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Association of Mycoflora with Coffee (*Coffea arabica* L.) Beans at Limmu Coffee Plantation, Southwestern Ethiopia

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ABSTRACT
Coffee (*Coffea arabica* L.) bean quality can be affected by a number of factors of which storage fungi are one of the major ones. In Ethiopia coffee is a number one export commodity supporting the national economy but there was little information about the association of mycoflora with coffee beans. Therefore, the objective of this study was to assess the associations of mycoflora with coffee seeds and their effect on coffee infection at Limmu Coffee Plantation. The comparison was done using two coffee berry disease resistant selections (74112 and 74110) with and without parchment and with and without surface treatment of coffee beans with 5% sodium hypochlorite and storing under two storage conditions (local cold house and corrugated iron warehouse). Thus, the experiment was laid down as 2×2×2×2 factorial experiment with four replications. The study showed the association of four fungal species (*Fusarium* spp., *Aspergillus* spp., *Penicillium* spp. and *Mucor* spp.) and some unidentified species in both blotting and agar plating techniques. In general, significantly higher infection percentage was found in coffee seeds without parchment and surface disinfection irrespective of the storage type and coffee selection. Thus, keeping coffee beans with parchment and disinfecting by disinfectants may reduce the association and prevalence of mycoflora on coffee and minimize postharvest problems.

Key words: Coffee bean, *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., storage fungi

INTRODUCTION

Coffee (*Coffea arabica* L.) is an important tropical cash crop, commercially cultivated in many countries of Africa, Asia and South America (Martins *et al*., 2003). It is the best described coffee species that accounts about 80% of the world coffee trade. The most dynamic growth of coffee production in African countries was observed in Ethiopia, which recorded an average annual growth rate of 2.6% during the last 50 years, increasing to 3.6%, since 1990 (ICO., 2014).

Fungal contamination and production of mycotoxins is one of the post harvest problems that influence the quality of coffee beans. The storage fungi, mainly several species of *Aspergillus* and *Penicillium* do not invade seeds to any appreciable degree before harvest but they can cause severe discoloration of seed in storage resulting in germination failure, discolored or otherwise damaged embryos or whole seeds (Malaker *et al*., 2008). Species of *Penicillium* are encountered at times, usually in seed lots stored at low temperatures and with above 16% moisture content. In the range of moisture content between 14.0 and 15.5% in coffee, a difference of only 0.2% may make a great difference in the rate of invasion of the seeds by storage fungi and in the damage caused to the seeds (Christensen and Kaufman, 1979). But preventing or retarding the invasion of these fungi and creating unfavorable conditions for their growth and multiplication will help in improving the quality of coffee seed. Mycoflora of coffee beans might be affected by type of variety and storage materials used. It might also be influenced whether the coffee beans are with or without parchment and treated or untreated with disinfectant. However, little work has been done on the assessment of mycoflora associated on coffee beans in Ethiopia. Therefore, the aim of present study was to assess mycoflora associated with coffee beans and their infection at Limmu Coffee Plantation, Southwestern Ethiopia.

MATERIALS AND METHODS

Sampling and study area: Samples for the research were collected from coffee beans stored at Limmu Coffee Plantation, Ethiopia, at different storage conditions. The collection was from stores of Gomma-II, Kossa, Gumer and Suntu farms of the plantation. From each store 300 g of samples were collected and transported to Plant Pathology Laboratory of Jimma University College of Agriculture and Veterinary Medicine (JUCAVM), Jimma, Ethiopia for seed purity and germination test, moisture content determination and mycological analyses.

Assessment of bean purity, germination, moisture content and weight: Samples of coffee seeds collected from different farms of Limmu Coffee Plantation (Gomma-II, Kossa, Gumer and Suntu) were physically inspected with eye on the basis of which they were separated into pure seeds, seeds of other crops and inert matter. For the assessment 300 g of each sample was used and pure seeds were separated from abnormal seeds and inert matter and each of these components were weighed and recorded. Seeds with physical abnormalities, like shriveling of the seed coat, reduction or increase in seed size, discoloration or spots in the seed coat were classified under abnormal seeds. Inert matter included soil, sand, stones and plant debris. The coffee seeds moisture contents were also measured by using a moisture meter and expressed as percentage.

