P-ISSN: 2305-6622; E-ISSN: 2306-3599
International Journal of Agriculture and Biosciences
www.ijagbio.com; editor@ijagbio.com

Research Article

Response of Groundnut (*Arachis hypogaea* L.) Genotypes to Aflatoxin Contaminations and *Aspergillus* species Seed Invasion as Influenced by Biocontrol Agent and Wheat Straw Mulching in Central Tigray, Northern Ethiopia

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Article History: Received: February 23, 2019 Revised: July 22, 2019 Accepted: August 16, 2019

ABSTRACT

Groundnut (*Arachis hypogaea* L.) is the 13th most important food and fourth most important oilseed crop of the world. Its kernel contains 40-50% oil, 20-50% proteins and 10-20% carbohydrates. However, due to lack of appropriate management practices, aflatoxin contaminations cause extremely serious health problems in human and animals. Trichoderma harzianum could suppress the aflatoxigenic Aspergillus species due to its antagonistic nature. Wheat straw mulches could increase the soil moisture retaining capacity to overcome terminal drought stress and decrease soil temperature. Therefore, a field experiment was conducted in Rama, Central Zone of Tigray, northern Ethiopia, from July to October 2016 to evaluate the effects of straw mulch and bio-inoculant T. harzianum on Aspergillus species seed infection and aflatoxin contamination of groundnut varieties (ICGV00308, ICIAR19BT, Werer-961 and Rama local). The treatments were arranged in a factorial randomized complete block design (RCBD) with three replications. Groundnut varieties, T. harzianum and wheat straw mulches highly and significantly ($P \leq 0.01$) decreased aflatoxin contamination levels and significant ($P \leq 0.05$) difference was recorded in seed invasion by Aspergillus flavus. The combined application of T. harzianum and straw mulch resulted in lower aflatoxin contamination levels of 28.58 and 28.78 ppb in Rama local and ICGV00308 varieties, respectively; and lower (18.52%) A. flavus seed invasion in Rama local and (22.22%) in ICGV00308. Application of T. harzianum and straw mulch suppressed Aspergillus species found in the soil and enhanced the tolerance of groundnut varieties to Aspergillus species as well as drought stress. Consequentially markedly decrease in aflatoxin contamination and seed invasion by A. flavus was recorded. The current findings suggested that integrated Aspergillus species management using drought tolerant groundnut genotypes together with the biocontrol T. harzianum isolate BD-13 and wheat straw mulching significantly reduced aflatoxin contamination level and Aspergillus seed invasion. Further screening of groundnut genotypes, biocontrol agents and mulching types would help to develop integrated aflatoxin management for sustainable production.

Key words: Aflatoxin, A. flavus and A. niger, T. harzianum and mulch

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an annual legume crop originated in North America and currently cultivated over 107 countries in six continents of the world (Wiess, 2000). It was introduced from Eritrea to Ethiopia through Hararghe by Italian explorers in 1920's (Daniel, 2009). Groundnut in Ethiopia covered an area of about 79,947.03 ha with a corresponding annual production of 112,088.724 Mt in 2013/2014 cropping season (CSA, 2014). However, its productivity is about 1.402 t ha^{-1}, which is lower than the average global yield of 1.52 t ha^{-1} but with good management, the potential of the crop can reach up to 3.0 t ha^{-1} (CSA, 2009).

Aflatoxin is a highly toxic cancer-causing fungal secondary metabolite that produces carcinogenic effects to human and animals. The occurrence of aflatoxin is highly favorable in the presence of terminal drought stress during groundnut production. Terminal drought increases the susceptibility of pods and seeds invasion because of lower soil moisture content. Low soil moisture lowers the physiological activities of groundnut (Azaizeh *et al*., 1989).

Cite This Article as: Weldu N and D Assefa, 2019. Response of groundnut (*Arachis hypogaea* L.) genotypes to aflatoxin contaminations and *aspergillus* species seed invasion as influenced by biocontrol agent and wheat straw mulching in central Tigray, Northern Ethiopia. Inter J Agri Biosci, 8(6): 282-290. www.ijagbio.com

Food and Agriculture Organization of the United Nations (FAO) estimated that 25% of the world food crops are affected by aflatoxin. More than 4.5-5.0 billion people in the developing countries are exposed to aflatoxin (Tiffany, 2013). According to World Health Organization (WHO), about 550,000-600,000 new cases are reported every year and 83% of them are from East Asia and Sub-Saharan Africa (SSA) (WHO, 2008). In 2004, 317 Kenyan people severely suffered from aflatoxin contamination and 125 people died because of acute aflatoxicosis (CDC, 2004). In Kenya also 20 cases were reported due to fatal outbreak of aflatoxicosis in 1981 (Ngindu *et al*. 1982). Similarly, 80 cases and 30 deaths in 2005 (Lewis *et al.*, 2005) and nine deaths in 2006 (Wagacha and Muthomi, 2008) were reported due to liver failure associated with consuming food contaminated with extremely high level of aflatoxin (Felica *et al*., 2011).

