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Isolation and Identification of Fungal Pathogens Associated with Cold Storage Type of (*Coffee Arabica.)* Seed, at Jimma Agricultural Research Center, Western Ethiopia

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Abstract
Coffee is the most important commercial crop in the national economy of Ethiopia. Coffee seed are subject to various operations of contamination by microorganisms during growth (while the beans are on trees), after harvesting (when the beans are de-hulled, washed and stored) and during storing. The aim of this research is to isolate and identify the fungal pathogens associated with cold storage type of coffee (coffee arabica L.) seed. Different fungi were associated with coffee seed under cold storage condition. These different fungal species was isolated and identified both in blotter and agar plate method. In blotter test method the identified fungi were; Aspergillus sp.; Pencillium sp.; Fusarium sp., and another some unidentified species were isolated and identified at genesis level from the two month coffee seeds storage. Among these, Aspergillus spp. had the highest (49.375%) frequency of occurrence, followed by Penicillium spp. (11.875%), Fusarium spp. (5.625%) and unidentified species (0.626%). In addition, in blotter test method the obtained result indicated that the infection mean percentage in blotting methods $W_0V_{74\text{-}1}$ (without parchment) followed by $W_0V_{74\text{-}110}$ (without parchment) were highly infected mean percentage 80% and 97.5% respectively. However the lowest fungal infestation was noted on both $W_1V_{74\text{-}1}$ and $W_1V_{74\text{-}110}$ (45 %) with parchment of coffee seed was verified. The results of germination test obtained in blotter plate method showed that the germination mean percentage of $W_0V_{74\text{-}110}$ (without parchment) were highly germinated with mean percentage of (98%). However the lowest germination mean percentage was noted on $W_1V_{74\text{-}1}$ (27.5%) with parchment of coffee seed. In agar plate method also the identified fungi were; Fusarium sp. Aspergillus sp. Pencillium sp. and another some unidentified species. Among these, Aspergillus spp. had the highest (31.25%) frequency of occurrence, followed by Penicillium spp. (10.625%) and Fusarium spp. (11.875%) and unidentified spp. (9.375%). Moreover, in agar plate method the maximum coffee seeds mean infection percentage were recor ded 82.5% and the mean minimum infection percentage were (52.5%) in the treatment $W_1V_{74\_110}$ (with parchmen t) and $W_0V_{74\_110}$ (without parchment) respectively. From total four treatments (62.5%) maximum fungal contam inations were recorded. The result of present study, storage fungi chiefly comprise several "group species" of the genera Aspergillus spp, Penicillium spp, Fusarium spp. and another unidentified species. The species identified in this study are among the most common species of fungi present in storage environments at high moisture. They can tolerate growth in different substrates and environmental conditions, and their complete elimination is difficult. However, the use of good hygiene practices and using optimum moisture of coffee seeds in storage management and can minimize mycroflora association of coffee seeds. Although the present study was carried out in one location in Jimma University College of Agriculture and Veterinary Medicine (JUCAVM)/Jimma Agricultural Research Center for three month in 2013 and, it has clearly indicated that different fungi were associated with coffee seed under storage condition, especially Aspergillus species. In general, further research is needed to identify all recovered fungal pathogens and evaluation of promising treatments for use in integrated disease management strategy to manage not only fungal but also other coffee seed diseases and also further investigation of storage temperature, relative humidity, periods of storage and storage types in wide range across the location that suitable for good supply of health coffee seeds.
Keywords: Coffee, seed, cold storage, mycoflora.

