction was very high when the seed antigens were reacted with PAb of *A. niger.* Results are presented in Table 37-39 and Plate XX. Variation were also obtained in seeds from different localities. *A. niger* was most prevalent in all three pulses i.e. *V. radiata, C. cajan* and *L. culinaris.* Colour intensity of reaction was very high in homologous reactions i.e. when antigens of *A. niger* reacted with PAb of *A. niger* (Plate (XXA, dot 43, 44, 45) and antigens of *A. flavus* were reacted with PAbs of *A. flavus* (Plate XX B. dot 43, 44, 45). Differences in colour intensity of dots was on indication of lesser association of the particular fungus with the seeds from a locality. The difference was more significant when probing was done with PAb of *A. flavus.*

Table 37: Dot-blot analysis of antigen from *V. radiata* reacted with PAbs of *A. niger* and *A. flavus*

Sample*	PAb of A_{405}	
	Aspergillus niger	***Aspergillus flavus***
V1	+++	++
V2	+++	++
V3	+++	++
V4	+++	++
V5	+++	++
V6	++	++
V7	+++	+
V8	+++	++
V9	++	++
V10	++	++
V11	+++	++
V12	+++	++
V13	+++	++

*Sample, 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 ;ink; ++ = Pink; +++ = Dark pink.