In Ethiopia, it is considered that there is little or no documented case of aflatoxicosis though toxigenic *Aspergillus* species and aflatoxin contaminations were reported from food grains, like sorghum (*Sorghum bicolor*), maize (*Zea mays*) barley (*Hordeum vulgare*), wheat (*Triticum* spp.) and tef (*Eragrostis tef)* (Amare *et al*., 2006) and sorghum, maize, tef and barley (Dawit and Berhanu, 1985). Recently Abdi *et al*. (2016) isolated eight *Aspergillus* species and two *A. flavus* (L and S) strains from groundnut seeds collected from eastern Ethiopia. Aflatoxin B_1 was previously reported in Ethiopia with concentrations of 34.7 and 105 ng g^{-1} from groundnut seeds and peanut butter, respectively (Besrat and Gebre 1981). Recently about 85% of the isolated *A. flavus* from groundnut in eastern Ethiopia produced aflatoxin contaminations ranging from 1 to more than 300 ng g^{-1} in liquid medium (Amare *et al*., 1995). Abdi *et al*. (2016) also reported 50% of the "Halawa" local cake prepared from groundnut was contaminated with aflatoxin B_1 with concentration up to 158.1 ng g^{-1} and groundnut seed samples collected from 2013/2014 and 2014/2015 production seasons with aflatoxin concentrations of 786 and 3135 ng g^{-1}, respectively.

Rama is one of the lowland areas of Tigray, agro-ecologically conducive for groundnut production, but the productivity of the crop is about 0.7 t ha^{-1} which is far below the national the national average 1.3 t ha^{-1} (CSA, 2013). Lower productivity is mainly due to the biotic and a biotic factor, including poor soil fertility and prevalence of soil borne *Aspergillus* species (Kahsay and Mewael, 2014). Dereje *et al*. (2012) reported the presence of aflatoxin contaminations of groundnut in the study area Rama with seed infection level of 41.5 and 12.3% by *A. flavus* and *A. niger*, respectively, and the aflatoxin B_1 contamination level ranged from 0.1 to 397.8 ppb, which is higher than the European Union standard level (5 ppb), FAO/WHO (15 ppb) and that of the maximum tolerable level set for USA (20 ppb).

Aflatoxin contamination is a complex problem and it is very difficult to protect with a single management practice. Integration of different management approaches and their compatibility may be very useful in reducing aflatoxin risk in groundnut seeds to human beings and animal health. Therefore, the present work focused on reduction of soilborne *Aspergillus* species in the soil and to reduce fungal infection and aflatoxin contamination of groundnut kernels through integrated management of groundnut genotypes, natural antagonistic *T. harzianum* isolate BD-13 and wheat straw mulching. The purpose of mulching is to retain soil moisture and provide benefit for the soil microorganisms with organic matter or as a primary source of carbon for the biocontrol agents, and to stabilize the soil temperature at late growth stage starting from pegging to pod maturation and harvesting of the groundnut. Hence, the study was carried out with the following specific objective to evaluate the effects of *Trichoderma harzianum* inoculant and wheat straw mulch alone or in combination and groundnut genotypes for the management of *Aspergillus* species and aflatoxin contamination.

MATERIALS AND METHODS

Description of the study area

The experiment was conducted at Rama, Mereb Leke District of Central Zone of Tigray, northern Ethiopia, during the 2016 main cropping season. Rama is located at 14º22'25" N latitude and 038º47'32" E longitude at an elevation of 1390 m. a. s. l. Rama is 258 and 1041 km away from Mekelle and Addis Ababa, respectively, towards the northern. It lies in the *kola* agro-ecological zone and the soil type is sandy clay loam. The mean annual rainfall ranges from 400 to 600 mm and the rainfall distribution is mono-modal with an erratic distribution beginning in late June and ending in the last week of August. The mean maximum and minimum temperature during 2016 production season was 33.9 and 18.7 ºC, respectively, and the average temperature of the study area was 26.3 ºC, while the total annual rainfall of the experimental site during 2016 main cropping season was 586.9 mm (NMA, 2017). Commonly grown crops are finger millet, sorghum, groundnut and maize. In addition to cereal and legume/oil crops, *Citrus* species and mango are also among the commonly grown fruit crops in the study area according to Mereb Leke District Office of Agriculture and Rural Development (DOARD, 2016).

Experimental materials

Planting materials

Four groundnut cultivars (two genotypes introduced from ICRISAT as drought tolerant and one variety collected from Werer Agricultural Research Center and one local variety as a drought susceptible check collected from farmers in the study area were used in the study. Those four groundnut cultivars together with *T. harzianum* and wheat straw mulch were evaluated for the management of aflatoxin contamination and toxicogenic *Aspergillus* species seed invasion at Rama, in 2016 main cropping season.**.** Seeds of those genotypes were obtained from Mekelle University RUFORUM Groundnut Project. Genotype Werer-961 was released from Werer Agricultural Research Center in 2004 and the other two genotypes ICGV00308 and ICIAR19BT were introduced from ICRISAT. Seeds of each genotype were planted with and without *T. harzianum* isolate BD-13 inoculation.

Trichoderma harzianum

T. harzianum isolate BD-13 was used as a biocontrol agent against groundnut seed infection by *Aspergillus* species that mainly cause the seed contamination by

aflatoxins both in pre-harvest and post-harvest conditions. *T. harzianum* isolate BD-13 was obtained from Mekelle University Plant Pathology Laboratory.