1. INTRODUCTION

Coffee belongs to the family Rubiaceae, which is widely distributed throughout the tropical region. Although there are many species of coffee, the only two commercially important ones are (*Coffea arabica*) and (*Coffea robusta)* (Pieters and Vander-Graff, 1980). Both species can grow best on deep, free- draining, loamy soils, with a good water holding capacity and a slightly acid soil (PH 5-6) (CTA, 1999; Kimani *et al*., 2002; Lewis Ivey *et al*., 2003) and soil fertility is important for good production. Coffee is the most important commercial crop in the national economy of Ethiopia, contributing 60% of its foreign exchange earnings and nearly 25% of Ethiopian population depends, directly or indirectly on coffee for a livelihood by involving in the production, processing, and marketing of coffee as the major contribution to the development of the rural and the national economy (CTA, 1999; Paulos Dubale and Demil Teketay, 2000). Currently, Ethiopia is a leading arabica coffee producer in Africa, ranking the fifth largest Arabica coffee producer and tenth in coffee export worldwide. Its total coffee

production and export respectively increased by 107% and 226% for the crop year 2009/10 and 2010/11 (ICO, 2011). In Ethiopia, coffee had been and still contributes to the Lion's share in its national economy being the leading source of foreign exchange earnings. Besides, the livelihood of a quarter of the Ethiopian population depends directly or indirectly on the different processes of production and marketing along the coffee value-chain (Girma *et al.,* 2008). Even though coffee is the second largest commodity after oil in the World Bank ranking (Smith, 1985), its production suffers from many constraints, including diseases, pests and weeds, resulting in substantial losses. According to Oerke *et al.,* (1994), losses in coffee production are estimated at 40% of the attainable harvest worldwide, broken down into 14.8% due to diseases, 14.9% due to insect pests and 10.3% due to weeds. To these important losses should be added post-harvest losses, which also affect very significantly the total yield obtained by the poor coffee farmers of the developing countries. Post-harvest problems of coffee adversely affect the quality of coffee beans because of fungal contamination and production of mycotoxins. Moreover, coffee quality is evaluated by key factors including the selection of the *Coffea arabica* variety, climatic conditions during growth, processing method and storage conditions. The main aim of coffee processing is removal of the pulp, mucilage, parchment and silver skin surrounding the coffee beans, which leaves the so called 'green' coffee beans. In Brazil and Ethiopia and for robusta coffees generally, the dry or natural method of fermentation is usually used. During the natural processing, coffee fruits are spread on the ground (earth, concrete or tarmac) in layers approximately 10 cm thick, heaped at night and re-spread each morning. During the course of 10-25 days of sun drying, the natural microbial fermentation that occurs can influence the final quality of the product (Silva *et al*., 2000). Microbial contamination can occur in the cherries and during harvesting, fermentation, drying and storage coffee beans (Silva *et al*., 2000). Bacteria, yeasts and filamentous fungi have been already reported in the pulp and beans of coffee processed in Brazil, India, Hawaii, Congo, Argentina, Colombia, Costa Rica, Ethiopia and Mexico (Avallone *et al*., 2001)). Filamentous fungi predominate at the end of the processing and during storage, and may affect the quality and safety of the final product due to production of mycotoxins (Batista *et al*., 2003). Several studies have reported the occurrence of toxin-producing fungi and ochratoxin in green coffee beans (Batista *et al*., 2003). Hence, the current study was meant to isolate and identify the fungal pathogens associated with cold storage type of coffee (*coffee arabica* L.) seed. Therefore, the objective of this study was conducted to isolate and identify the fungal pathogens associated with cold storage type of coffee seed.

3. MATERIALS AND METHODS

3.1. Study Area and Period

The field study was conducted in Jimma zone Ethiopia which is found at about 345 km from Addis Ababa in South west and lies between 36° 10´E longitude and 7° 40´ N latitude. The two varieties of coffee arabica such as variety one (V_{74_1}) and variety two (V_{74_110}) coffee seeds sample from cold storage type stored at the 100% purity, 70% relative humidity, 12% moisture content and temperature (8-10^{0}c) were collected from Jimma Agricultural Research Center (JARC) February 2005 and the experiment were carried out for three months (February, March and April) in 2013 at Jimma University College of Agriculture and Veterinary medicine (JUCAVM) plant pathology laboratory. Jimma which is a zone for coffee production and supplier to the other towns. This area experiences annual average rainfall of 1000 mm for 8 to 10 months. The zone has an elevation ranging from 880 to 3360 masl. The temperature of Jimma zone varies from 8-28°C. The average annual temperature is 20°C [15] (Haile A. and Tolemariam T, 2008).

