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Research Paper

Fungi Present in Stored Cowpea Seeds and its Effect on Seed Quality

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A B S T R A C T

A laboratory study was conducted to study the fungi present in stored cowpea seeds. Freshly harvested cowpea seeds were stored for twelve months to determine the fungi appeared during storage. Sampling was made for isolation of fungi at zero day, 3, 6, 9 and 12 months of storage. Results revealed that, before storage i.e., at zero day of storage, four fungal strains were isolated. At the end of the three months, nine fungal species were isolated. After six months, fourteen fungal species were isolated from the stored cowpea seeds. Nineteen and twenty-three fungal species were isolated from nine- and twelve-months stored cowpea seeds respectively. The number of storage fungi isolated from stored cowpea seeds increased with increase in storage time. These include different species of *Aspergillus, Penicillium, Fusarium, Mucor, Cephalosporoim, Chaetomium, Sprotrichum, Zygosporoum* and *Geotrichum*. The most predominant fungus isolated was *Aspergillus flavus*. All these fungi resulted in the reduction of protein content of the seeds.

***Key words*:** Mycoflora, Post-harvest, Protein, Storage, Spoilage

A grain saved is a grain produced. About 40% of food is lost in India because of problematic storage. The greatest losses during storage of cereals, pulses and other durable commodities are caused by fungal contamination. Pulses can remain in edible condition for several years if properly stored. However, they are more difficult to store than cereals and suffer much greater damage from microorganisms and insects. Storage fungi mostly invade the seeds due to improper storage and unhygienic condition, even the smallest amount of inoculums can spoil the whole stored product. Cowpea is one of the important annual food crops grown mainly as pulse, vegetables and fodder. It is mainly consumed as a food in the form of dried seed. The mature cowpea seeds contain 24.8% protein, 63.6% carbohydrate, 1.9% fat, 6.3% fiber, 0.00074% thiamine, 0.00042% riboflavin and 0.00281% niacin. As the seeds contain high protein and several vitamins and minerals, it can provide a cheap source of dietary protein for low income urban and rural populations. Among various factors that affect seed health, the most important are the fungi. There is a loss in the quality of grain which can be seen in change in colour, smell, taste and nutritional value. Fungi can cause all these deleterious changes in stored seeds. Storage of seed is certainly the most important post-harvest operation and the losses incurred are great. Production of cowpea seeds in India is about 0.3 mmt and a major part of these (10.4%) is lost due to fungal contamination. In storage, cowpea seeds are attacked by pests and diseases leading to their deterioration and loss of nutritive value. The fungi that invade stored products are generally known as storage fungi. *Aspergillus* and *Penicillium* are the most dominant storage fungi which attack the stored products [1]. These fungi are known to be producers of mycotoxins which are secondary metabolites that are known to cause a lot of deleterious effects when consumed in food by human. Due to the production of toxic chemical substances the seeds become unfit for human consumption and there is reduction of its market value. Aflatoxins are biologically active secondary metabolites produced by members of the *Aspergillus* sp [2]. Aflatoxin B1 alters the physiology of seeds and seedlings. It also restricts plant growth by inhibiting seed germination, seedling growth and other physiological processes of plants [3]. Mycotoxins have the ability to decrease the quality and nutritive value of seed. As a result, foodstuffs become unfit for consumption [4]. In cognizance of the above a study was undertaken to determine the fungi associated with stored cowpea seeds and their impact on quality of seeds.

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MATERIALS AND METHODS

Freshly harvested dried cowpea seeds were collected during the month of October, 2018 and stored for twelve months to determine the fungi appeared during storage. The average room in room temperature and humidity recorded were 32 ± 2°C and 86 ± 2% respectively. Sampling was made for isolation of fungi at zero day 3, 6, 9 and 12 months of storage. Fungi associated with stored cowpea seeds were isolated by using following two standard method.