Preparation of *Trichoderma* inoculum and inoculation techniques

Culture of *T. harzianum* isolate BD-13 was prepared on potato dextrose agar (PDA) plates under dark room at natural condition and under fluorescent light until sporulation was visible. The conidial inoculum was harvested by washing plates with 10 ml distilled sterile water and filtered the suspension through nylon mesh into a tube. The concentration of the spore suspension was standardized via haemacytometer count and the spore suspension was adjusted with distilled sterile water to produce a spore suspension of 10^6 spores per millimeter and finally a drop of Tween 20 was added to the adjusted spore suspension at a rate of 0.5% to disperse the spores and to increase the effectiveness of inoculation by attaching the spores with the seeds.

Seeds were inoculated by seed priming techniques (soaking seeds in the spore suspension) for three hours just before planting by counting 70 seeds of groundnut for each plot. Seeds were inoculated at a rate of 50 ml of spore suspension for each 70 seeds and the inoculated seeds (Tr1) were air dried for 40 minutes under shade and similarly the remaining non-inoculated seeds (Tr0) were soaked with sterile distilled water for similar duration and air dried in the same manner to the *T. harzianum* inoculated seeds. Additionally, spore suspension of the *T. harzianum* isolate BD-13 prepared in a similar manner with the abovementioned procedure was sprayed to the soil of groundnut seedling plots that were first planted with inoculated seeds. Similarly, the non-inoculated seedlings were sprayed with sterile distilled water only. One time spraying was done around the root zone (rhizosphere) of the seedlings 59 days after planting, when the groundnut growth stage reached the first pegging stage. The rate of application was 50 ml per plot using a hand sprayer (atomizer).

Wheat straw mulching material

Wheat straw was used as a source of mulching to increase the soil moisture holding capacity and to reduce terminal drought stress and soil temperature, which is scientifically justified as conducive environment for *Aspergillus* species to invade groundnut seeds. Mulching can create conducive microclimatic conditions for the atoxigenic soil microorganisms and this helps them to suppress *Aspergillus* species living in the soil. Wheat straw was first chopped into pieces of size 5 - 10 cm to cover the plots properly. Mulch was applied at a rate of 12 t ha^1 in split application of two times first half in 27 days after planting and the second half was applied 49 days after planting or 22 days after the first mulching based on the treatment randomizations. Un-mulched (ML0) plots were used as control.

Treatment Combinations and Experimental Design

The treatment combinations consisted of three factors: four groundnut varieties (ICGV00308, ICIAR19BT, Werer-961 and Rama local, RL), two *T. harzianum levels* with and without inoculation (Tr_1 and Tr0) and two wheat straw mulching with (ML_1) and without mulching (ML0) (Table 1). The experimental plot size was 2 m by 3.6 m with a plot size of 7.2 m^2 with six rows per plot. Groundnut varieties were sown in rows maintaining 60 and 20 cm spacing between rows and between plants, respectively. The net harvestable area was 4.8 m (2.4 m x 2.0 m) leaving one outermost row in both sides as borders. Each treatment was assigned to the plots randomly. The treatments were arranged in a randomized complete block design (RCBD) with three replications. One meter and 1.5 m spacing were maintained between plots and replications, respectively.

Table 1: List of treatments and treatment combinations of groundnut genotypes, wheat straw mulch and *T. harzianum* inoculum at Rama in 2016 main cropping season

Treatment combination[1]	Groundnut genotypes	*T. harzianum* inoculants	Wheat straw Mulch
(T1) = WTr1ML1	W	Tr1	ML1
(T2) = WTr1ML0	W	Tr1	ML0
(T3) = WTr0ML1	W	Tr0	ML1
(T4) = WTr0ML0	W	Tr0	ML0
(T5) = RLTr1ML1	RL	Tr1	ML1
(T6) = RLTr1ML0	RL	Tr1	ML0
(T7) = RLTr0ML1	RL	Tr0	ML1
(T8) = RLTr0ML0	RL	Tr0	ML0
(T9) = IRTr1ML1	IR	Tr1	ML1
(T10) = IRTr1ML0	IR	Tr1	ML0
(T11) = IRTr0ML1	IR	TR0	ML1
(T12) = IRTr0ML0	IR	Tr0	ML0
(T13) = IVTr1ML1	IV	Tr1	ML1
(T14) = IVTr1ML0	IV	Tr1	ML0
(T15) = IVTr0ML1	IV	Tr0	ML1
(T16) = IVTr0ML0	IV	Tr0	ML0

[1] W = Werer-961 groundnut variety, RL = Rama local, IV = ICGV00308 groundnut genotype, IR = ICIAR19BT groundnut genotype =, Tr1 = Seeds with *Trichoderma harzianum* inoculated, Tr0 = Seeds without *Trichoderma harzianum* inoculation, ML1 = plots with wheat straw mulched, ML0 = Plots without wheat straw mulching.