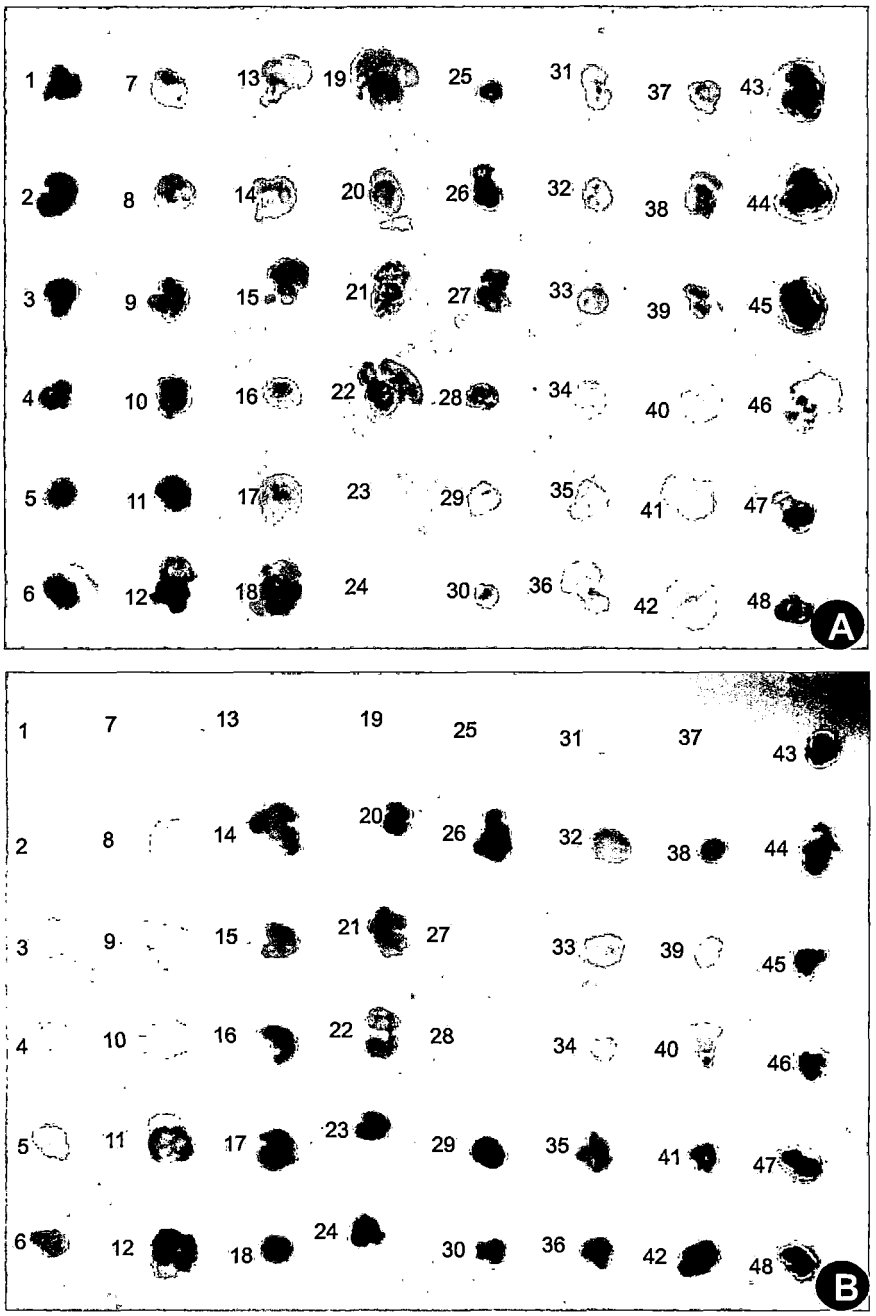

Plate-XX: A. Dot-immunobinding assay of PAb of *A.niger* with seed antigens. Seed antigens : *V. radiata* (1-13); *L. culinaris* (14-26); C. cajan (27-42); Fungal antigens: *A. niger* - homologous (43-45) and *A. flavus* (46-48). Seeds obtained from different localities: Raiganj (1, 14 & 27); Malda (2,15, 28);Rohini (3,16, 29); Siliguri (4, 17, 30); Matigara (5,18,31), Bagdogra (6,19,32; Jalpaiguri (7, 20, 33); Berhampore (8, 21,34); Burdwan (9, 22, 35); Bidhannagar (Kolkata) (10, 23,36); Khidirpur (11, 24, 37, 38); Behala (12, 25, 39, 40); Gangtok (Sikkim) (13, 26, 41, 42);

B. Dot-immunobinding assay of PAb of *A.flavus* with seed antigens. Seed antigens : *V. radiata* (1-13); *L. culinaris* (14-26); C. cajan (27-42); Fungal antigens: *A. flavus* - homologous (43-45) and *A. niger* (46-48). Seeds obtained from different localities: Raiganj (1, 14 & 27); Malda (2,15, 28);Rohini (3,16, 29); Siliguri (4, 17, 30); Matigara (5,18,31), Bagdogra (6,19,32; Jalpaiguri (7, 20, 33); Berhampore (8, 21,34); Burdwan (9, 22, 35); Bidhannagar (Kolkata) (10, 23,36); Khidirpur (11,24,37,38); Behala (12,25,39,40); Gangtok (Sikkim) (13,26,41, 42).

Table 38: Dot-blot analysis of antigen from *Cajanas cajan* reacted with PAbs of *A. niger* and *A. flavus*

Sample*	*Aspergillus niger*	*Aspergillus flavus*
C1	+++	++
C2	+++	++
C3	++	++
C4	+++	++
C5	+++	++
C6	++	++
C7	+++	++
C8	+++	++
C9	++	++
C10	++	+
C11	++	++
C12	++	+
C13	+++	+

*Sample, 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

Table 39: Dot-blot analysis of antigens from *Lens culinaris* reacted with PAbs of *A.niger* and *A. flavus.*

Sample*	*Aspergillus niger*	*Aspergillus flavus*
L1	+++	++
L2	+++	++
L3	++	++
L4	+++	++
L5	+++	++
L6	++	++
L7	+++	++
L8	+++	++
L9	+++	++
L10	++	++
L11	++	++
L12	++	++
L13	++	++

*Sample, 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

4.9. Immunoflorescence

Florescence antibody lebeling with fluorescein isothiocyanate (FITC) is known to be one of the techniques to determine cross reactivity between fungal and host tissues. In the present study mycelia and spores of *A. flavus* and *A. niger* were treated with homologous PAbs stained indirectly with FITC labeled antibodies and observed under florescent microscope (Leica Leitz Biomed microscope). Bright yellow florescence was observed in both mycelia and spores. (Plates XXI and XXII).

Cross sections of one variety each of stored *V. radiata, C. cajanas* and *L. culinaris* were made and treated with PAb of *A. niger* and *A. flavus* separately followed by reaction with FITC. Observations under florescence microscope showed florescence in all cases which was limited mostly to the seed coat and peripheral regions. (Plates XXII and XXIV).

4.10. Immunocytochemical staining

Another approach was used finally to study the invasion of seed tissues by hyphae of *A. niger* or *A. flavus.* Immunocytochemcial staining based on specific PAbs produced against *A. niger* and *A. flavus* provided a means of visualizing hyphae within the seed tissues. Cross section of inoculated seeds were stained immunocytochemicaly as described under materials and methods and observed under bright field microscope. Presence of fungi within the seed tissues was evident on the basis of deeply stained regions. (Plates XXV-XXVII).