Agar plate method

In this method, 15 ml of sterilized Potato Dextrose Agar (PDA) media was poured in pre sterilized Petri dishes. On cooling, ten seeds cowpea per plate of the sample were placed aseptically. The plates were incubated at 25° ± 2°C for seven days. Five replicates were taken for isolation. On seventh day of incubation, seeds were first examined under

stereoscopic microscope for determining the various fungal growths.

Seed washates method

In this isolation technique, 100 seeds were taken in conical flask with sterile distilled water for their soaking. The flasks were subjected to mechanical shaker for 5–10 minutes. 1ml of seed washing, thus obtained was spread on PDA medium for growth of individual spore of fungus. Five replicates were taken for isolation. The plates were incubated at 25° ± 2°C for seven days for development of colonies and observations were made.

The colonies were immediately transferred to PDA slants for further study. Isolated fungi were identified both macroscopically and microscopically. The fungal colonies were counted and identified on the basis of colony characters, morphology and reproductive characteristics. The percentage incidence of occurrence of each fungus was calculated.

Estimation of protein

Standard Bradford protein estimation method was used to estimate the protein content of the cowpea seeds before storage (zero day) and after storage (12 month). For protein extraction, 1g of seed was homogenized in a mortar and pestle in 3 ml extraction buffer containing 50 mM Tris HCl (pH 7.5), 150mM NaCl, 1% NP-40 and 1mM Phenyl Methyl Sulfonyl Fluoride (PMFS). The homogenate was centrifuged at 15000 rmp for 10 minutes and the supernatant was used as crude protein extract. The crude protein content was estimated using Bradford method for protein estimation. Bradford reagent was prepared by dissolving Coomassie Brilliant Blue G250 (100 mg) in 50 ml of 95% ethanol. 100 ml of concentrated ortho phosphoric acid was added to make the volume up to 200 ml with distilled water. Various concentrations of standard protein solutions were prepared from the stock solution (0.2, 0.4, 0.6, 0.8 and 1.0 ml) by dissolving in distilled water. A tube with 1 ml of water served as blank. 5.0 ml of Bradford reagent was added to each tube and vortexed. After 15 minutes, reading was taken by spectrophotometer at 595 nm for each sample. The absorbance of the standards was plotted versus their concentration. From standard curve the amount of protein in unknown sample was calculated.

Statistical analysis

To minimize experimental errors and attainment of proper degrees of freedom five replications were taken. Duncan Multiple Range Test (DMRT) was done using SPSS software.

RESULTS AND DISCUSSION

Cowpea seeds were studied for presence of fungi before storage (zero day) and at different time intervals (3, 6, 9 and 12 months) of storage. Before storage, four fungal species belonging to three genera were isolated from the cowpea seeds (Table 1). In agar plate technique the percentage incidence of *Helminthosporium solani* (40.2%) was highest followed by *Mucor mucedo* (23.8%), *Fusarium solani* (18.8%), *Fusarium moniliforme* (17.7%). Similarly, in seed washate method only *Helminthosporium solani* (100%) was isolated, but *Fusarium solani, F. moniliforme* and *Mucor mucedo* were not isolated by this technique.

Table 1 Percentage incidence of fungi associated with cowpea seeds before storage (zero day)

Isolated fungi	Percentage incidence	
	Agar plate	Seed washate
Fusarium moniliforme	$17.7^{d} \pm 1.7$	$0.0^{b} \pm 0.0$
Fusarium solani	$18.8^{c} \pm 1.1$	$0.0^{b} \pm 0.0$
Helminthosporium solani	$40.2^{a} \pm 1.3$	$100^{a} \pm 0.0$
Mucor mucedo	$23.3^{b} \pm 1.8$	$0.0^{b} \pm 0.0$