Management of Experimental Field

Groundnut seeds were sown on 13/07/ 2016. Each plot was divided into six planting rows and data were collected only from the harvestable central four rows. During planting diammonium phosphate (DAP) fertilizer was applied for all plots uniformly at a rate of 100 kg ha^{-1} in the form of P_2O_5 as a source of phosphorus (P) and, similarly, all the other management practices (hand weeding and hoeing) were done manually to all treatments.

Data collection

Data were collected on identification of *Aspergillus* species from groundnut kernels and incidence of kernel invasion by *Aspergillus flavus* and *Aspergillus niger* and aflatoxin contamination level. For each collected datum, the means obtained were recorded in the data collection sheet and were subjected to statistical analyses.

Isolation of *Aspergillus* species from groundnut seeds: Groundnut seed samples harvested from the central four rows were carefully hand-shelled and 45 seeds from each plot were taken and surface-sterilized by soaking in 10% Chlorox (NaOCl) solution for three minutes and rinsed three times with sterile distilled water. Then the seeds were plated on *Aspergillus flavus* and *Aspergillus parasiticus*

agar medium (AFPA) with a composition of peptone 10 g, yeast extract 20 g, ferric ammonium citrate 0.5 g, dichloran 0.002 g and agar 15 g that were mixed and heated with a volume of 1.0 L distilled water, with adjusted pH to 6.3 using hydrochloric acid (HCl) and autoclaved at 121ºC for 20 minutes according to (Dorner, 1998). After adjusting the pH of the medium to 6.3 using HCl, the antibiotic Chloramphenicol 0.1 g/L was added. After cooling the medium to 50 ºC three replications, containing 15 seeds per plate of 14.9 cm diameter. Seed-plated Petri dishes were incubated at 27 ºC for five to seven days. The growing colonies were visually evaluated and recorded.

Fungal identification was conducted based on macro morphological system (reverse and surface coloration of colonies, presence/absence of pigment and colony texture) and micro morphological characteristics: conidial size, conidial head and shape of vesicle (Klich, 2002). Incidence of *Aspergillus* species was calculated based on the formula described by González *et al*. (1995) as follows. Groundnut seed samples were diagnosed for fungal infection with emphasis on the prevalence of *Aspergillus* species.

$$\text{Incidence of } Aspergillus \text{ specie}(\%) = \frac{\text{Number of } Aspergillus \text{ sp. infected samples}}{\text{Total number of samples examined}} \text{x}100$$

Identification of *Aspergillus* species: *Aspergillus* species were identified by growing groundnut seeds on *Aspergillus flavus* and *Aspergillus parasiticus* (AFPA) agar medium. Groundnut seeds plated on AFPA agar medium were incubated for five to seven days at 27ºC. Morphological characteristics, macroscopic and microscopic examinations were performed according to Kahsay and Mewael (2014). Isolated *Aspergillus* species were identified to the species level using taxonomic system of *Aspergillus* done by Klich (2002).

Aflatoxin analysis: Aflatoxin contamination was analyzed in Plant Pathology Laboratory of School of Plant Sciences, Haramaya University, as per the manufacturer's protocol using Standard Aflatoxin Test Protocol of Neogen Reveal Q+m Reader (Mobile Assay Inc; Neogen Corporation, USA) according to Chauhan *et al*. (2016). One hundred grams of properly shelled groundnut seeds collected from plants harvested from the middle four rows was weighed using sensitive balance and the seeds were ground with a grinder. The flour was thoroughly mixed and five grams of the representative blended groundnut flour was prepared in 50 ml of falcon tube and 25 ml of 65% ethanol was added, which was five times the groundnut flour. Five grams of groundnut flour mixed with 25 ml of 65% ethanol was vigorously shaken manually for three minutes and, after shaking, the sample was filtered into another falcon tube, using funnel with pre-plated filter paper of Whatman No. 4 for the transfer of liquid extraction.

After the completion of extraction, 500 µL of sample diluent was added to the red dilution cup, which was supplied with the strip and another 100 µL of sample extract was added to red dilution cup and the mixture of 500 µL and 100 µL were mixed five times by turning up and down, using pipette and then 100 µL of sub-sample was transferred to another light sample cup, which was supplied with strips. Finally, the aflatoxin test strip was inserted into the light sample cup by keeping the red arrow to downward and the strip was incubated for six minutes, using the stopwatch for accuracy. Immediately after six minutes, the strip incubated in the light sample cup was removed and automatically inserted into the strip reader and then taken to the devices carefully with proper position of the line of strip perpendicular to the control line. Finally, the device was taken a photo after the clicking the capture options on the mReader for aflatoxin software and after a while the valid result was displayed.

Data analysis: All collected data were subjected to analysis of variance (ANOVA) using SAS version 9.1.3 computer software (SAS Institute Inc., 2004). The least significant difference (LSD) was used to separate and compare treatment means at 5% probability level.