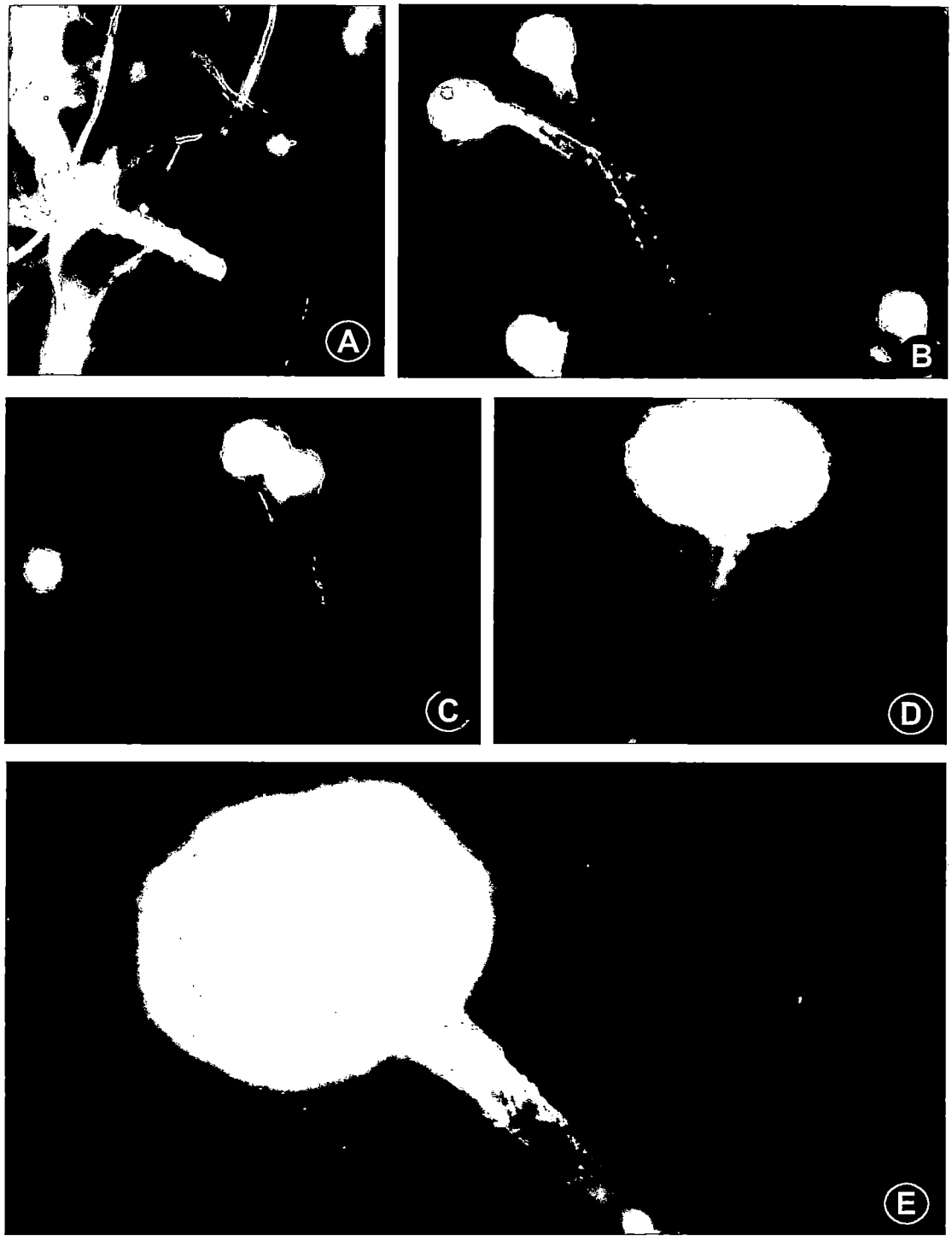

Plate-XXI: (Fig. A-E)
Fluorescence of hyphae (A), Conidiophores and conidia (B-E) of *A. flavus* after staining with FITC-Con A.