3.2. Blotter Tests Methods

The blotter method was used to isolate the fungal pathogens associated with the coffee seeds were as to determine the health seeds after the storage periods. The samples were tested according to (ISTA, 1981). A total of 340 coffee seeds of the two varieties (V_{74_1}) and (V_{74_110}) and the total treatment combination was 4 (2x2), in each four replicates, were tested from each sample. The sample with and without parchment and disinfection then seeds were plated directly on top of three layers of well-soaked blotter paper. Ten seeds were plated on per plastic Petri-dish of 9cm diameter by surface disinfection. The plated coffee seeds were incubated at 22-25°C for 7 days under alternate cycles of 12h daylight and darkness. After incubation each coffee beans were observed under different magnifications in the stereomicroscope for fungal growth. Pathogenic fungi developing on coffee seeds were isolated. Seed borne pathogen and the micro flora associated with seeds were cultured on Potato dextrose agar media and identified to geneses level following the descriptions of (Singh *et al.*, 1991 and Samson *et al.,* 1995).The infection level (%) is determining as the ratio of infected seeds over the total number of coffee seed tested.

3.3. Agar Plate Method

For agar plate method the two varieties of cold storage types of coffee seeds were equal placed aseptically on PDA in 9cm Petri plate 10 seeds per plate 40 seeds per 4 treatment with four replication. In comparable set the seeds with and without parchments and surface sterilization with 5% sodium hypochlorite for three minutes washed in sterilized distilled water before plate on the PDA medium and the plated coffee seeds were incubated

for 7-10 days at 25°c under 12hr alternate cycles of light and darkness. At the end of the incubation period, fungi growing out from the seeds on the agar medium were examined and identified. Identification was done by preparation of slide mounts of spores or other bodies in a drop of water and examine under a compound microscope for shape, size and color colony characters and morphology of sporulation structures (Mathur and Kongsdal, 2003).

3.4. Germination Test

With and without parchment of coffee seeds were collected and socked for 48 hours for imbibitions. Carefully saturated the absorbent material for each of ten (10) days each day check that absorbent material remains moist record the number of germinated seeds.

$$\text{Germination (\%)} = \frac{\text{Number of seeds germinated}}{\text{Number of seeds on petri plate}} \times 100$$

3.5. Statistical analysis

Microsoft Excel software programs were used in the calculations of treatment means and summary tables presented wherever required.

4. RESULT AND DISCUSSION

4.1. Detection and identification of seed borne fungi of coffee seed by blotter plate method

This study showed that coffee seeds could be attacked by several economically important post harvest fungal pathogens under storage condition. A total of four post harvest fungal disease (mould fungi) of different genera were identified. High number of fungal mycoflora was also associated with coffee bean seeds (Table 1 and 2). In blotting methods coffee seed are subjected to contamination and consequent colonization by microorganism during different phases of development, harvesting, preparation, transport and storage were tested. In this study the results showed that four fungal species including *Aspergillus sp.; Pencillium sp.; Fusarium sp.,* and another some unidentified species were isolated and identified at genesis level from the two month coffee seeds storage (Table 1). The results showed that the occurrence of these fungal species was somewhat heterogeneous and *Aspergillus spp.* had the highest (49.375%) frequency of occurrence, followed by *Penicillium spp.* (11.875%), *Fusarium spp.* (5.625%) and unidentified species (0.626%). Photographic images of these isolates have been depicted below. Among these isolates species of the main toxigenic fungal genera (*Aspergillus, Penicillium* and *Fusarium*) are natural coffee contaminants and are present from the field to the warehouse (Bokhari *et al*., 2002). These toxic genera consist of some species which produce Aflatoxin (AFB1), Ochratoxin (ATO), *P. variadile, P. verrucosum, P. viridcatum, P. chrysognum, P. commune, P. palis and P. cyclopium* (Frysvad *et al*., 1990). Furthermore, the climatic conditions of some areas of the countries like Ethiopia, where temperature and relative humidity are high, as well as the poor conditions and long duration of coffee storing could promote fungi, *Aspergillus Fusarium* and *Penicillium*, in particular, to invade coffee beans during storage (Bokhari *et al*., 2002).