For germination test the parchment of coffee seeds were removed and socked in water for 48 h for imbibition. Water absorbent material was placed inside the waterproof plastic for germination. Samples were randomly taken from each seed lot and mixed in a container by taking at least four seed samples from the mixed coffee seeds. Hundred seeds from each sample were counted and placed on absorbent material inside the plastic tray. The absorbent materials were carefully saturated ten days by keeping each day absorbent material remains moist. Finally germination percentage was calculated as follows (Olmez *et al*., 2006):

$$\text{Germination (\%)} = \frac{\text{No. of seeds germination}}{\text{No. of seeds sample}} \times 100$$

Mycological analyses: In order to evaluate the effect of different factors on the association of mycoflora on coffee beans, the experiment was laid down as 2×2x2x2 factorial experiment where the first factor was coffee selection (74112 and 74110), the second factor was storage type (cold house storage and corrugated iron warehouse), the third factor was coffee seed parchment (with and without parchment) and the fourth was surface disinfection (with and without surface disinfection) by 5% sodium hypochlorite. Each of the factor level was combined to create 16 treatment combinations (Table 1). All the treatment combinations were arranged as factorial experiment in completely randomized design with four replications and the experiment was repeated. For mycological assessment, isolation of fungi associated with coffee beans was carried out with blotting and agar plating techniques.

Blotter test method: The blotter test method was used to isolate the fungal pathogens associated with coffee selections (74112 and 74110) stored for seven months. The samples were tested according to ISTA (1981). A total of 640 coffee seeds of 16 treatments, in four replicates, were tested from each sample. The sample with and without parchment were disinfected and non-disinfected then plated directly on top of three layers of well-soaked blotter paper. Ten seeds were plated per plastic on petri-dish of 9 cm diameter by surface disinfection. The plated coffee seeds were incubated at 22-25°C for 7 days under alternate cycles of 12 h daylight and darkness. After incubation each coffee bean was observed under different magnifications in the stereo microscope for fungal growth. Pathogenic fungi developing on coffee seeds were isolated. Then microflora associated with seeds were cultured on potato dextrose agar media and identified to genus level using morphological characters such as spore size,

Table 1: Treatment combinations of the different factors for the mycological analyses

Treatment combinations	Description of the treatment combination
$V_1 S_1 P_1 T_0$	Coffee seeds selection74112 stored in local cold house with parchments and without surface disinfection
$V_1 S_1 P_1 T_1$	Coffee seeds selection74112 stored in local cold house with parchments and with surface disinfection
$V_1 S_1 P_0 T_0$	Coffee seeds selection74112 stored in local cold house without parchments and without surface disinfection
$V_1 S_1 P_0 T_1$	Coffee seeds selection74112 stored in local cold house without parchments and with surface disinfection
$V_2 S_1 P_1 T_0$	Coffee seeds selection74110 stored in local cold house with parchments and without surface disinfection
$V_2 S_1 P_1 T_1$	Coffee seeds selection74110 stored in local cold house with parchments and with surface disinfection
$V_2 S_1 P_0 T_0$	Coffee seeds selection74110 stored in local cold house without parchments and without surface disinfection
$V_2 S_1 P_0 T_1$	Coffee seeds selection74110 stored in local cold house without parchments and with surface disinfection
$V_1 S_2 P_1 T_0$	Coffee seeds selection74112 stored in corrugated ironware house with parchments and without surface disinfection
$V_1 S_2 P_1 T_1$	Coffee seeds selection74112 stored in corrugated ironware house with parchments and with surface disinfection
$V_1 S_2 P_0 T_0$	Coffee seeds selection74112 stored in corrugated ironware house without parchments and without surface disinfection
$V_1 S_2 P_0 T_1$	Coffee seeds selection74112 stored in corrugated ironware house without parchments and with surface disinfection
$V_2 S_2 P_1 T_0$	Coffee seeds selection74110 stored in corrugated ironware house with parchments and without surface disinfection
$V_2 S_2 P_1 T_1$	Coffee seeds selection74110 stored in corrugated ironware house with parchments and with surface disinfection
$V_2 S_2 P_0 T_0$	Coffee seeds selection74110 stored in corrugated ironware house without parchments and without surface disinfection
$V_2 S_2 P_0 T_1$	Coffee seeds selection74110 stored in corrugated ironware house without parchments and with surface disinfection