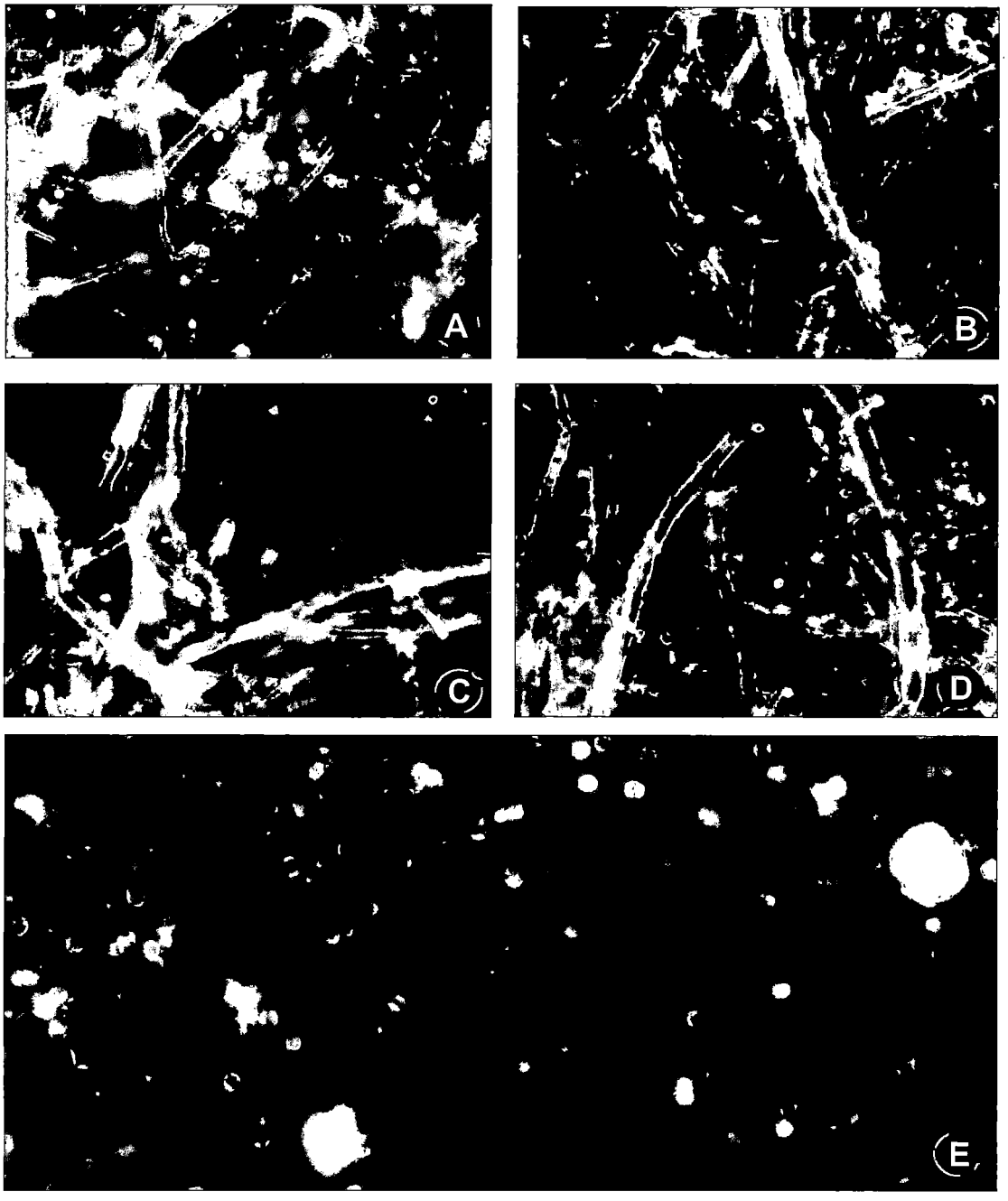

Plate-XXII: (Fig. A-E)
Fluorescence of hyphae (A-D) and conidia (E) of *Aspergillus niger* after staining with FITC-Con A.

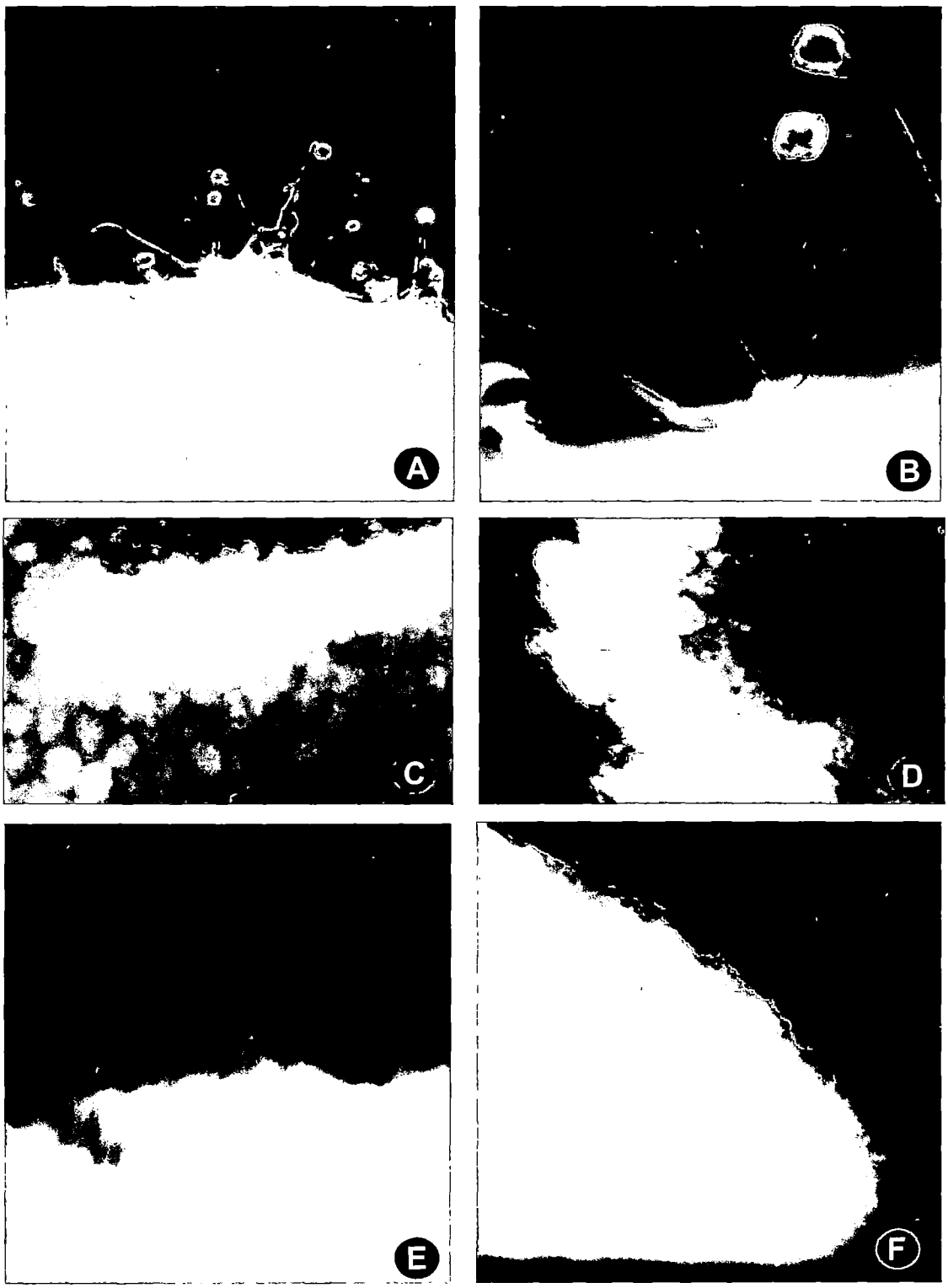

Plate-XXIII: (Fig.. A-F)