RESULTS AND DISCUSSION

Effect of *T. harzianum*, wheat straw mulching and groundnut genotypes on *Aspergillus* species seed invasion

Two *Aspergillus* species, namely *A. flavus* and *A. niger*, were isolated, using *A. flavus* and *A. parasiticus* agar medium (AFPA). from groundnut seeds tested for their reaction to the aflatoxigenic fungi. The result from the analyses of variance revealed that the main effect *T. harzianum* and the interaction effect of *T. harzianum* and wheat straw mulching highly and significantly ($P \leq 0.01$) influenced *A. flavus* and *A. niger* groundnut seed invasion. The main effect of genotypes, wheat straw mulch and interaction effect of genotypes with *T. harzianum* and three-way interaction effect of genotypes, wheat straw mulching and *T. harzianum* significantly ($P \leq 0.05$) influenced groundnut seed invasion by *A. flavus* and *A.niger*. The analyses of variance showed significant ($P \leq 0.05$) difference among the groundnut genotypes seed invasion by *A. flavus* and *A. niger*.

The highest (53.52%) *A. flavu*s seed infection was recorded on Werer-961, followed by ICIAR19BT with seed invasion of 43.70%, while the lowest (34.54%) seed infection by *A. flavus* was recorded on ICGV00308, followed by Rama local (40.32%). Seed infection of Werer-961 by *A.flavus* was higher by 18.98 and 13.2% than both ICGV00308 and Rama local, respectively. The *A. flavus* seed invasion of non-inoculated plots was 23.82% higher than the *T. harzianum*-inoculated plots.

Similarly, seeds from non-mulched plot had 10.28% higher *A. flavus* infection than the straw mulched (Table 2). The analyses of variance of the three way interaction effects of *A. flavus* seed invasion showed significant ($P \leq 0.05$) difference among genotypes, straw mulch and *T. harzianum* treatment combinations. The highest *A. flavus* seed invasions of 75.55 and 61.48% were recorded in Werer-961 and Rama local varieties, respectively, over the control treatments, which were significantly different from Werer-961 and Rama local treated with *T. harzianum* in combination with straw mulch. However, the lowest *A. flavus* seed invasions of 18.52, 22.22, and 25.18% were recorded in RLTr1ML1, IVTr1ML1 and IRTr1ML1 treatment combinations, respectively (Table 2). Thus, application of straw mulch along with *T. harzianum* inoculation of the genotypes ICGV00308, ICIAR19BT and Rama local variety effectively suppressed *A. flavus* seed

infection by about 20.15, 31.12 and 42.96% over the control. There were significant differences among the groundnut genotypes in *A. niger* seed invasion. The highest (33.89%) *A. niger* seed invasion was observed on ICIAR19BT, followed by Rama local with seed invasion of 25.74% and the lowest (17.96%) invasion was on Werer-961. Similarly, application of *T. harzianum* as biocontrol agent showed highly significant (P≤0.01) reduction of groundnut seed invasion by *A. niger*. The highest (36.58%) percentage of seed invasion was recorded from the non-inoculated seeds, while the lowest (14.07%) was from inoculated. Application of bio-inoculant reduced *A. niger* seed infection by 22.51% over the non-inoculated control treatment. Similarly, the main effect of wheat straw mulching also significantly reduced *A. niger* seed invasions. The highest (34.35%) seed invasion was recorded from the non-mulched plots, whereas the lowest (16.29%) was obtained from mulched seeds. Application of straw mulch reduced *A. niger* seed invasions by 18.06% over the control treatment. This may be due to the effect of mulching on improving soil moisture level and soil temperature towards the last growth stages of groundnut (Table 3).

Aspergillus infection and aflatoxin contamination are mostly influenced by the occurrence of drought during the late seed filling duration. In a field study in Niger, Craufurd *et al.* (2006) established that infection and aflatoxin concentration in groundnut could be related to the occurrence of soil moisture stress during pod filling when soil temperatures are near optimal for *A. flavus*.

Two-way interaction effect of *T. harzianum* by straw mulch significantly (P≤0.05) reduced percentage of *A. niger* seed invasion. The highest (49.80%) seed invasion was observed in non-*T. harzianum* inoculated by no-mulched treatment combinations, followed by 23.33 and 18.88% in no-*T. harzianum* inoculated (Tr0ML1) and no-straw mulched (Tr1ML0), respectively, while the lowest (9.25%) seed invasion was recorded from the treatment combination Tr1M1. Treatment combination Tr1M1 was significantly different from Tr0M0, Tr0M1 and Tr1M0. The interaction effect of *T. harzianum* by wheat straw mulch reduced the percentage of seed invasion by 40.55% as compared to the control (Table 3).The three-way interaction effects of genotypes by *T. harzianum* and by wheat straw mulching significantly ($p \leq 0.05$) influenced *A. niger* seed invasion. The highest 72.59 and 50.37% seed invasions were recorded in IRTr0ML0 and IVTr0ML0 treatment combinations, while the lowest 5.19 and 7.41% were recorded in IVTr1ML1 and WTr1ML1 treatment combinations, respectively (Table 2).Thus application of *T. harzianum* and wheat straw mulch in the genotypes ICIAR19BT, ICGV00308 and Rama local variety significantly reduced *A. niger* seed invasion by 57.78, 45.18 and 36.30%, respectively, as compared to the control treatment (Table 2).