V_1: Coffee selection 74112, V_2: Coffee selection 74110, S_1: Local cold house storage, S_2: Corrugated iron warehouse storage, P_0: Without parchment, P_1: With parchment, T_0: Without surface disinfection, T_1: With surface disinfection

shape, color and their arrangement on the conidiophores and morphology of the mycelium (Nelson *et al*., 1983; Sivanesan, 1987; Singh *et al*., 1991; Samson *et al*., 1995; Watanabe, 2002; Leslie and Summerell, 2006) as cited by Utobo *et al.* (2011).

Agar plate method: For agar plate method the two coffee selections (74112 and 74110) were equally placed aseptically on PDA in 9cm petri dish, 10 seeds per plate replicated four times. In comparable set the seeds with and without parchments were surface sterilized with 5% sodium hypochlorite for three minutes and washed in sterilized distilled water before plating on the PDA medium. The plated coffee seeds were incubated for 7-10 days at 25°Cunder 12 h 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. For identification preparation of slide mounts of spores or other bodies in a drop of water was examined under a compound microscope for shape, size and color and morphology of sporulation structures (Mathur and Kongdal, 2003).

Fungi infection percentage: The infection percentage (%) of coffee beans by fungi was determined as the ratio of infected seeds over the total number of coffee seeds tested in both agar plate and blotting methods as follows:

$$\text{Mean ratio of seed infection} = \frac{\text{No. of seed on which a fungal species identified}}{\text{No. of seed tested}} \times 100$$

Data analysis: Descriptive data analysis was used to summarize information about germination percentage and physical inspection of the seeds and fungi infection percentage using micros of Excel software program. However, mycoflora assessment was done by running Analyses of Variances (ANOVA) using General Linear Models (GLM) procedure of SAS software (9.2 version) (SAS., 2008). Mean values among treatments were compared by the Tukey test at a = 0.05% level of significance.

RESULTS AND DISCUSSION

Bean purity, germination, moisture content and weight: The physical analysis of the coffee sample (Table 2) indicated that the percentage of normal seed was 72, 78, 93 and 74% at Gomma-II, Kossa, Gumer and Suntu, respectively. Abnormal seed occurs in the range of 0-20% while the damaged seeds constituted to 2-6% (Table 2).

The moisture content of the coffee seed samples was higher at Gomma -II (16%) followed by Gumer coffee farm (14%) (Table 2). Germination percentage of the sample from different coffee farms, Gomma-II, Kossa, Gumer and Suntu was 21, 65, 78 and 60%, respectively (Table 2). The highest (78%) and lowest (21%) germination percentage were observed from samples taken at Gummer and Gomma-II, respectively (Table 2).

The level of moisture content in stored coffee seeds affects both its grade and storability and has been designated as an essential pre-requisite for microbial activity which enhances the rate of damage. Seed stored in humid and warm environments tend to absorb moisture from the surroundings, leading to increased seed moisture content until equilibrium is established. The present finding showed high moisture content at Gomma-II coffee farm with low mean germination percentage indicating moisture content favored development of micro-organisms and resulted in decreased germination percentage. This result was in line with other workers (Rahman *et al*., 1985; Malaker *et al*., 2008) who reported that there was decrease in seed germination with increasing moisture content. However, the storage physiology of seeds from *Coffea* is complex and the literature is often conflicting (Eira *et al*., 2006) and thus further researches that explicitly confirm the result are required.

Mycoflora associated with coffee seeds: In connection with the investigations of mycroflora associated with coffee seeds, the isolation with blotting method and agar plating technique recovered four fungal species which were identified at genus level and some unidentified species from coffee seed stored for seven months. The fungal species that belong to genus *Fusarium, Aspergillus, Penicillium* and *Mucor* were identified (Table 3 and 4).