Fluorescent antibody staining of cross section of seeds (stored for 12 months) of various pulses. Seed tissues treated with PAb of *A. flavus*. A (x10) & B (x45); *Cajanas cajan*; C&D (x10); *Vigna radiata*; E&F *Lens culinaris* (x10).

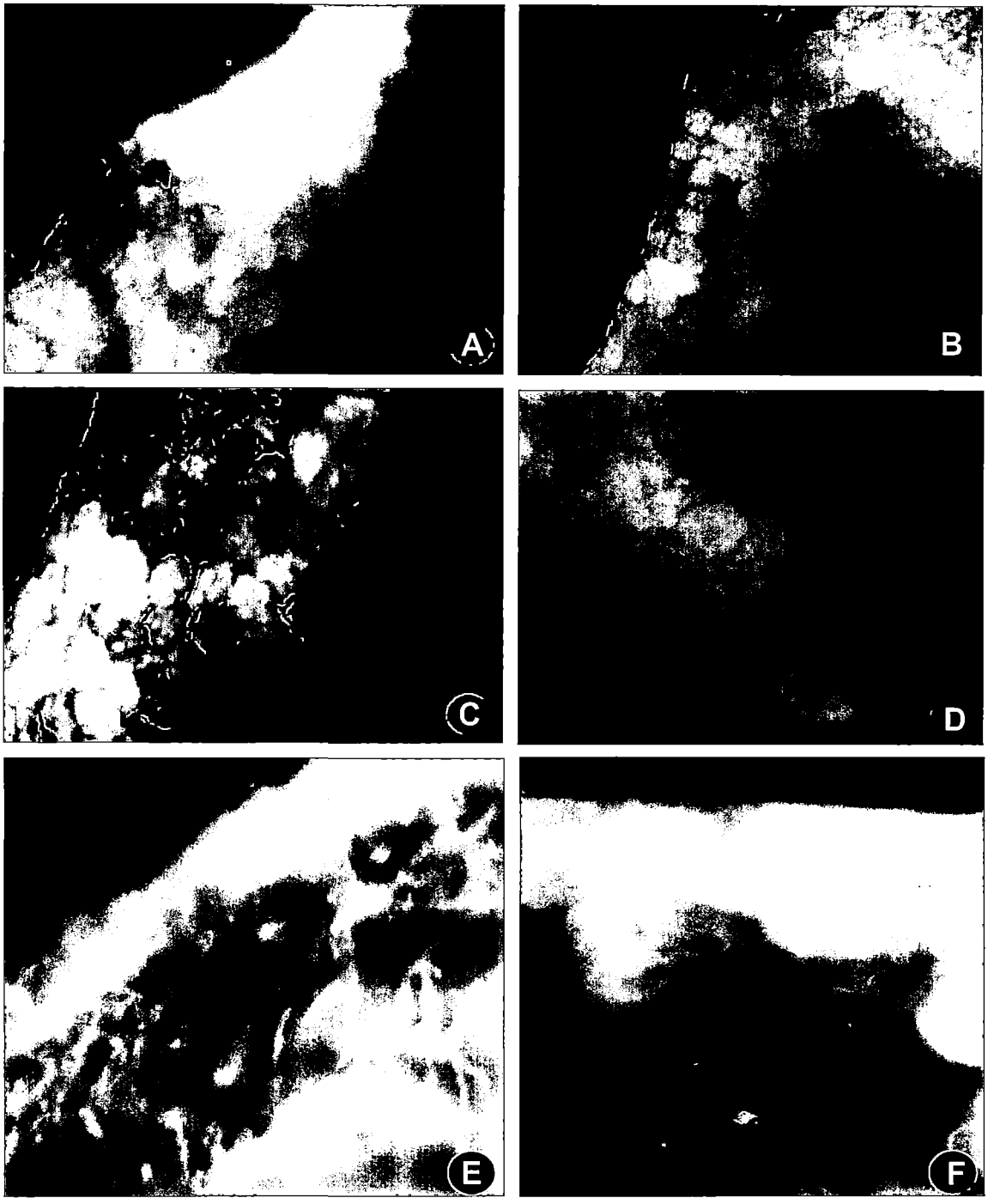

Plate-XXIV: (Fig.. A-F)

Fluorescent antibody staining of cross section of seeds (stored for 12 months) of various pulses. Seed tissues treated with PAb of *A. niger* A(x10) & B (x10) *Vigna radiata*; C(x45) & D (x45) *Lens culinaris*; E (x45) & F (x45) *Cajanas cajan.*