At the end of the three month of storage nine fungal species belonging to four different genera (Table 2) were isolated from cowpea seeds. In agar plate technique the percentage incidence of *Aspergillus flavus* (38.6%) was highest followed by *Aspergillus niger* (24.8%), *Aspergillus ochraceus* (14.3%), *Aspergillus terreus* (7.1%), *Fusarium solani* (5.8%), *Penicillium chrysogenum* (4.5%), *Penicillium citrinum* (2.5%), *Fusarium moniliforme* (1.3%), *Mucor mucedo* (1.3%), Similarly in seed washate method the highest percentage incidence was of *Aspergillus flavus* (36.6%). The remaining isolated fungi were found in the range between 2.5-18.1% while *Mucor mucedo* and *Penicillium citrinum* could not be isolated by this technique.

Table 2 Percentage incidence of fungi associated with cowpea seeds after three months of storage

Isolated fungi	Percentage incidence	
	Agar plate	Seed washate
Aspergillus flavus	$38.6^{a} \pm 4.7$	$36.6^{a} \pm 3.5$
Aspergillus niger	$24.8^{b} \pm 2.6$	$18.1^{b} \pm 2.4$
Aspergillus ochraceus	$14.3^{c} \pm 1.6$	$16.3^{bc} \pm 3.2$
Aspergillus terreus	$7.1^{d} \pm 2.1$	$13.4^{bc} \pm 0.96$
Fusarium moniliforme	$1.3^{e} \pm 1.2$	$2.5^{d} \pm 2.5$
Fusarium solani	$5.8^{d} \pm 1.6$	$10.6^{c} \pm 2.8$
Mucor mucedo	$1.3^{e} \pm 1.2$	$0.0^{d} \pm 0.0$
Penicillium chrysogenum	$4.5^{d} \pm 2.8$	$2.5^{d} \pm 2.5$
Penicillium citrinum	$2.5^{d} \pm 1.5$	$0.0^{d} \pm 0.0$

Table 3 Percentage incidence of fungi associated with cowpea seeds after six months of storage

Isolated fungi	Percentage incidence	
	Agar plate	Seed washate
Aspergillus flavus	$34.6^{a} \pm 3.3$	$34.3^{a} \pm 2.2$
Aspergillus niger	$22.5^{b} \pm 1.8$	$19.9^{b} \pm 2.3$
Aspergillus ochraceus	$11.7^{c} \pm 1.6$	$14.0^{c} \pm 1.9$
Aspergillus terreus	$6.0^{d} \pm 0.2$	$5.5^{de} \pm 1.3$
Chaetomium globosum	$2.4^{d} \pm 1.5$	$3.0^{def} \pm 1.8$
Fusarium moniliforme	$2.6^{d} \pm 1.6$	$4.2^{def} \pm 1.7$
Fusarium solani	$4.6^{d} \pm 1.2$	$7.2^{d} \pm 0.3$
Mucor mucedo	$2.4^{d} \pm 1.4$	$3.1^{def} \pm 1.9$
Penicillium chrysogenum	$4.7^{d} \pm 2.1$	$4.4^{def} \pm 1.8$
Penicillium citrinum	$2.4^{d} \pm 1.5$	$0.0^{f} \pm 0.0$
Penicillium expansum	$1.1^{d} \pm 1.1$	$1.7^{ef} \pm 1.7$
Penicillium frequentans	$1.3^{d} \pm 1.3$	$1.3^{ef} \pm 1.3$
Penicillium notatum	$2.4^{d} \pm 1.5$	$1.3^{ef} \pm 1.3$
Penicillium oxalicum	$1.3^{d} \pm 1.3$	$0.0^{f} \pm 0.0$

After six months of storage, fourteen fungal species belonging to five genera were isolated from cowpea seeds (Table 3). In both agar plate and seed washate technique highest percentage incidence was of *Aspergillus flavus* i.e., 34.6% and 34.3% respectively followed by *Aspergillus niger* (22.5% and 19.9% respectively). Additionally, five other fungal species i.e., *Penicillium notatum* (2.4%), *Chaetomium globosum* (2.4%), *Penicillium oxalicum* (1.3%), *Penicillium frequentans* (1.3%), *Penicillium expansum* (1.1%), were

isolated by agar plate method. However, *Penicillium citrinum* and *Penicillium oxalicum* were not isolated in seed washate method and remaining fungi were found between the range of 1.3-14.0%.