Coffee cherries and beans are subjected to contamination and consequently colonization by microorganisms during different phases of development from harvesting to storage. The contamination of coffee beans by fungi affects both the quality in terms of flavor and aroma of the beverage and presents a safety risk to the final product due to the production of toxic secondary metabolites, the mycotoxins, which can be harmful to consumers at certain concentrations (Bennett and Klich, 2003; Vilela *et al*., 2010; Rezende *et al*., 2013).

Table 2: Purity, germination percentage, moisture content and seed weight of coffee (*Coffea arabica* L.) beans stored for seven months at limmu coffee plantation

	Purity test (%)							
Coffee farms	Normal	Bean bear	Damaged	Light bean	Wrinkled bean	Germination (%)	Moisture content (%)	Average. seed weight (g)
Gomma-II	72.00	12.00	4.00	8.00	4.00	21.00	16.00	0.21
Kossa	78.00	14.00	6.00	0.00	2.00	65.00	13.50	0.18
Gumer	93.00	05.00	2.00	0.00	0.00	78.00	14.00	0.21
Suntu	74.00	20.00	4.00	2.00	0.00	60.00	13.80	0.19
Mean	79.00	13.00	4.00	2.00	2.00	56.00	14.33	0.20

Table 3: Frequency of fungal mycoflora associated with stored coffee seeds at Limmu Coffee Plantation (seed health test by blotting technique)

Treatment combination	Storage fungal species (%)					
	Aspergillus	*Penicillium*	*Fusarium*	*Mucar*	Unidentified	Mean
$V_1S_1P_1T_0$	2.50	0.00	2.50	45.00	0.00	10.00^{b}
$V_1S_1P_1T_1$	20.00	0.00	0.00	12.50	0.00	6.50^{c}
$V_1S_1P_0T_0$	50.00	2.50	12.50	25.00	0.00	18.00^{a}
$V_1S_1P_0T_1$	17.50	0.00	7.50	50.00	0.00	15.00^{ab}
$V_2S_1P_1T_0$	0.00	0.00	0.00	20.00	0.00	4.00^{d}
$V_2S_1P_1T_1$	42.50	0.00	2.50	7.50	5.00	11.50^{b}
$V_2S_1P_0T_0$	13.10	0.00	7.50	5.00	0.00	5.10^{dc}
$V_2S_1P_0T_1$	15.00	0.00	2.50	2.50	0.00	4.00^{d}
$V_1S_2P_1T_0$	2.50	0.00	0.00	35.00	0.00	7.50^{bc}
$V_1S_2P_1T_1$	2.50	0.00	0.00	10.00	0.00	2.50^{d}
$V_1S_2P_0T_0$	7.50	2.50	10.00	45.00	0.00	13.00^{ab}
$V_1S_2P_0T_1$	2.50	7.50	2.50	12.50	0.00	5.00^{dc}
$V_2S_2P_1T_0$	7.50	0.00	0.00	55.00	0.00	12.50^{ab}
$V_2S_2P_1T_1$	15.00	0.00	10.00	20.00	0.00	9.00^{bc}
$V_2S_2P_0T_0$	57.50	0.00	7.50	35.00	0.00	20.00^{a}
$V_2S_2P_0T_1$	20.00	37.50	10.00	32.50	0.00	20.00^{a}
Mean	17.20	3.10	4.70	25.80	0.30	
SD (±)	4.70	4.00	4.70	4.70	4.60	

Mean of the same letters are not significant different at alpha 5%, V_1: Coffee selection 74112, V_2: Coffee selection 74110, S_1: Local cold house storage, S_2: Corrugated ironware house storage, P_0: Without parchment, P_1 With parchment, T_0: Without surface disinfection, T_1: With surface disinfection

Table 4: Frequency of fungal mycoflora associated with stored coffee seeds at Limmu Coffee Plantation (seed health test by agar plating technique).