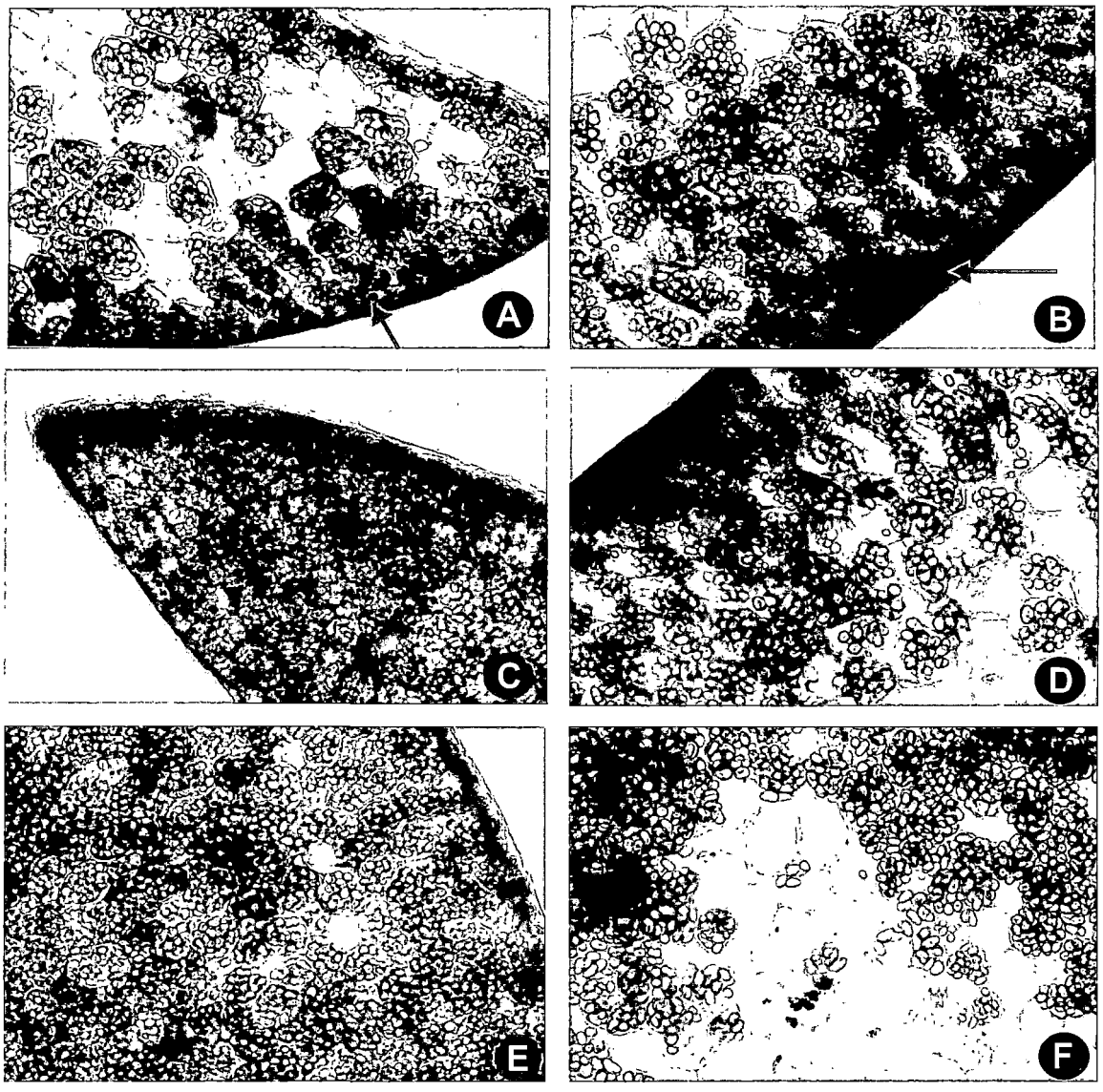

Plate-XXV: (Fig. A-F)
Immunocytochemical staining of section *Lens culinaris* (stored for 12 months) treated with PAb of *A. flavus* (A-D) and *A. niger* (E&F).