Table 1. Fungal pathogens associated with stored coffee seeds at JARC (seed health test by blotting techniques) at jimma in 2014.

TRT	Fungal flora observed and its Percentage of occurrence			
	Aspergillus spp.	*Penicillium spp.*	*Fusarium spp.*	UN
$W_0V_{74\_1}$	37.5	20	22.5	0
$W_1V_{74\_1}$	27.5	17.5	0	2.5
$W_0V_{74\_110}$	97.5	0	0	0
$W_1V_{74\_110}$	35	10	0	0
Mean	49.375	11.875	5.625	0.626

TRT = Treatment, $W_0V_{74\_1}$=Variety one wituout parchement, $W_1V_{74\_1}$ = Variety one with parchement, $W_0V_{74\_110}$ = Variety two wituout parchement, and $W_1V_{74\_110}$ = Variety two with parchment.

4.2. Detection and identification of seed borne fungi of coffee seed by agar plate method

Coffee beans are subject to various operations of contamination by microorganisms during growth (while the beans are on trees), after harvesting (when the beans are de-hulled, washed and stored) and during storing. In Agar plate methods also coffee seed are subjected to contamination and consequent colonization by microorganism during different phases of development, harvesting, preparation, transport and storage. In this study the results showed four fungal species including *Fusarium sp. Aspergillus sp. Pencillium sp.* and another some unidentified spp. were isolated and identified at genesis level from the two month coffee seeds storage (Table 2). The results showed that the occurrence of these fungal species was somewhat heterogeneous. *Aspergillus spp.* had the highest (31.25%) frequency of occurrence, followed by *Penicillium spp.* (10.625%) and *Fusarium spp.* (11.875%) and unidentified spp. (9.375%). Bokhari (2007a) reported that coffee seeds were highly contaminated with toxigenic fungal isolates and toxins especially ochratoxin A. The occurrence of fungal contamination and mycotoxin production started at the beginning of harvest due to high moisture content of the seeds and increased during transport, storage, or marketing. Within the last decade, significant advances have

been made in mycotoxin detection methods and control strategies as well as in studying the effect of environmental factors on toxin synthesis (Patterson, 1984; Nakajima *et al*., 1997).

Table 2. Fungal pathogens associated with stored coffee seeds at JARC (seed health test by agar plat techniques) at jimma in 2014.

TRT	Fungal flora observed and its Percentage of occurrence			
	Aspergillus spp.	*Penicillium spp.*	*Fusarium spp.*	UN
$W_0V_{74\_1}$	35	7.5	0	10
$W_1V_{74\_1}$	30	15	0	5
$W_0V_{74\_110}$	45	7.5	5	0
$W_1V_{74\_110}$	20	7.5	42.5	2.5
Mean	32.5	9.375	11.875	4.375

TRT = Treatment, $W_0V_{74\_1}$=Variety one wituout parchement, $W_1V_{74\_1}$ = Variety one with parchement, $W_0V_{74\_110}$ = Variety two wituout parchement, and $W_1V_{74\_110}$ = Variety two with parchment.

4.3. Detection of seed infection percentage in both blotter and agar plate method

The result showed that an infection mean percentage in blotting methods Variety one ($W_0V_{74\text{-}1}$) without parchment followed by Variety two ($W_0V_{74\text{-}110}$) without parchment were highly infected mean percentage of 80% and 97.5% respectively. However, the lowest fungal infestation was noted on both with parchment of Variety one ($W_1V_{74\text{-}1}$) and Variety two ($W_1V_{74\text{-}110}$) 45 % of coffee seed. In agar plate methods the result showed that the maximum coffee seeds mean infection percentage were recorded 82.5% and the mean minimum infection percentage were 52.5% in the treatment Variety two ($W_1V_{74\_110}$) with parchment and Variety two ($W_0V_{74\_110}$) without parchment respectively.