In this study, *A. flavus* was found to be the most dominant fungal pathogen with reference to groundnut seed invasion with mean invasion ranges of 34.54 to 54.93%, followed by *A. niger* with mean invasion ranges of 17.96 to 33.89%. This finding is in agreement with the findings of Dereje *et al.* (2012) and Gebreselassie *et al*. (2014) who reported that *A. flavus* was the dominant species identified from groundnut seeds in Tigray. Similarly, Abdi *et al*. (2016) reported that *A. flavus* was the dominant species identified from groundnut seeds in East Haraghe Zone. The combined application of *Trichoderma* and mulching reduced *A. niger* seed infection by about 81% and *T. harzianum* alone by 62% compared to the control (non- mulched and non-inoculated with *T. harzianum* (Table 2). Phillips *et al*. (2005) discussed that application of biocontrol agents for the reduction of pre-harvest seed infection of groundnut once a year reduced aflatoxin-producing *Aspergillus* species by 80% in Arizona and Texas.

Table 2: Three-way interaction effect of groundnut genotypes, *T. harzianum* and wheat straw mulching on seed invasion by *A. flavus* and *A. niger* and aflatoxin contamination level at Rama in 2016 cropping season

Treatment combination	Aflatoxin contamination level	*Aspergillus* species seed invasion (%)	
		A. flavus	*A. niger*
Werer961*Tr1*ML1	81.20^{bc}	33.34 (5.71)$^{b-e}$	7.41 (2.70)^{f}
Werer961*Tr1*ML0	88.13^{abc}	45.93 (6.80)$^{a-d}$	14.81 (3.85)$^{d-f}$
Werer961*Tr0*ML1	91.33^{abc}	59.26 (7.68)^{ab}	19.26 (4.46)$^{d-f}$
Werer961*Tr0*ML0	109.07^{ab}	75.55 (8.66)^{a}	30.37 (5.35)$^{b-d}$
ICGV00308*Tr1*ML1	28.78^{e}	22.22 (4.34)^{e}	5.19 (2.33)^{f}
ICGV00308*Tr1*ML0	42.10^{ed}	29.82 (5.27)$^{c-e}$	17.04 (4.06)$^{d-f}$
ICGV00308*Tr0*ML1	61.66^{cd}	43.74 (6.59)$^{a-d}$	22.22 (4.70)$^{c-e}$
ICGV00308*Tr0*ML0	95.77^{ab}	42.37 (6.47)$^{a-e}$	50.37 (6.94)^{ab}
ICIAR19BT*Tr1*ML1	84.00^{bc}	25.18 (4.92)^{de}	14.81 (3.90)$^{d-f}$
ICIAR19BT*Tr1*ML0	89.37^{abc}	40.06 (6.30)$^{b-e}$	20.00 (4.51)$^{c-e}$
ICIAR19BT*Tr0*ML1	92.4^{ab}	53.33 (7.31)$^{a-c}$	28.15 (5.32)$^{b-d}$
ICIAR19BT*Tr0*ML0	118.53^{a}	56.30 (7.48)$^{a-c}$	72.59 (8.45)^{a}
Rama local*Tr1*ML1	28.58^{e}	18.52 (4.28)^{e}	9.63 (2.99)^{ef}
Rama local*Tr1*ML0	62.51^{cd}	33.85 (5.81)$^{b-e}$	23.70 (4.81)$^{b-e}$
Rama local*Tr0*ML1	93.64^{abc}	47.41 (6.84)$^{a-d}$	23.70 (4.71)$^{c-e}$
Rama local*Tr0*ML0	112.03^{ab}	61.48 (7.69)^{ab}	45.93 (6.59)$^{a-c}$
LSD (0.05)	32.78	2.24	2.13
CV (%)	24.65	21.16	27.13

*****=Stands for interaction, LSD = Least significant difference, CV = Coefficient of variation; Means followed by the same small letter(s) in the same column are not significantly different from each other at 5% probability level. Numbers within the parentheses are transformed means for the *A. flavus* and *A. niger* seed invasions.

Table 3: Two-way table of interaction effect of *T. harzianum* by wheat straw mulching for the management of *Aspergillus niger* seed invasion at Rama during 2016 main cropping season

T. harzianum (Tr)*Straw mulch (ML)		*A. niger* seed invasion (%)
With *T.harzianum*	With wheat straw mulch	2.98 (9.25)c
	Without Wheat straw mulch	4.31 (18.88)b
Without *T.harzianum*	With wheat straw mulch	4.78 (23.33)b
	Without Wheat straw mulch	6.83 (49.80)a
LSD (0.05)		1.07
CV (%)		27.50

Where Tr0 = stands for seeds without *T. harzianum*, Tr1, for seeds treated with *T. harzianum*, ML0, plots without straw mulch and ML0 plots with straw mulch. LSD = Least significant difference, CV = Coefficient of variation. Means followed by the same letter in the same column are not significantly different at 5% probability level. Numbers within the parentheses are untransformed means for *A. niger* seed invasion.