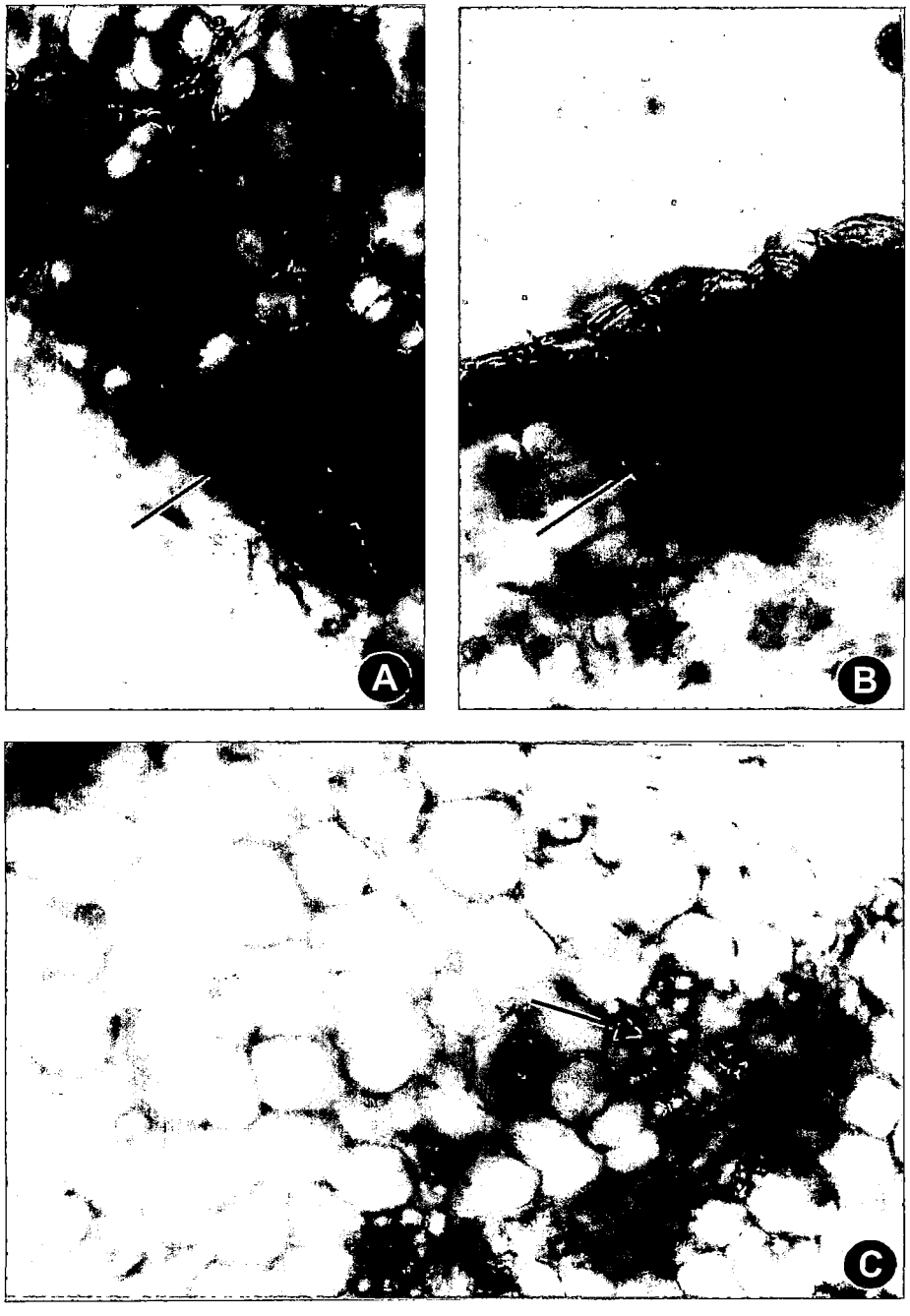

Plate-XXVI: (Fig. A-C)
Immunocytochemical staining of section *Vigna radiata* (stored for 12 months) treated with *Aspergillus flavus* (A & B) and *Aspergillus niger* (C).