After nine months of storage, twenty fungal species belonging to ten genera were isolated from cowpea seeds (Table 4). *Aspergillus flavus* was the dominant fungi with highest percentage of incidence in both the isolation techniques i.e., agar plate and seed washate (27.0 and 29.4% respectively) followed by *Aspergillus niger* (16.9 and 15.9% respectively). *Aspergillus fumigatus* (4.1%), *Cladosporium herbarum* (3.3%), *Cephalosporium gramineum* (1.9%), *Geotrichum candidum* (0.7%), *Zygosporium* sp. (0.6%) and *Sporotrichum* sp. (0.6%) were additional fungus isolated from agar plate isolation technique. *Cephalosporium gramineum, Sporotrichum* sp. and *Zygosporium* sp. were not isolated in seed washate method and remaining isolated fungi were found in the range between 0.8- 8.0%.

Table 4 Percentage incidence of fungi associated with cowpea seeds after nine months of storage

Isolated fungi	Percentage incidence	
	Agar plate	Seed washate
Aspergillus flavus	$27.0^{a} \pm 1.6$	$29.4^{a} \pm 1.5$
Aspergillus fumigatus	$4.1^{defg} \pm 0.7$	$4.5^{def} \pm 0.2$
Aspergillus niger	$16.9^{b} \pm 1.3$	$15.9^{b} \pm 0.9$
Aspergillus ochraceus	$8.8^{c} \pm 1.8$	$8.0^{c} \pm 1.8$
Aspergillus terreus	$6.0^{cde} \pm 1.1$	$5.2^{cdef} \pm 0.6$
Cephalosporium gramineum	$1.9^{fgh} \pm 0.8$	$0.0^{h} \pm 0.0$
Chaetomium globosum	$2.7^{efgh} \pm 0.6$	$3.6^{efg} \pm 0.9$
Cladosporium herbarum	$3.3^{defgh} \pm 1.1$	$2.0^{fgh} \pm 1.2$
Fusarium moniliforme	$4.7^{def} \pm 1.3$	$5.2^{cdef} \pm 1.6$
Fusarium solani	$6.1^{cd} \pm 1.3$	$7.1^{cd} \pm 1.8$
Geotrichum candidum	$0.7^{gh} \pm 0.7$	$1.0^{gh} \pm 1.0$
Mucor mucedo	$2.7^{efgh} \pm 0.7$	$3.5^{efgh} \pm 0.9$
Penicillium chrysogenum	$5.3^{de} \pm 0.8$	$6.1^{cde} \pm 0.8$
Penicillium citrinum	$2.0^{fgh} \pm 0.8$	$2.5^{fgh} \pm 1.0$
Penicillium expansum	$1.4^{fgh} \pm 0.8$	$1.8^{fgh} \pm 1.1$
Penicillium frequentans	$1.4^{fgh} \pm 0.8$	$1.0^{gh} \pm 1.0$
Penicillium notatum	$2.7^{efgh} \pm 0.7$	$2.5^{fgh} \pm 1.0$
Penicillium oxalicum	$1.4^{fgh} \pm 0.8$	$0.8^{gh} \pm 0.8$
Sporotrichum sp.	$0.6^{h} \pm 0.6$	$0.0^{h} \pm 0.0$
Zygosporium sp.	$0.6^{h} \pm 0.6$	$0.0^{h} \pm 0.0$