Treatment combination	Storage fungal species (%)					
	Aspergillus	*Penicillium*	*Fusarium*	*Mucor*	Unidentified	Mean
$V_1S_1P_1T_0$	05.00	0.00	40.00	15.00	10.00	14.00^{c}
$V_1S_1P_1T_1$	32.50	5.00	30.00	15.00	12.50	19.00^{ab}
$V_1S_1P_0T_0$	75.00	12.50	27.50	12.50	7.50	27.00^{a}
$V_1S_1P_0T_1$	22.50	0.00	35.00	20.00	10.00	17.50^{c}
$V_2S_1P_1T_0$	45.00	5.00	25.00	12.50	22.50	22.00^{b}
$V_2S_1P_1T_1$	30.00	7.50	35.00	7.50	12.50	18.50^{bc}
$V_2S_1P_0T_0$	75.00	5.00	22.50	25.00	15.00	28.50^{a}
$V_2S_1P_0T_1$	25.00	42.50	25.00	2.50	5.00	20.00^{b}
$V_1S_2P_1T_0$	12.50	0.00	22.50	15.00	15.00	13.00^{cd}
$V_1S_2P_1T_1$	25.00	2.50	45.00	7.50	15.00	19.00^{bc}
$V_1S_2P_0T_0$	27.50	5.00	42.50	57.50	12.50	29.00^{a}
$V_1S_2P_0T_1$	22.50	0.00	50.00	12.50	7.50	18.50^{bc}
$V_2S_2P_1T_0$	12.50	0.00	2.50	32.50	0.00	9.50d
$V_2S_2P_1T_1$	20.00	0.00	47.50	2.50	5.00	15.00^{c}
$V_2S_2P_0T_0$	25.00	0.00	37.50	25.00	0.00	17.50^{c}
$V_2S_2P_0T_1$	22.50	52.50	15.00	10.00	5.00	21.00^{b}
Mean	29.80	8.60	31.40	17.00	9.70	
SD (+)	4.60	2.20	1.40	4.70	4.60	

The mean the same letters are no significant difference at alpha 5%, V_1: coffee selection 74112, $\mathbf{V_2}$- Coffee selection 74110, S_1: Local cold house storage, S_2: Corrugated ironware house storage, P_0: Without parchment, P_1: With parchment, T_0: Without surface disinfection, T_1: With surface disinfection

In the present study, coffee beans stored at different farms with different storage conditions have shown to have contaminated mainly by the genera of *Aspergillus*, *Penicillium Fusarium* and *Mucor*. Many species of fungi identified in this work had been already detected in cherries and grains of coffee beans in other studies. Girma *et al*. (2008) reported fungal species belonging to the genera of *Aspergillus* and *Penicillium*, while studying the occurrence and distribution of mold species associated with Ethiopian dried coffee cherries collected from the ground and those picked from coffee trees and parchment coffee samples taken from drying table at Gera, Jimma and Teppi coffee production areas. Fungi identified in this work had been already detected in coffee seeds with parchment by different authors elsewhere (Batista *et al*., 2003; Taniwaki *et al*., 2003; Pardo *et al*., 2004). Similarly, Silv *et al*. (2000) reported the occurrence and diversity of these groups of fungi on the surface of coffee cherries and beans as natural coffee contaminants from the field to the warehouse conditions. In other studies it has been shown that *Aspergillus*, *Penicillium* and *Fusarium* genera are natural coffee contaminants and are present from the field to warehouse (Bokhari, 2007; Silv *et al*., 2000) in coffee cherries and beans. Fungal genera identified in this study had already been recorded in coffee (Batista *et al*., 2003) and from coffee bean samples from Brazil (Urbano *et al*., 2001).

Considering the effect of different treatment combinations on the mean incidence of the microbes on coffee beans, our study showed that there was significant difference ($p<0.001$) between the treatment combinations in both blotting technique and agar plating method. In blotting method, the highest mean incidence (20%) was recorded on coffee seed selection74110 stored in corrugated iron warehouse without parchments and with and without surface disinfection while the lower mean incidence (2.5%) was on coffee seeds selection 74112 stored in corrugated iron warehouse with parchment and with surface disinfection (Table 3).

With the agar plating technique, significantly higher mean mycoflora associations were recorded on the coffee seeds selection 74112 stored in corrugated iron warehouse without parchments and without surface disinfection (29%) followed by coffee seeds selection74110 stored in local cold house without parchments and without surface disinfection (28.5%). While significantly lower mean mycoflora (9.5%) was found on coffee selection 74112 stored in corrugated warehouse with parchment and without surface disinfection (Table 4).