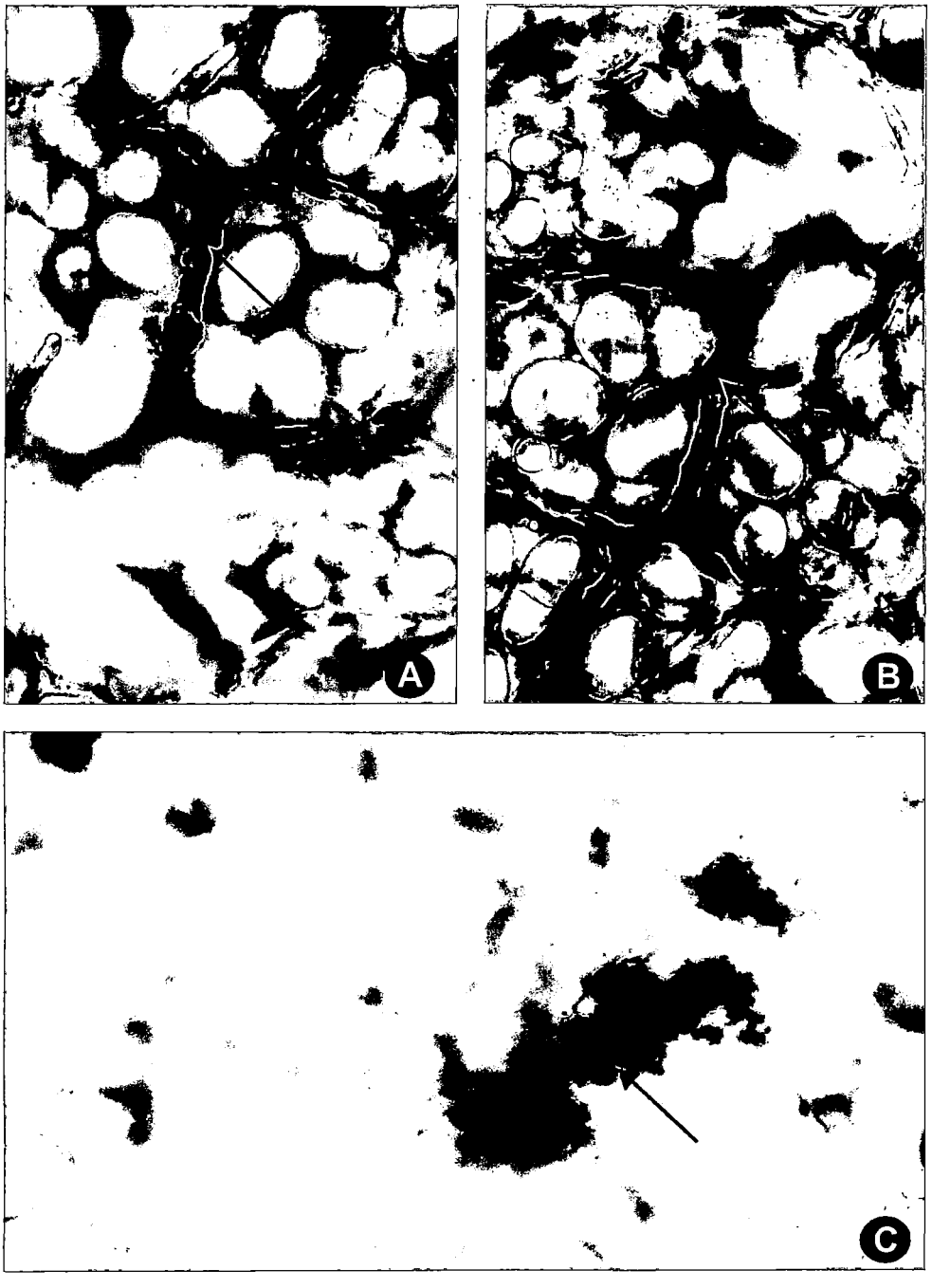

Plate-XXVII: (Fig. A&B)
Immunocytochemical staining of section *Cajanas cajan* (stored for 12 months) treated with *Aspergillus flavus* (A&B) and A*spergillus niger* (C).

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.

SUMMARY

1. A brief review of literature pertaining to the present line of investigation has been presented. The review mainly deals with the storage fungi of seeds and their harmful effects i.e. bio-deterioration of seeds by storage fungi.
2. Materials used in this investigation and the experimental procedures followed have also been described in detail.
3. Twenty six (26) fungal species were isolated from the three pulses- *Cajanus cajan, Lens culinaris* and *Vigna radiata* and these were characterised and identified. Species of *Aspergillus* was predominant in all the seeds with other genera like *Penicillium, Alternaria* and *Fusarium* showing lesser frequency.
4. Viability of seeds was shown to depend 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.
5. Effect of seed storage on two major seed components i.e. proteins and carbohydrates were determined. It was observed that after a period of storage of one year when analysis were done, significant reduction in both protein and total sugar contents were observed in all cases. This was farther confirmed by analyzing seeds artificially inoculated with *A. niger* and *A. flavus.* In this case also significant reduction was obtained. *A. falvus* was found to cause 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.

6. SDS PAGE analysis of protein pattern also showed significant reduction in the number of protein bands both during storage as well as artificial inoculation.

7. Activities of amylase as well as protease showed declining trend during storage.

8. Polyclonal antibodies (PAbs) were raised against antigen preparations from mycelia and spores of *Aspergillus niger* and *Aspergillus flavus*. These were purified by ammonium sulphate precipitation followed by DEAE cellulose chromatography. IgG obtained in each case was used for immunodiffusin and ELISA tests.

9. Agar gel double diffusion tests were performed using crude antibody as well as purified IgG prepared after four different bleedings collected for the pathogen. Strong precipitin reactions were observed from 2^{nd} bleed onwards.

10. Optimization of ELISA by using PAbs of *A. niger* and *A. flavus* and angiten preparations at variable concentration were performed. ELISA values showed both PAbs to have very high titer of which *A. flavus* gave higher absorbance values. A_{405} values decrease with antigen and IgG dilutions.

11. DAC-ELISA tests were performed separately using PAbs raised against mycelia and spore antigens of *A. niger* and *A. flavus* and antigens of seeds (Pulses) obtained from 13 different localities of West Bengal and Sikkim.

12. When seeds of all three pulses obtained from various localities were tested against PAbs of *A. niger* and *A. flavus* much higher A_{405} values were obtained with PAb of *A. niger*.

13. These results were also confirmed by Dot-Blot assays. Among the three pulses, *V. radiata* showed most reactivity to the PAbs.

Differences in ELISA values as well as intensity of colour of the dots in dot-blots also varied with the locations.

14. Mycelia and conidia of *A. niger* and *A. flavus* when treated with homologous antisera followed by FITC, bright fluorescence was noticed on young hyphae and throught the surface of conidia.

15. Fluorescence staining and immunocytochemical staining techniques were used to determine the presence of fungi within seeds. For these immunoassays 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.

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