After twelve months of storage, four additional fungal species were isolated from cowpea seeds. Altogether, twenty-three species belonging to twelve genera were isolated (Table 5). In case of agar plate method, incidence of *Aspergillus flavus* was highest (27.8%) followed by *Aspergillus niger* (15.9%), *Aspergillus ochraceus* (8.3%), *Fusarium solani* (5.7%), *Aspergillus terreus* (5.6%), *Penicillium chrysogenum* (5.0%), *Fusarium moniliforme* (4.3%), *Aspergillus fumigatus* (3.8%), *Cladosporium herbarum* (3.1%), *Penicillium notatum* (2.5%), *Mucor mucedo* (2.5%), *Cephalosoprium gramineum* (2.5%), *Chaetomium globosum* (1.8 ± 0.7%), *Penicillium citrinum* (1.8 ± 0.8%), *Penicillium oxalicum* (1.3 ± 0.8%), *Penicillium expansum* (1.3%), *Trichoderma viride* (1.3%), *Penicillium frequentans* (1.3%), *Geotrichum candidum* (1.2%), *Sporotricchum* sp. (1.2%), *Trichoderma harzianum* (0.7%), *Botrytis cinerea* (0.6%) and *Zygosporium* sp. (0.6%). In seed washates method, *Aspergillus flavus* (28.9%), gave highest percentage incidence followed by *Aspergillus niger* (15.9%), *Aspergillus ochraceus* (8.9%), *Fusarium solani* (6.5%), *Aspergillus terreus* (5.6%), *Penicillium chrysogenum* (4.4%), *Aspergillus fumigatus* (4.3%), *Fusarium moniliforme* (3.5%), *Mucor mucedo* (3.0%) while *Penicillium expansum, Penicillium frequentans*, *Penicillium citrinum, Penicillium notatum, Penicillium oxalicum, Cladosporium herbarum, Cephalosporium gramineum, Trichoderma viride, Trichoderma harzianum, Chaetomium globosum, Geotrichum candidum* were found to be intermediate within the range of 0.7 -2.9%. *Botrytis cinerea*, *Sporotrichum* sp. and *Zygosporium* sp. were not isolated from this technique.

Table 5 Percentage incidence of fungi associated with cowpea seeds after twelve months of storage

Isolated fungi	Percentage incidence	
	Agar plate	Seed washate
Aspergillus flavus	$27.8^{a} \pm 1.2$	$28.9^{a} \pm 0.9$
Aspergillus fumigatus	$3.8^{defg} \pm 0.6$	$4.3^{def} \pm 0.6$
Aspergillus niger	$15.9^{b} \pm 1.4$	$15.9^{b} \pm 1.1$
Aspergillus ochraceus	$8.3^{c} \pm 1.9$	$8.9^{c} \pm 2.4$
Aspergillus terreus	$5.6^{cd} \pm 1.0$	$5.6^{de} \pm 1.2$
Botrytis cinerea	$0.6^{h} \pm 0.6$	$0.0^{h} \pm 0.0$
Cephalosporium gramineum	$2.5^{efgh} \pm 0.6$	$2.9^{efgh} \pm 0.7$
Chaetomium globosum	$1.8^{fgh} \pm 0.7$	$2.1^{fgh} \pm 0.8$
Cladosporium herbarum	$3.1^{defgh} \pm 0.9$	$2.9^{efgh} \pm 1.3$
Fusarium moniliforme	$4.3^{def} \pm 1.2$	$3.5^{defg} \pm 1.0$
Fusarium solani	$5.7^{cd} \pm 1.2$	$6.5^{cd} \pm 1.4$
Geotrichum candidum	$1.2^{fgh} \pm 0.7$	$1.3^{fgh} \pm 0.8$
Mucor mucedo	$2.5^{efgh} \pm 0.6$	$3.0^{efgh} \pm 0.7$
Penicillium chrysogenum	$5.0^{de} \pm 0.7$	$4.4^{def} \pm 0.8$
Penicillium citrinum	$1.8^{fg} \pm 0.8$	$2.2^{fgh} \pm 0.9$
Penicillium expansum	$1.3^{fgh} \pm 0.8$	$1.5^{f} \pm 0.9$
Penicillium frequentans	$1.3^{fgh} \pm 0.8$	$1.4^{fgh} \pm 0.9$
Penicillium notatum	$2.5^{efgh} \pm 0.6$	$2.2^{fgh} \pm 0.9$
Penicillium oxalicum	$1.3^{fgh} \pm 0.8$	$1.4^{fgh} \pm 0.9$
Trichoderma harzianum	$0.7^{gh} \pm 0.7$	$0.8^{gh} \pm 0.8$
Trichoderma viride	$1.3^{fgh} \pm 0.8$	$0.7^{gh} \pm 0.7$
Sporotrichum sp.	$1.2^{fgh} \pm 0.7$	$0.0^{h} \pm 0.0$
Zygosporium sp.	$0.6^{h} \pm 0.6$	$0.0^{h} \pm 0.0$