Effect of Bio-inoculant *T. harzianum*, Wheat Straw Mulching and Groundnut Genotypes on Aflatoxin Contamination Level in Groundnut (*Arachis hypogaea* L.) Seeds

The analysis of variance (ANOVA) of aflatoxin contamination level revealed highly and significantly ($P \leq 0.01$) different influence due to the main effect of *T. harzianum*, interaction effect of genotypes by *T. harzianum*, *T. harzianum* by straw mulching; and by the interaction effects of the three factors, namely genotypes, *T. harzianum* and straw mulching. However, the main effects of groundnut genotypes, wheat straw mulching and two-way interaction effect of groundnut genotypes by mulching showed significant ($P \leq 0.05$) difference on aflatoxin contamination level. The current research result indicated that there was highly significant ($P \leq 0.01$) difference between the *T. harzianum* inoculated and non-inoculated groundnut seeds. *Trichoderma*-treated seeds exhibited the lowest (63.09 ppb) aflatoxin contamination level, while the highest (96.08 ppb) was recorded from non-inoculated groundnut seeds.

Harman *et al.* (2004) stated that *T. harzianum* was the most known biocontrol agent protecting crops from different fungal pathogens starting from 1930s onwards all over the world. The difference in aflatoxin contamination level between biocontrol-inoculated and non-inoculated seeds in the current study was attributed to the antagonistic nature of *T. harzianum* to suppress the aflatoxigenic *Aspergillus* species as also stated by Kucuk and Kivanc (2008) who reviewed that *Trichoderma* species, having several fungal cell wall degrading enzymes (CWDE), like chitinase and glucanase, were considered as the most common fungal biocontrol agents throughout the world.

There was a significant ($P \leq 0.05$) difference among the tested four groundnut genotypes in aflatoxin contamination levels. The genotype ICGV00308 and Rama local were significantly different from the other two genotypes with relatively lower aflatoxin contamination with corresponding values of 57.08 and 74.19 ppb than that of ICIAR19BT and Werer -961 with 96.08 and 92.43 ppb, respectively. Aflatoxin contamination level in ICGV00308 was lower by 39.00, 35.35 and 17.11 ppb than the genotypes ICIAR19BT, Werer-961 and Rama local genotype, respectively. Similarly, straw mulch significantly lowered the level of aflatoxin contamination to 70.20 ppb as compared to the non-mulched plots with 89.69 ppb. The current finding indicated that straw mulch reduced the level of aflatoxin contamination by 19.49 ppb. The reduction of aflatoxin contamination level with the application of wheat straw mulches could be attributed to considerable availability of soil moisture, which could avoid the occurrence of late drought stress that leads to lower aflatoxin contamination than in non-mulched plots (Okello *et al*., 2010). Previously it was reported that straw-mulched soils with a thickness of 3.81 cm reduced the soil water evaporation rate by 35% as compared to the bare soil (Anonymous, 2007).

Thus, the current finding revealed that mulched-soils can have greater moisture retention ability than the non-mulched ones. To this effect, in the current study mulching helped in retaining soil moisture in groundnut at its critical period, thereby resulting in reduced *Aspergillus* species infection rate and, might have consequently lead to lower aflatoxin contamination than the non-mulched plots. That means the reduction of aflatoxin contamination level with the application of wheat straw mulch could be positively associated with the availability of soil moisture, which can avoid the occurrence of late drought stress that leads to lower aflatoxin contamination level (Okello *et al*., 2010).The level of aflatoxin contamination increased as the soil temperature also increased but straw mulching dampened the environment around the mulched plots due to the retained soil moisture and could stabilize soil temperature (Li *et al*., 2013). This finding agrees with the investigation of Lal (2000) who reported that mulch enhances the biological activities of beneficial microorganisms living in the soil. Ghosh *et al*. (2006) also reported that straw mulch had been commonly exercised as an important pest management tool in many countries of the world.

The three-way interaction effects highly and significantly ($P \leq 0.01$) influenced aflatoxin contamination level. The highest aflatoxin contaminations levels were 118.53, 112.03, 109.07 and 95.77 ppb detected from the control treatment combinations of ICIAR19BT, Rama local, Werer 961 and ICGV00308, respectively, while the lowest contamination levels 28.58 and 28.78 ppb were detected from Rama local and ICGV00308 treated with *T. harzianum* and wheat straw mulches, respectively, followed by 42.1 ppb from ICVG00308 genotype treated with *T. harzianum* only. Integrated management of aflatoxin using groundnut genotypes, bio-inoculant *T. harzianum* and wheat straw mulch showed highly significant ($P \leq 0.01$) reduction in aflatoxin contamination risk of groundnut kernels. Thus, interaction effect of genotypes, *T. harzianum* and straw mulch significantly reduced the aflatoxin contamination level by 83.45, 66.99, 34.53 and 27.87 ppb on Rama local, ICGV00308, ICIAR19BT and Werer-961 genotypes, respectively as compared to the control treatments.

Among the tested four groundnut genotypes for aflatoxin accumulation level when combined with biocontrol agent and wheat straw mulching, seeds from ICGV00308 and Rama local had relatively lower accumulation than the other treatment combinations. Although the levels of aflatoxin contamination detected in Rama local and ICGV00308 were higher than the permissible limit of the European Union standard (5 ppb), FAO/WHO (15 ppb) and USA (20 ppb), it is less than the maximum tolerable limit of India 30 ppb. The level of contamination was generally found less than the treatment contaminations in ICIAR19BT and Werer-961 genotypes and it was found nearly safe. The variation in the aflatoxin contamination levels among the tested groundnut genotypes might have resulted from the inherent aflatoxin tolerant genetic content and drought tolerant characteristics of the genotypes in combination with the bio-inoculant and straw mulching treatments.