From total four treatments 62.5% maximum fungal contaminations were recorded. Detection of major seed borne pathogens using the standard blotter method gave the higher amount of seed infection as compared to the agar plate method (Figure 2 and 3). This might be due to the availability of more free moisture on the moistened blotter papers than the agar plates. The coffee bean seed coat absorbs more water from the wet blotters and enables the fungi to easily grow and sporulate. Whereas on the agar plates, the coffee bean seeds remain dry and sporulation of the fungus was reduced as compared to the standard blotter method. Blotter method is the easiest, efficient and economical detection technique for *Fusarium sp. Aspergillus sp. Pencillium sp.* and another some unidentified species from naturally infected coffee bean seeds under storage condition.

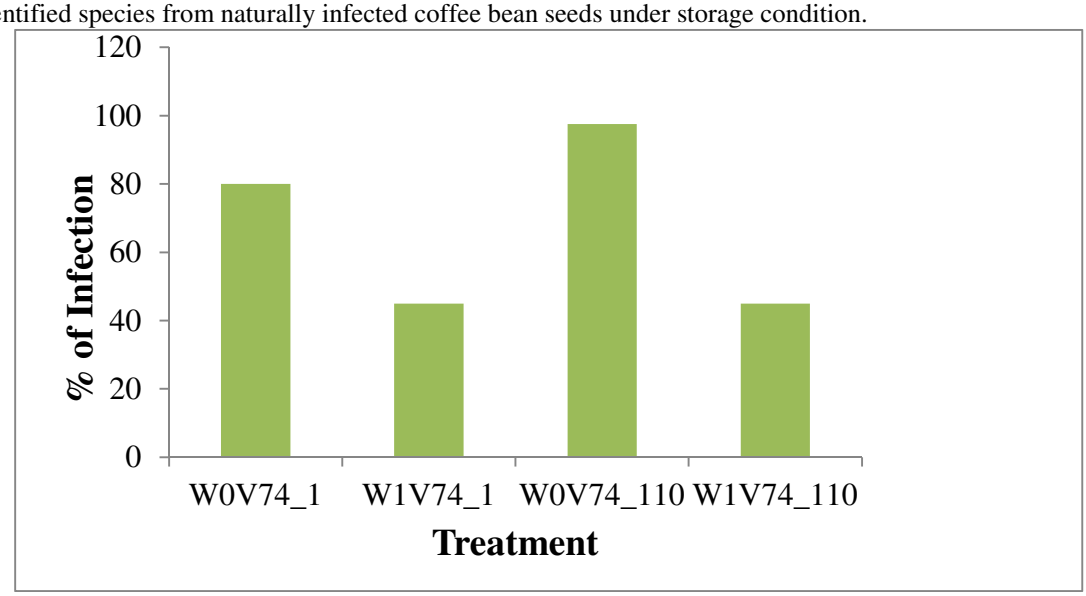

Figure 1.The infection percentage of coffee seeds by blotter plating method.