The number of storage fungi isolated from stored cowpea seeds increased with increase in storage time. Lowest numbers of fungi were found at zero day followed by three months of storage and subsequently number of fungi increased with the increase in storage period (Fig 1). Three months stored seeds were associated with lowest number and also different types of fungal species in comparison to seeds stored for twelve months which possessed highest number and some additional types of fungal species. This may be probably due to the fact that it is difficult to maintain environmental conditions which are not conductive for fungal growth during storage [5]. Similarly, the fungal incidence of three varieties of stored greengram seeds as reported by [6]. In their initial evaluation (zero day of storage), the seed samples had minimum incidence of storage fungi (*Aspergillus flavus*, 1% and *A. niger*, 1%). However, after three months of storage, a gradual increase of the storage fungal spectrum was observed. The storage period of seeds increases, the pathogen activity also gradually increases in seeds [7]. After storing the crop there is a gradual decrease in percentage field fungi and simultaneous increase in storage fungi accompanied by a reduction in germinability of seeds as the storage period proceeds. An increase of *Aspergillus flavus* from 40-78% after 10 months of storage of soybean and groundnut seeds [8]. There is a progressive increase of *Aspergillus niger* in groundnut with the increase in storage period [9].

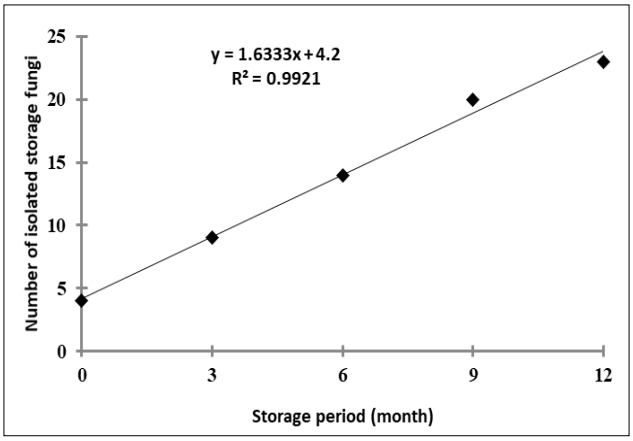

Fig 1 Number of fungi associated with stored cowpea seeds at different storage periods