The result from this current research indicated that planting ICGV00308 and Werer-961 with the combined application of biocontrol agent and wheat straw mulching significantly minimized the aflatoxin contamination level. Chet *et al*. (1997) reviewed the inhibiting mechanism of *Trichoderma* species as a biocontrol agent (BCA) and found that the mechanism was due to their high reproductive nature, the ability to survive under different unfavorable conditions, their capacity to modify the rhizosphere of the crops, the efficient utilization of nutrients from the soil and their aggressiveness against phytopathogenic fungi. Similarly, Harman *et al*. (2004) also stated the mechanism of *Trichoderma* species to inhibit aflatoxigenic fungal species. Some of the well-known controlling mechanisms of BCAs include antibiosis, parasitism, enhancing host-plant resistance, competition for food and space, promoting plant growth performance and strengthening the defense mechanism(s) of crops and mycoparasitism.

These current findings agree with the observations of Bandyopadhyay *et al.* (2016) who reported that biocontrol agents can minimize the aflatoxin contamination level by 74.3 up to 99.9% as compared to the untreated plots. However, this variation in aflatoxin reduction/suppression depends on the type of biocontrol agent and inoculum rate of the BCA as well as the strain of the *Aspergillus* species. Al-Othman *et al*. (2013) described that percent inhibition by *T. harzianum* to aflatoxin B_1 production ranged from 68.8 to 100% caused by culture filtrate and the inhibition percentage increased whenever concentration of the culture filtrate increased. About 100% aflatoxin B_1 inhibition was recorded when 150 ml kg^{-1} culture filtrate was applied on cashew seeds.

Means followed by the same small letter(s) in the same column are not significantly different from each other at 5% probability level. Numbers within the parentheses are transformed means for the *A. flavus* and *A. niger* seed invasions.

Conclusions

Rama is one of the lowland areas of Tigray, agro-ecologically ideal for groundnut production. Groundnut is the major crop in Rama but the productivity is extremely low in quantity and quality, mainly due to biotic and abiotic factors. Despite the fact that groundnut is the major source of income it is vulnerable to aflatoxin contaminations due to geographical and climatic conditions. Limited researches have been reported in relation to management options of aflatoxin. Therefore, the present research attempted reduce the level of aflatoxin contaminations of groundnut kernels through integrated management of groundnut genotypes, natural antagonistic strain of *T. harzianum* isolate BD-13 and wheat straw mulch. A field experiment was carried out at Rama in 2016/2017 main cropping season with the objective of to evaluate the effects of groundnut genotypes, *T.harzianum* and straw mulch alone or in combination for the management of *Aspergillus* species and aflatoxin contamination. The current research findings indicated that seed treatment with *T. harzianum* and application of wheat straw mulch significantly reduced aflatoxin contamination levels up to 28.58 and 28.78 ppb on Rama local and ICGV00308 genotypes, respectively. Similarly, seed treatment with *T. harzianum* and straw mulching lowered *A. flavus* seed invasion to 18.52, 22.22 and 25.18% on Ram local, ICGV00308 and ICIAR19BT, respectively; and lowered *A. niger* seed invasion to 5.19 and 7.41% on ICGV00308 and Werer-961, respectively.

Production of completely aflatoxin-free groundnut seeds is too difficult to achieve, but application of wheat straw mulching and treatment with *T. harzianum* together with moderately resistant groundnut genotypes highly and significantly reduced seed invasion by *Aspergillus* species and the associated aflatoxin contamination level, especially in Rama local and ICGV00308 groundnut genotypes with concentrations of 28.58 and 28.78 ppb, which is in fact lower than the Indian maximum tolerable limit, i.e. 30 ppb. Similarly, the levels of seed invasion by *A. flavus* and *A. niger* were highly suppressed by the interaction effects of the wheat straw mulching, *T. harzianum* and groundnut genotypes. The newly introduced groundnut genotype ICGV00308 was highly superior in quantitative and qualitative yield and yield related parameters compared to other tested genotypes. Wheat straw mulched soils retained higher soil moisture than non-mulched plots at harvest and the maturity period of genotypes on mulched plot extended by five days than non-mulched plots and it is recommended that farmers should use locally available mulching materials at a rate of 12 t ha^{-1} to overcome terminal drought stress, forced maturity of groundnut and concomitant aflatoxin contamination problem in the study area and other similar drought-prone agro-ecologies. Finally, it is suggested that further extensive research should be conducted to verify the current research findings in the study area and other areas having similar agro-ecological conditions to come up with a final and conclusive recommendation for successful *Aspergillus* species and aflatoxin contamination management and sustainable groundnut production.

Acknowledgments

We acknowledge the financial support of the Regional University Forum for Capacity Building (RUFORUM) Mekelle University Groundnut Project for funding and giving the planting materials of groundnut genotypes to the research work, and Axum Agricultural Research Center of Tigray Agricultural Research Institute for supporting transportation expenses and research facilities. The work is also part of an MSc thesis research at Haramaya University and appreciation goes to the Plant Pathology Laboratory

staff members of School of Plant Sciences for their unreserved support during the laboratory works.

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