Seed infection percentage: For seed infection percentage the analysis of variance indicated that there was significant difference ($p<0.0001$) between treatment combinations in infection percentage for both blotting method and agar plating technique. With blotting method, the highest seed infection percentage (100%) were recorded from seeds without parchment and without surface disinfection irrespective of the coffee selection and type of store used, while the lowest infection percentages were recorded from seeds stored with parchment and with surface disinfection (Fig. 1).

In the agar plating method, the maximum fungal contaminations(100%) were recorded from majority of the treatment combinations (Fig. 2). On the other hand, the minimum infection percentage (80%) was recorded from treatment combination of coffee selection stored in both cases without parchments and with surface disinfection (Fig. 2). In a study made by Nega (2014) also coffee seed infection percentage was found to be higher for coffee beans without parchment than with parchment when experimented in both agar plating and blotting methods.

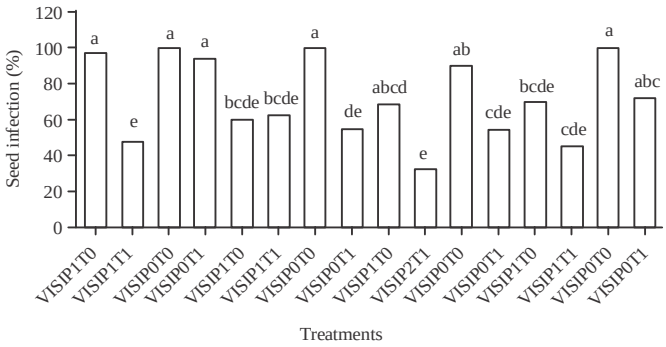

Fig. 1: Infection percentage of coffee (*Coffea arabica*) seeds by blotting method. Mean with the same letters is not significantly different at alpha 5%. V_1: Coffee selection 74112, V_2: Coffee selection 74110, S_1: Local cold house storage, S_2: Corrugated ironware house storage, P0-: Without parchment, P_1: With parchment, T_0: Without surface disinfection, T_1: With surface disinfection

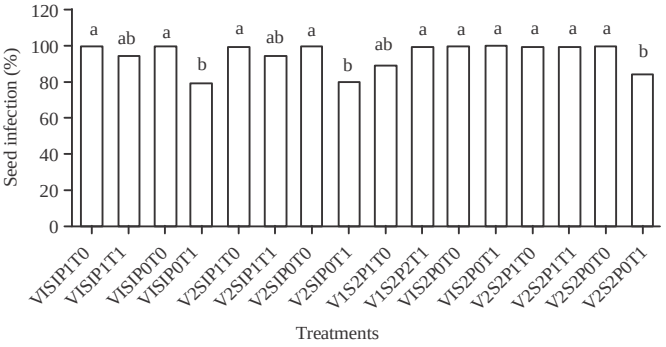

Fig. 2: Infection percentage of coffee (*Coffea arabica*)seeds by agar plating method. Mean with the same letters is not significantly different at alpha 5%. V_1: Coffee selection 74112, V_2: Coffee selection 74110, S_1: Local cold house storage, S_2: Corrugated iron warehouse storage, P_0: Without parchment, P_1: With parchment T_0: Without surface disinfection, T_1: With surface disinfection

Generally, the difference in the prevalence and incidence of the mycoflora on the coffee beans was more associated with the presence and absence of parchment on the coffee seeds and surface disinfection than the type of coffee selection and storage type. Because in most cases significantly lower pathogen association with the coffee beans was found in coffee beans with parchment and surface disinfection. Possible explanations could be the parchment on coffee beans may protect entrance and prevent invasion of the beans by fungi. Furthermore, as parchment represents a dead and relatively dry sclerenchymatic tissue, gases present around the seeds such as CO_2, O_2 or H_2O (Valio, 1980) may not diffuse and help germination of fungi.

In conclusion, storing coffee beans with parchment coupled with surface disinfectant may reduce the association of microfolra with coffee beans and help to reduce surface contamination and postharvest problems. However, the commercial viability of storing coffee beans with parchment needs further investigation.

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