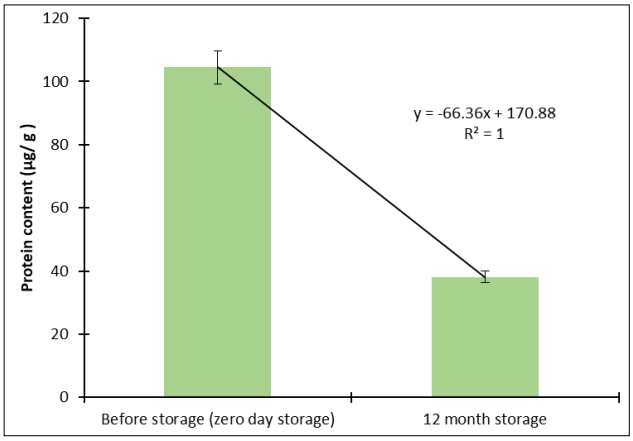

Fig 2 Protein content of seeds before and after storage

Twenty-three different seed borne fungal species belonging to twelve genera were isolated from cowpea after a year of storage. Of these *Aspergillus flavus* (27.8-29.4%) was the most dominant followed by *Aspergillus niger* (15.9-16.9%), *Aspergillus ochraceus* (8-8.3%), *Fusarium solani* (6.1-7.1%), *Penicillium chrysogenum* (5.3- 6.1%). Therefore, *Aspergillus flavus* was found to be the major destroyer of the post-harvest stored cowpea seeds in the present study. *Aspergillus niger, A. flavus, Cladosporium* sp., *Penicillium* sp., *Fusarium oxysporum, F. solani, F. semitectum, Trichoderma viride, Curvularia lunata*, *Mucor* sp., *Rhizoctonia solani* and *Verticillium* sp. were the most dominant pathogens isolated from stored cowpea seeds [10]. Incidence of *Aspergillus flavus* was highest in peanut (58.6%) followed by other species of *Aspergillus* (11.7%), *Cladosporium* (12.2%), *Penicillium* (6%), *Alternaria* (0.1%) and *Fusarium* (0.1%) [11]. The predominance of *Aspergillus flavus* may be due to the great adaptation of this fungus to the substrate, especially during storage [12]. Isolation different species of fungi from three varieties of stored groundnut seed and reported that among the fungi *Aspergillus flavus* was the most preponderant one (29-76.3%) followed by *A. niger* (8-25.6%), *Fusarium moniliforme* (9.3-14.7%), *Penicillium citrinum* (12.7-14.7%), *A. fumigatus* (4-13%), *A. terreus* (9.3-12.7%), *A. nidulans* (5.7-11.3%), *A. tamari* (0-2%) [13]. *Helminthosporium solani* was isolated from the cowpea seeds before storage i.e., zero day of storage. However, this fungus was totally absent at three, six, nine and twelve months of storage. This may be due to the fact that, *Helminthosporium solani* is unable to survive in stored condition. Species of *Helminthosporium* invade seeds as they are developing on the plants in the field or after they have matured, but before they are harvested [14]. This fungus usually does not continue to grow in seeds after harvest, but may remain alive for years in grains stored at low moisture content and low temperature [15].

The protein content of the cowpea before storage was 104.52 µg/ g seed and after 12 months of storage it was 38.16 µg/g seed (Fig 2). Protein content of the seeds was significantly reduced by 63.5% after a year of storage. Storage fungi viz. *Aspergillus niger, A. terreus, A. parasiticus, A. fumigatus* were responsible for reducing seed protein content of soybean [16]. Storage fungi were responsible for decrease in protein content in oil seeds [17]. The reason for decrease in protein content might be due to hydrolysis of protein by fungal proteolytic enzymes [18].

Data were statistically analyzed using SPSS programme. It was observed that percentage incidence of different fungi in both the isolation method at all the storage period differ significantly at 0.05 level. *Aspergillus flavus* gave significantly highest percentage incidence in comparison to other isolates in both the isolation techniques.

CONCLUSIONS

There was a variation in number of fungi isolated at different storage intervals. Number of fungal species gradually increased with the increase in storage time. Among the storage fungi *Aspergillus flavus* was the most dominant fungus. Result of fungal contamination reduces the quality of the seeds. Hence, there is need to investigate the best way of storage of cowpea seeds that could be used for ambient storage which could reduce fungal infection. However, it is to be expected that any effort to effectively manage the fungal pathogens associated with stored cowpea seeds will have to include components that address the issue of safe storage.

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