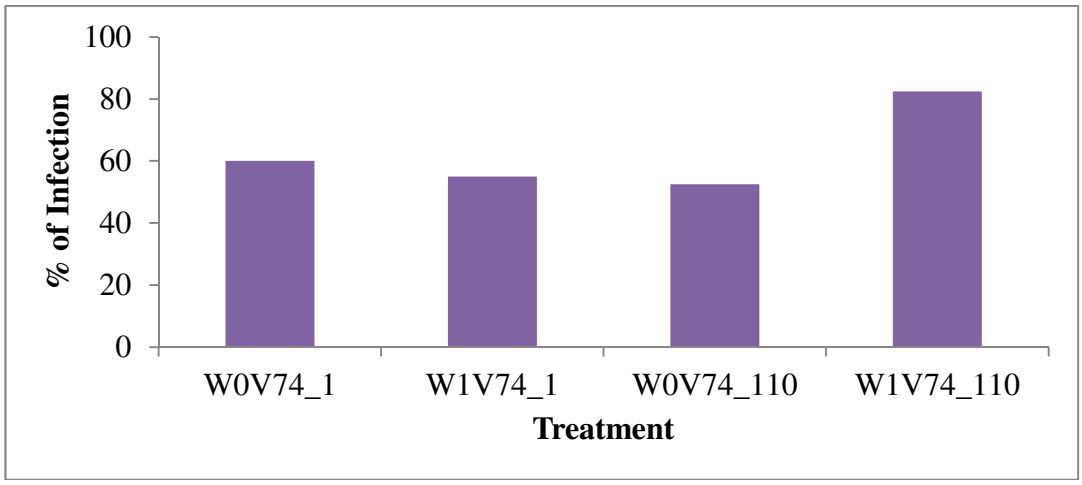

Figure 2.The infection percentage of coffee seeds by agar plating method.

4.4. Germination test

Most crop seed is stored for some period of time. During this time, seed may deteriorate considerably. Good storage conditions can slow the rate of deterioration, but seed germination and vigor cannot be improved, regardless of the storage facilities. Many costly storage problems actually begin during field exposure, harvesting, and conditioning of the seed. Excessive harvesting delays, mechanical injuries, and improper drying techniques, followed by poor storage conditions, can lead to rapid deterioration of seed germination and vigor. Fast drying decreases the vigor of seeds independent from where they dry. In cold chamber it is possible to store regular seeds or slowly dried seeds for up to nine months. In a regular warehouse environment the vigor is affected independently from the way they are dried. The result showed that the germination mean percentage of Variety two ($W_0V_{74\text{-}110}$) without parchment were highly germinated with mean percentage of 98%. However the lowest germination mean percentage was noted on Variety one ($W_1V_{74\text{-}1}$) (27.5%) with parchment of coffee bean seed were recorded in blotter plate method (Fig. 3).

Wooton (1970) conducted a study to determine the best storage conditions for parchment coffee for a period up to one year. He found that conventional storage of coffee in sisal bags stacked on wooden pallets, exposed coffee to storage pests and possible spoilage depending on moisture content. Drying to a low moisture content and hermetic sealing of coffee bag was found to be a desirable and necessary condition for storage.

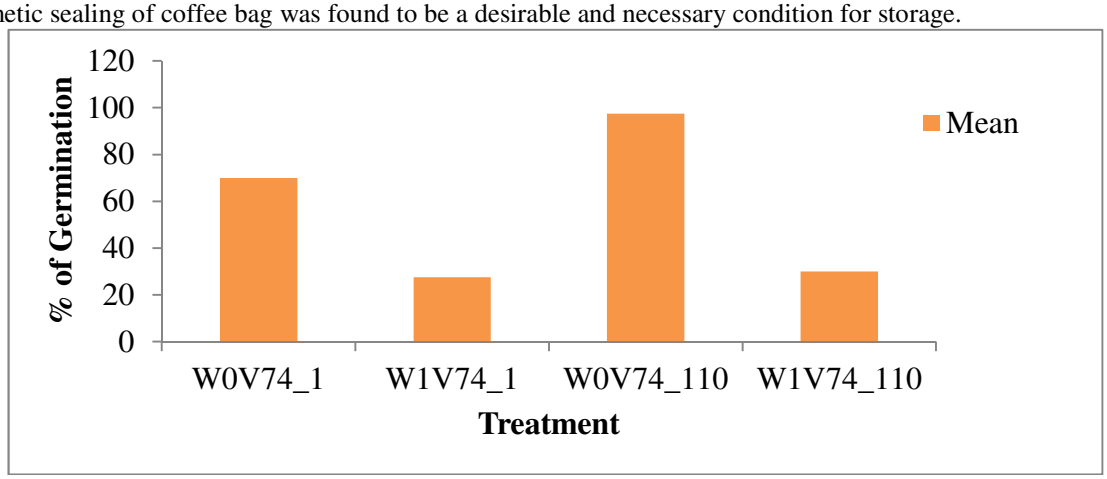

Figure 3.The germination percentage of coffee bean seeds.

5. CONCLUSION

Coffee is the most important commercial crop in the national economy of Ethiopia. Coffee beans are subject to various operations of contamination by microorganisms during growth (while the beans are on trees), after harvesting (when the beans are de-hulled, washed and stored) and during storing. The loss of seed viability, what makes it difficult to keep the high physiological quality, is one the greatest problems faced by the coffee seed producers during seed storage. For all storage systems, good store management aims to maintain the coffee within the 'safe' range of moisture content over the required duration of storage, to protect the product from damage by insects or other pests, and to prevent cross contamination or new contamination from other sources. It also facilitates identification and handling of coffee lots. Different fungi were associated with coffee seed under

cold storage condition. These different fungal species was isolated and identified both in blotter and agar plate method. In blotter test method the identified fungi were; *Aspergillus sp.; Pencillium sp.; Fusarium sp.,* and another some unidentified species were isolated and identified at genesis level from the two month coffee seeds storage. Among these, *Aspergillus spp.* had the highest (49.375%) frequency of occurrence, followed by *Penicillium spp.* (11.875%), *Fusarium spp.* (5.625%) and unidentified species (0.626%). In addition, in blotter test method the obtained result indicated that the infection mean percentage in blotting methods $W_0V_{74\text{-}1}$ (without parchment) followed by $W_0V_{74\text{-}110}$ (without parchment) were highly infected mean percentage 80% and 97.5% respectively. However the lowest fungal infestation was noted on both $W_1V_{74\text{-}1}$ and $W_1V_{74\text{-}110}$ 45 % (with parchment) of coffee bean seed were verified. The results of germination test obtained in blotter plate method showed that the germination mean percentage of $W_0V_{74\text{-}110}$ (without parchment) were highly germinated with mean percentage of (98%). However the lowest germination mean percentage was noted on $W_1V_{74\text{-}1}$ (27.5%) with parchment of coffee seed. In agar plate method also the identified fungi were; *Fusarium sp. Aspergillus sp. Pencillium sp.* and another some unidentified species. Among these, *Aspergillus spp.* had the highest (31.25%) frequency of occurrence, followed by *Penicillium spp.* (10.625%) and *Fusarium spp.* (11.875%) and unidentified spp. (9.375%). Moreover, in agar plate method the maximum coffee seeds mean infection percentage were recorded 82.5% and the mean minimum infection percentage were 52.5% in the treatment $W_1V_{74\_110}$ (with parchment) and $W_0V_{74\_110}$ (without parchment) respecti vely. From total four treatments 62.5% maximum fungal contaminations were recorded. The result of present study, storage fungi chiefly comprise several "group species" of the genera *Aspergillus spp, Penicillium spp, Fusarium spp.* and another unidentified species. The species identified in this study are among the most common species of fungi present in storage environments at high moisture. They can tolerate growth in different substrates and environmental conditions, and their complete elimination is difficult. However, the use of good hygiene practices and using optimum moisture of coffee seeds in storage management and can minimize mycroflora association of coffee seeds.

Although the present study was carried out in one location in Jimma University College of Agriculture and Veterinary Medicine (JUCAVM)/Jimma Agricultural Reserch Center (JARC) for three month in 2014 and, it has clearly indicated that different fungi were associated with coffee bean seed under storage condition, especially *Aspergillus spp.* In general, further research is needed to identify all recovered fungal pathogens and evaluation of promising treatments for use in integrated disease management strategy to manage not only fungal but also other coffee bean seed diseases and also further investigation of storage temperature, relative humidity, periods of storage and storage types in wide range across the location that suitable for good supply of health coffee seeds.

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